Investigating Function and Evolution of Genes and Proteins Involved in Metabolic Control in Mammals

by

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Abstract

The duck-billed platypus and the short-beaked echidna represent the most basal lineage of living mammals and therefore provide important information about mammalian evolution. Monotremes have also undergone remarkable anatomical, physiological and genetic changes. One of the most radical changes involves the monotreme digestive system and genes associated with metabolic control. I have investigated several genes that are vital for metabolic control (specifically genes in the ghrelin and incretin pathways) as well as the histology of the monotreme pancreas.

Given the glandless gut in monotremes, I first sought to investigate genes in the appetite regulating ghrelin pathway. Surprisingly, I discovered that genes encoding ghrelin and ghrelin *O*-acyl transferase (GOAT) are missing in the platypus and echidna genome, whilst, its receptor, growth hormone secretagogue receptor 1a (GHS-R 1a) is present. This is the first report suggesting the loss of ghrelin in a mammal. The conservation of the ghrelin receptor gene despite the lack of the ghrelin and GOAT genes in platypus suggests that another ligand maybe acting via this receptor in monotremes (Chapter 2).

Ghrelin is expressed in human pancreatic ε-cells. Hence the lack of ghrelin in platypus led us to investigate in more detail the structure of the monotreme pancreas, another key organ in both metabolic control and digestion (Chapter 3). Generally, the monotreme pancreata share the basic characteristics of other mammalian pancreata, including both endocrine islets and exocrine acini. I performed immunohistochemical analysis to reveal the detailed architecture of the platypus and echidna endocrine islets of Langerhans. The unique phenotypes of the PP-lobe, smaller size of islets, and the

abundance of α-cells indicate the monotreme pancreata have more resemblance to that of birds and marsupials than eutherian species.

One of the key functions of the pancreas is to release insulin upon food intake. Glucagon-like peptide 1 (GLP-1), a hormone released from the small intestine upon food intake, triggers insulin release via the GLP-1 receptor (GLP-1R) in β-cells of the pancreas. In human, GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4) and thereby has a very short serum half-life (<2 min). Searching for long-acting GLP-1 analogs to improve insulin sensitivity has been a key strategy in Type 2 diabetes (T2D) treatment. We identified and characterised *Glp-1*, *Glp-1r* and *Dpp-4* and found both *Glp-1* and *Dpp-4* are expressed in gut and pancreas as expected, and interestingly also in venom. Importantly, evolutionary changes in monotreme GLP-1 sequences led us to predict that it would be resistant to enzymatic degradation. Extensive biochemical analysis of monotreme GLP-1 revealed that this variant is in fact resistant to DDP-4 degradation, however can be degraded by other enzymes (trypsin-like) in their own sera. Moreover, we demonstrated that monotreme GLP-1s can bind and activate both pGLP-1R and hGLP-1R with similar potency and stimulate insulin release in isolated mouse islets (Chapter 4).

Together this work provides fundamental new insights into the molecular and anatomical characteristics of the monotreme digestive system, the evolution of metabolic control and potential novel avenues for diabetes treatment based on monotreme GLP-1.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of Publications

Four publications will arise from this thesis. One has been published, one has been submitted and two are in preparation and will be submitted soon.

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1. He C, Tsend-Ayush E, Myers MA, Forbes BE, Grützner F (2013) Changes in the ghrelin hormone pathway maybe part of an unusual gastric system in monotremes. Gen Comp Endocrinol 191:74-82

Accepted for publication:

1. He C, Myers MA, Forbes BE, Grützner F. Immunohistochemical analysis of pancreatic islets of platypus (*Ornithorhynchus anatinus*) and echidna (*Tachyglossus aculeatus ssp*)
Journal of Anatomy

Manuscripts in preparation:

1. Tsend-Ayush E, He C (co-first author), Myers MA, Andrikopoulos S, Wong N, Sexton PM, Wootten D, Forbes BE, Grützner F. In monotremes glucagon-like peptide 1 (GLP-1) is dipeptidyl peptidase 4 (DPP-4) resistant and expressed in venom
Prepared for submission to *Nature*

2. He C, Forbes BE, Grützner F Monotremes provide unique insights into the evolution of metabolic control in mammals
Prepared for submission to *BioEssays*

Chapter 1

Introduction

Monotremes provide unique insights into the evolution of metabolic control in mammals

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Signature		Date	

Abstract

Metabolic control is central to two health issues the world is now facing: obesity and diabetes. A better understanding of anatomical, physiological and molecular aspects in different mammalian lineages is vital to be able to understand the evolutionary dynamics of metabolic control and has implications for the treatment of metabolic diseases.

Monotremes, including the enigmatic egg-laying platypuses and echidnas, are key species in mammalian comparative genomics as they represent the oldest surviving mammalian lineage. Already we have learned much about mammalian biology through studying these unique animals including sex chromosome evolution and sex determination in vertebrates. Monotremes have evolved remarkable changes to their digestive system on the anatomical and molecular levels. In this review, we highlight the importance of monotremes in our endeavour to understand the evolution of metabolic control in mammals.

In this review we put into context the unique anatomy of the monotreme stomach, small intestine, pancreas and brain with novel research of metabolic control molecules to highlight novel insights into the evolution of metabolic control in mammals as well as potential applications for human health and diseases.

Introduction

Metabolic control involves many organs and is key to the biggest disease burden in humans

Metabolic control is essential for the maintenance of cellular and organismal life. Mammalian metabolic homeostasis involves the peripheral and central control processes of blood glucose, body temperature and energy expenditure and is achieved by the brain, liver, blood, gastrointestinal (GI) tract and pancreas (Fig. 1). Metabolic control is also central to two health issues the world is facing: obesity and diabetes [1]. A greater understanding of anatomical, physiological and molecular aspects in different mammalian lineages is vital to be able to understand the evolutionary dynamics of metabolic control and has implications for the treatment of metabolic diseases.

The duck-billed platypus (*Ornithorhynchus anatinus*), together with two genera of echidnas (*Zaglossus sp* or long-beaked echidna and *Tachyglossus aculeatus ssp* or the short-beaked echidna), represent the most basal lineage amongst living mammals.

The weird combination of mammalian and reptilian anatomy has confused scientists and inspired cartoonists for centuries. Monotremes lay eggs and yet possess a placenta. They feed their young through secretion of milk onto the abdominal surface [2]. Other peculiar traits, like the soft and sensitive bill, webbed feet and spurs for venom delivery, define these animals as an evolutionary oddity. Increasingly, the molecular changes underlying this fascinating biology are unraveled, and in particular, the sequencing of the platypus genome and coding and non-coding transcriptomes provided important insights into the molecular basis of the unusual monotreme biology [3-6].

There are key anatomical and molecular features of monotremes, which make them a unique species in which to study metabolic control. They possess an extremely small stomach [7] and maintain their body temperature at 32 °C rather than 37 °C for other mammals [8]. Moreover, in the echidna the unusual thermoregulation enables a deep torpor, which saves energy and reduces metabolism in winter. Interestingly, the torpor can be interrupted by numerous endothermic arousals. These arousals are generated without brown fat, which is present in hibernating placental mammals but absent in monotremes and marsupials [9,10].

The understanding of monotreme metabolic and digestive processes has been advanced by important morphological, physiological and anatomical work in the 1970s, predominately by Griffiths, Krause and colleagues [11-19]. More recently accessibility of monotreme material, antibodies and in particular genomic information revealed major insights into the molecular basis of these processes in monotremes [6,20]. Still many aspects of monotreme metabolic control are uncharted territory but the novel insights gained promise that we can learn a lot about metabolic control from this distant mammalian lineage. This review will provide an overview of the anatomical and histological features of the organs involved in metabolic control and will relate these to the more recently derived understanding of specific genes and proteins involved in their function.

The monotreme stomach, an esophagus in disguise?

Platypus is a semiaquatic animal that feeds on invertebrates. Mud and gravel are ingested along with the prey and probably help grind the food [21]. The stomach of platypus is extremely small [16], thin-walled, lacks gastric glands, has a neutral pH and no peptic digestion (Fig. 2A) [7]. These drastic anatomical and physiological differences between platypus and other vertebrates can be explained by changes at molecular level. A recent study by Ordonez *et al* [20] reported that genes required for the formation of acidic gastric juice, including *GAST* (gastrin), *ATP4A* and *ATP4B* (H^+/K^+-ATPase) are absent from platypus genome (Table 1). In human, gastrin stimulates the secretion of gastric acid (HCl) and H^+/K^+-ATPase pumps H^+ into the stomach cavity. Therefore, both gastrin and H^+/K^+-ATPase are key proteins responsible for the acidification of the stomach. The lack of these genes in the platypus is striking as they are generally highly conserved in vertebrates, but explains why the stomach of platypus has a neutral pH instead of an acidic environment as observed in other mammals. Remarkably monotremes have lost a key gene required for the development of endocrine cells in GI tract, the neurogenin-3 (NGN-3) gene (*Ngn3*) [20] (Table 1). NGN-3 deficiency in mice results in a considerably smaller stomach lacking gastrin-secreting G cells [22,23]. Hence, the lack of *NGN3* in monotremes could explain, at least in part, the small stomach and the absence of gastrin-secreting G cells in monotremes. Moreover, all genes encoding pepsin proteases, which are responsible for the initial digestion of proteins in the stomach, including *PGA* (pepsinogen A) and *PGC* (pepsinogen B/C), are deleted from the platypus genome (Table 1).

As a result of this absence of key genes, the platypus stomach has no digestive functions at all. This removes the need for food to remain in the stomach. In mammals

the function of the pylorus is normally to retain food in the stomach until it has been broken down. The lack of a distinct pylorus in the platypus stomach is consistent with the loss of a functional stomach [7] and suggests a faster passage rate of food from the stomach to the small intestine. What could be the function of such a rudimentary stomach? In most vertebrate species, much of the stomach is lined with a columnar epithelium, whereas the lining of the esophagus is a stratified squamous epithelium as it is subjected to considerable abrasion by the food particles. The histological structure of the platypus stomach shows more resemblance to the esophagus in most other vertebrates, as their stomachs are lined completely with stratified squamous epithelium rather than simple columnar epithelium. Krause also described the platypus stomach as "a dilatation of the esophagus before joining to the small intestine" [16].

The gastric glands that normally secrete hydrochloric acid and pepsinogen are missing in the platypus stomach. Interestingly, the histology of the glandless stomach of platypus closely resembles three of the four chambers of the ruminant stomach (rumen, reticulum and omasum) with a lining of an esophageal type of epithelium and an absence of gastric glands. These three chambers have no digestive functions, but rather to ferment food and absorb organic acids, water and many of the inorganic mineral elements into the blood stream [24]. Only the ruminant abomasum is lined with columnar epithelium and has gastric glands. We speculate that the vestigial stomach of the platypus has adopted more absorptive properties while loosing digestive functions.

The evolution of a digestive stomach is thought to coincide with species feeding intermittently on larger food [25]. If an animal feeds on very small particles, or if the food is finely ground in the mouth, a stomach may be secondarily lost [26]. The

primitive hagfishes, lampreys, chimaeras, lungfishes [25] and some teleost fish lack stomachs and their esophagus empties directly into the small intestine where most of the digestion and absorption of nutrients occurs. In platypus, the roles of the stomach seemed to be carried out by cheek pouches, and then passed to the adjacent keratinous horny grinding pads on the maxillae and lower jaws for fine grinding [27], which may have driven the loss of digestive function of the platypus stomach and genes underlying digestion.

The echidna's diet consists of ants and termites but also includes beetles, larvae and worms. Similar to the platypus, its stomach is small, thin-walled and its contents are maintained at a pH of 6.2 to 6.5 [12,28]. Also, its stomach is lined completely with stratified squamous epithelium and lacks gastric glands. However, different from platypus, echidna stomach has a pylorus, which may indicate a slower passage rate of food from stomach into small intestine compared to the platypus (Fig. 2 B), which maybe related to the more rigid nature of its pray.

New tricks for an old dog? Brunner's glands may have a new function in monotremes.

Brunner's glands are mammalian specific glands that secret an alkaline fluid and mucus in order to protect the proximal duodenal mucosa from gastric acid, pepsin, and acidic chyme entering from the stomach, provide an alkaline condition for the intestinal enzymes to be active as well as lubricate the intestinal walls [29]. In other mammals, the Brunner's glands are confined primarily to the submucosa of the proximal duodenum, whereas in platypus and echidna, these glands are present in the submucosa of the distal stomach near its junction with the intestine (Fig. 2) [12,13]. Given the

absence of gastric glands and neutral pH of the stomach in the monotremes, one would predict that these glands are obsolete in monotremes. The presence of these glands raises the possibility that they have adopted a novel function in monotremes [7,13,15]. Maybe these glands secrete components that can influence the motility or the nutritional supply of the intestinal tract. Alternatively they may help with the formation of an aseptic lining for the intestinal mucosa [13]. Future genomic and proteomic approaches may explore the composition and effects of the secretion of these glands in monotremes. In addition, the retention of these glands may play roles other than the prevention of mechanical damage and ulcerating effects of acid and pepsin.

The platypus and echidna intestine: viva la difference

The GI tract of platypus is relatively short and simple and shows more resemblance to that of amphibians and reptiles than to that of mammals [28]. Also there are no finger-like villi which are present in birds and mammals, but numerous transverse surface folds [16,30] (Fig. 2 A) as seen in reptiles and fishes. Krause [16] confirmed the appearance of intestinal glands (crypts of Lieberkühn) in the mucosa of the small intestine in the platypus. Another obviously reptilian feature in monotremes is the cloaca. The large intestine discharges through a cloaca, an orifice possessed by all amphibians, reptiles, birds and monotremes, but not placental mammals.

The surface area of the small intestine of platypus may be less than that of other mammals with intestinal villi. Therefore, so as to ensure thorough digestion and absorption in the small intestine, more active smooth muscle movements that lead to the contraction and expansion of the surface folds are expected. An immunohistochemical analysis done by Yamada *et al.* observed the substance P, vasoactive intestinal peptide (VIP) and gastrin releasing peptide (GRP) immunoreactive nerve fibers penetrating or surrounding the smooth muscle fibers of the surface folds, and 5-HT, motilin immunoreactive cells in the intestinal mucosa in the platypus [17]. All these peptides and 5-HT are known to be involved in regulating the contraction and expansion of surface folds, the active movement of which may compensate for the lack of sufficient surface area for absorption and lead to high passage rate of food in the small intestine of platypus.

The echidna has a relatively long GI tract (over 3.5m in length in adult echidnas with a body length of around 40 cm). The small intestine is covered by finger-like villi and

possesses crypts of Lieberkühn (Fig. 2B) as found in the intestine of other vertebrates [11,14]. In the echidna, the existence of a pylorus and a long intestine supports a slow passage of food, which would allow ample time for complete food digestion. An adult echidna can ingest a 200g meal of termites within minutes but it takes up to 2 days to be cleared [31]. The differences between platypus and echidna GI tract (Fig. 2, Table 2) might be due to their different diet and metabolic rates as platypus is an active animal that seems to feed constantly whereas echidnas are known to enjoy “life in the slow lane” [10].

Monotremes do have a pancreas

The pancreas is absolutely central to metabolic control, and glucose homeostasis. It contains distinct endocrine islets and exocrine tissue, that evolved hundreds of millions of years ago in early vertebrates, when endocrine cells migrated out of the gut tube and clustered to form distinct islets [32].

In protochorades (amphioxus and tunicates), four types of endocrine cells (insulin, glucagon, somatostatin and PP-producing) are detected in the gut mucosa without aggregation into a functional islet organ [33]. The cyclostomes (hagfish and lamprey) are the first species to form a primitive pancreas-like organ where insulin producing cells (99%) and somatostatin producing cells (1%) aggregate into a separate cluster and bud out from the bile duct; however, no exocrine pancreatic tissue is observed [34,35]. It is with cartilaginous and bony fishes that the first pancreas consisting of both endocrine and exocrine tissue appeared. Their endocrine islets comprise all four classical hormone-producing cell types (α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), PP-cells (pancreatic polypeptide)) and the exocrine pancreas is formed by branching and folding of pancreatic ducts [36,37]. The appearance of the fifth cell type, ghrelin-producing ε-cells was first reported in catfish. The function of ghrelin in this fish species is to stimulate growth hormone (GH) gene expression and GH release [38]. Amphibian, reptiles, birds and mammals share high level of similarities in gross anatomy and histology of the pancreas; however, the islet architecture differs greatly among species, reviewed by [39,40]. This may reflect evolutionary adaptations to different diets or other environmental conditions although the stringent control of blood glucose levels and the regulation of metabolism within a tight range is of such fundamental importance that it has been evolutionarily conserved [41].

The anatomy and histology of the monotreme pancreas

At first the overall anatomy of the platypus pancreas appears diffuse (observed by F. Grützner and A. Casey during dissection). Such differences are also observed in therian mammals: rodents and lagomorphs also feature a diffuse pancreas [42], while the structure of pancreas is more defined in carnivores [43]. Several histological studies show that the pancreas of echidna has distinct endocrine and exocrine parts (He *et al.* submitted) [19]. NGN-3 is required for the development of endocrine cells in pancreas [23,44,45]. In mice in addition to a small group of NGN-3 independent, multi-potent ε-cells [46], the five different pancreatic endocrine cell populations (α-, β-, δ-, PP- and ε-cells) are derived from NGN-3 expressing progenitor cells in a time-dependent manner during embryonic development [47,48]. *Ngn3* deficiency in mice results in the lack of pancreatic islets due to the inability to generate pancreatic α- and δ-cells [22,23].

Ngn3 is absent from the platypus genome [20]. In addition, another islet gene that is missing is the ghrelin gene [49], a marker of ε-cells. Despite losing these "islet genes", the other four pancreatic endocrine cell types (α-, β-, δ- and PP-cells) are present in both platypus and echidna. We found islets in monotremes to be generally smaller than those of other mammalian species, but larger than those of birds [50,51] (Fig. 3). Overall this indicates a trend towards fewer islets with enlarged size during mammalian evolution. The enlarged islets may be related to an increase of β-cell mass to compensate insulin demand in the body, whereas decreased islets numbers may indicated an enhancement in islets function through evolution.

Monotremes also contain a specific category of islets in echidna where α- and β-cells were juxtaposed to form two distinct hemispheres (Fig. 3 D). What this unique

arrangement of endocrine cells means in terms of islets function, especially insulin secretion, remains unknown. However, several studies have suggested that the cytoarchitecture of pancreatic islets is functionally related to the microvasculature within the islets [52-54]. In order to address the functional significance of islet cell arrangement, the organization of endocrine cells and blood vessels within islets needs to be investigated. The structural analysis will delineate the role of blood vessels in the maintenance of islet architecture and function. In addition, determining which cell types tend to locate adjacent to blood vessels may inform us of how the release of hormones into blood stream is controlled.

Long lasting GLP-1? Insight into glucose homeostasis in the monotremes

Insulin is a peptide hormone secreted by pancreatic β-cells, and it is crucial for the regulation of blood glucose level. Gastric inhibitory polypeptide (GIP), secreted by K-cells of the duodenum and jejumun, and glucagon-like peptide-1 (GLP-1) produced shortly thereafter from L-cells of the ileum and colon, are known as incretin hormones as they stimulate insulin secretion by the pancreatic β-cells [55,56]. These incretin signals are mediated through separate G-protein couple receptors, the GLP-1 receptor (GLP-1R) and GIP receptor (GIPR). Although GLP-1 and GIP share a high degree of similarity in terms of peptide sequences and structure, GLP-1 does not bind to GIPR and vice versa [57].

Within minutes of their synthesis both GLP-1 and GIP are proteolysed by dipeptidyl peptidase 4 (DPP-4), which cleaves C-terminally of residue 8 of GLP-1 and residue 2 of GIP to produce a truncated inactive form (Fig. 5 A). Rapid inactivation tightly controls the insulinotropic activity of incretin hormones. Further cleavage by neprilysin (NEP

24.11), which cleaves at the N-terminal side of aromatic and hydrophobic residues (Fig. 5 A), allows rapid renal clearance of the incretin metabolites [58].

We have shown that platypus GLP-1 (pGLP-1) has a serine at residue 8 (Fig. 5 A), which renders it resistant to human DPP-4 cleavage and is more stable than hGLP-1 in human serum. Nevertheless, our recent findings suggest in fact pGLP-1 is cleaved in its endogenous serum by a tryptic-like protease.

Surprisingly, pGLP-1 has a low affinity and potency for both hGLP-1R and its platypus GLP-1 receptor (pGLP-1R) compared to hGLP-1 to hGLP-1R (Fig .5). The lower affinity of pGLP-1 for the pGLP-1R raises the question whether pGLP-1 is acting as an incretin hormone, and if it is, how its activity is regulated. There are three possible scenarios (Fig. 5 B): 1) another endogenous platypus ligand(s) acts via pGLP-1R to promote insulin secretion. Our analyses of the pGLP-1R sequence suggest it has a conserved GLP-1 ligand binding pocket but binding of other glucagon-like peptides has not been investigated; 2) the pGLP-1 is the natural pGLP-1R ligand but in order to achieve sufficient potency circulating pGLP-1 levels are higher, and its lower affinity may have been selected as a compensation for increased stability. pGLP-1 serum levels have not yet been measured; and 3) pGLP-1 can bind and active another G-protein coupled receptor (GPCR) to promote insulin secretion. Due to the sequence similarity of pGLP-1 with other glucagon related peptides, such as GIP, it is possible that pGLP-1 acts via one of their cognate receptors.

Interestingly, attempts to identify GPCRs failed to identify the pGIPR sequence in the platypus genome, suggesting there may be further differences in the incretin hormone systems in the platypus.

Exocrine pancreatic juice in monotremes: alkaline or neutral?

Pancreatic juice released from the exocrine pancreas is composed of two elements vital to proper digestion: digestive enzymes and bicarbonate. The enzymes are synthesised and secreted from the exocrine acinar cells, whereas bicarbonate is secreted from the epithelial cells lining small pancreatic ducts.

The structure and histology of exocrine pancreatic tissue is overall conserved between mammals and birds, where cells that synthesise and secrete digestive enzymes are arranged in grape-like clusters called acini [59,60].We demonstrated that this conservation of the exocrine pancreas histology is maintained in the monotremes, and also extend to the molecular level, as previous studies have demonstrated amylase, lipase and trypsin-like activities in pancreatic juice [11].

Human pancreatic juice is alkaline due to the high concentration of bicarbonate ions. Bicarbonate is critical to neutralizing the gastric acid coming into the small intestine from the stomach, and thereby allowing for effective enzymatic action. Considering the absence of gastric glands and neutral pH of the stomach in the monotremes, it would be interesting to consider the pH of pancreatic juice in monotremes as no neutralizing function is required. Although little is known about the concentration of bicarbonate ions and pH of pancreatic juice in monotremes, no secretin (*SCT*) gene seems to be annotated in the platypus genome (He *et al.* unpublished data, Table 1). In addition,

previous immunohistochemical studies reported by Yamada *et al.* failed to observe secretin immunoreactive cells in the mucosa of the small intestine of platypus [17]. The human secretin is released from the small intestine to adjust the pH of the duodenum by inhibiting secretion of gastric acid from the stomach, and also stimulating the release of bicarbonate from the exocrine pancreas [61]. Therefore, the potential loss of *SCT* may be related to the altered biological activities of gastric juice in the platypus, and may affect the bicarbonate secretion and therefore the pH of pancreatic juice.

Appetite regulation in monotremes without ghrelin and NGN-3

The hypothalamus effectively links the nervous system to the endocrine system via the pituitary gland. It plays vital roles in regulating metabolism including adjusting body temperature and controlling appetite. The arcuate nucleus (ARC) of the hypothalamus is the major appetite-controlling centre of the human brain. It consists of two appetite controlling cell types: the pro-opiomelanocortin (POMC) appetite-inhibiting neurons and the neuropeptide Y (NPY) and agouti-related peptide (AgRP) appetite-stimulating co-expressing neurons [62,63]. Upon receiving signals from the periphery/gut including leptin, insulin, peptide YY (PYY) and ghrelin, the ARC produces neuropeptides that act on the downstream neurons to control feeding and energy expenditure [64].

In monotremes the histology of the hypothalamus and pituitary has been reported in detail in several studies [31,65,66]. Generally, the histology of the hypothalamus is similar to therians and with some unique features such as a small supraoptic and paraventricular nucleus. Whereas the anatomy of the monotreme pituitary is more reptilian than mammalian, in that the infundibular cavity extends into pars nervosa, the morphology of which is more like a sac rather than a knob. However, little is known about the expression and action of appetite controlling and growth promoting molecules in monotremes. Anthwal *et al.* have shown that early embryonic hypothalamic inactivation of *Ngn3* led to post weaning obesity in mice as a result of loss of expression of appetite-inhibiting POMC neurons in ARC [67]. How the lack of *Ngn3* [20] in the platypus affects the POMC neuron function is unclear but it may indicate the existence of a different appetite regulating mechanism in monotremes.

Different growth hormone secretion and appetite regulating mechanisms in monotremes

Ghrelin (encoded by *GHRL*) is a 28 amino acid peptide produced predominately in stomach [68] that has been identified as the endogenous ligand for the growth hormone secretagogue (GHS) receptor 1a (GHS-R 1a) [69]. In addition to stimulation of GH secretion, ghrelin exerts an important role in several other aspects, including stimulating food intake and body weight gain, increasing gastric motility and acid release, decreasing lipid metabolism, and various neuronal functions in a variety of vertebrate species [reviewed by 70,71]. Acylation at the third serine residue (Ser^3) of unacylated ghrelin (UAG) is essential for GHS-R 1a binding and consequent biological activities [69]. This process is catalyzed by ghrelin *O*-acyl transferase (GOAT), which belongs to the membrane-bound *O*-acyl transferase (MBOAT) family and is encoded by *MBOAT4* (summarised in Fig. 4 A). Orthologs of ghrelin pathway genes, including *GHRL*, *GHSR* and *MBOAT4*, are highly conserved and have been identified in a broad range of vertebrate species [69,72-76]. However, monotremes *Ghrl* and *Mboat4* have been lost whereas *Ghsr* is present, conserved and expressed as in other mammals [49] (Fig. 4B, Table 1).

The loss of ghrelin questions the metabolic control in monotremes, especially in terms of GH release and appetite regulation. The effect of ghrelin deficiency on growth varies among species. For instance, zebrafish that lack ghrelin showed impaired growth [77], whereas, *Ghrl* knockout (KO) mice have a normal growth rate, body composition and size [78-81]. Interestingly, growth hormone releasing hormone (GHRH) is an agonist of the ghrelin receptor GHS-R 1a [82]. GHRH is important in promoting GH release and then insulin-like growth factor 1 (IGF-1) production and is highly conserved amongst

vertebrate species. The normal growth of *Ghrl* KO mice may be explained by the redundancy in GH regulating pathways compensation for the loss of ghrelin by GHRH. Surprisingly, together with *Ghrl*, *Ghrh* also appears to be missing in the platypus genome (unpublished data), whereas partial sequence of growth hormone releasing hormone receptor (GHRHR), missing the N-terminus is found in the NCBI database (accession No. XP_001505263.1). The simultaneously loss of *Ghrl* and *Ghrh* suggests a novel mechanism to regulate GH release in monotremes.

The role of ghrelin in controlling appetite varies between species from having no effect in mice and pigs to an appetite stimulant in humans and rats or a suppressant in chickens (reviewed by [71]). Using *Ghrl*$^{-/-}$ mice models, Wortley *et al.* demonstrated a role for ghrelin in selection of fat over carbohydrate as a substrate to maintain an energy balance [83]. In this context, it is possible that there is no need for ghrelin considering the restricted diet and high metabolic turnover in monotremes.

The use of comparative studies for the treatment of human metabolic disorders

Appetite control and obesity

According to the World Health Organization (http://www.who.int), more than 1.4 billion adults are overweight (body mass index (BMI) ≥ 25 kg/m^2). Of these over 200 million men and nearly 300 million women are obese (BMI ≥30 kg/m^2). Obesity is most commonly caused by a combination of excessive food intake, lack of exercise and genetic susceptibility [84,85]. It is associated with various genes that regulate appetite, such as, *GHRL* (ghrelin), *LEP* (leptin) and *FTO* (fat mass and obesity-associated protein). Moreover, obesity is a major feature in several syndromes, such as Prader-Willi syndrome (PWS), Bardet-Biedl syndrome (BBS), and Alström syndrome [86]. The most frequent of these syndromes is PWS, the genetic basis of which involves dysregulation of imprinting of several genes on chromosome 15 [87]. Excessive appetite in PWS leads to severe obesity. Interestingly, ghrelin has been shown to be the candidate peptide that may mediate the severe hyperphagia of PWS, as elevated plasma ghrelin levels were observed in PWS [88].

Controlling appetite and exercise are the most important approaches to treat obesity. The appetite is regulated in the central nerves system (CNS) via the brain-gut axis, with a series of GI peptides playing crucial roles including the satiety stimulating hormones leptin, insulin, PYY, GLP-1, OXM, CCK and PP, as well as hunger-stimulating peptide ghrelin. Ghrelin is the only known appetite-stimulating hormone that acts via the vagus nerve, the brainstem as well as NPY and AgRP neurons in the ARC of the hypothalamus [89]. In this context, ghrelin has been identified as a target for the treatment of obesity. Blocking or neutralizing ghrelin's activity may be a feasible

approach to control appetite and reverse obese state. Ghrelin neutralization leads to reduced food intake and weight loss in diet-induced obese mice [90]. Nevertheless, there remain many unsolved mysteries regarding ghrelin related biology. Especially, ghrelin KO animal models showed no obvious abnormalities in terms of feeding behaviour [78-80], suggesting that ghrelin antagonist might only have a limited effect on obesity treatment. The unexpected mild phenotypes of ghrelin KO animal models indicate redundancy in the appetite control pathway. Specifically, the existence of additional ligands acting via ghrelin receptor GHS-R 1a in other species has been directly postulated by several studies [91-93]. The monotreme scenario where GHS-R 1a is present in despite of the lack of ghrelin, further supports this.

Monotremes are so far the only mammals known to lack ghrelin and GOAT. The lack of ghrelin in the other non-mammalian spices has been reported [94]. Initially Wang *et al.* claimed that the ghrelin gene was lost in turtle species, the Chinese soft-shell turtle (*Pelodiscus sinensis*) and the green sea turtle (*Chelonia mydas*) [94]. However, recently, ghrelin genes in these turtles were characterised by two other independent studies [95-97]. Hence, monotremes provide unique and valuable models to gain better understandings of the ghrelin pathway and potentially elucidate the unknown ligand for GHS-R 1a.

Blood glucose homeostasis and T2D

347 million people worldwide have diabetes [98], among which, 90% suffer from T2D [99], a disease where insulin release and function are compromised. There are several classes of anti-diabetic medications available, including metformin, sulfonylureas, nonsulfonylurea secretagogues, alpha glucosidase inhibitors, thiazolidinediones, insulin

as well as incretin hormone based treatments including glucagon-like peptide-1 (GLP-1) analogs, and dipeptidyl peptidase-4 (DPP-4) inhibitors [100].

Incretin hormones, GLP-1 and GIP stimulate insulin release by pancreatic β-cells. GLP-1 has also been shown to have cardioprotective and neuroprotective effects, to inhibit gastric emptying, decrease food intake and therefore is associated with weight loss [101]. Therefore incretin based therapy has attracted much interest recently as key treatment option in T2D. One of the key drugs used in the treatment of T2D, called exenatide, has been derived from a GLP-1 paralog, exendin-4 (Ex-4), which was isolated from venom of the lizard gila monster (*Heloderma suspectum*) [102]. It is considered to be the most successful example of utilizing comparative studies in drug discovery.

The isolation of Ex-4 from venom was sparked when Eng *et al.* learned that the gila monster is able to slow down metabolism and maintain constant blood glucose levels during starvation [102]. The toxic bioactivity of Ex-4 in venom is to induce hypotension mediated by relaxation of cardiac smooth muscle [103]. Ex-4 shows similarities to GLP-1 in both structure and function, which includes stimulating insulin release and improving insulin sensitivity [102]. More importantly, while circulating hGLP-1 has a short half life (<2 min) [104] and Ex-4 remains active for hours [105], suggesting that it could be a long-acting, effective T2D treatment. The evolutionary origin of *exendin-4* genes has not been fully resolved but it seems likely that exendins evolved by duplication from a glucagon-like peptide gene precursor [103].

Excitingly, monotreme GLP-1 peptides are the only naturally occurring GLP-1 orthologs that are resistant to DPP-4-mediated degradation, which may make this a potentially novel T2D treatment option. Remarkably, changes in both of the incretin hormones, GIP and GLP-1 indicate further differences in incretin hormone regulation in monotremes. Hence, detailed comparative studies between monotreme incretin systems with those of other mammals will shed light on the evolutionary dynamics of the incretin biology, and provide insights into insulin releasing mechanisms.

Conclusion

Monotremes occupy a special phylogenic position within vertebrates. Comparison between monotremes, birds and eutherian mammals has shed light on mammalian metabolism. In this review, we compared the anatomy, histology as well as the molecular basis of the most vital organs involved in metabolic control and digestion, including stomach, small intestine, pancreas and brain in vertebrates, focusing on the unique biology of monotremes.

While possessing a unique GI tract, the overall histology of the monotreme pancreas is similar to that of other vertebrates. In addition to the differences at both morphological and histological levels, the observation of the lack of several key genes and the changes in amino acid sequences of important peptide hormones is striking. The lack of *Ngn3* and *Ghrl* may indicate evolution of a unique mechanism involved in pancreatic development, appetite and growth regulation. Remarkably, changes in amino acid sequences of incretin peptide hormones indicate that monotreme GLP-1 peptides might be promising new treatments for T2D and shed light on the evolution of incretin hormones.

With the completeness and improvement of both monotreme genome and transcriptome databases, further understandings of metabolic control and digestion in monotremes will be gained.

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Table 1 Summary of genes and peptides involved in metabolic control and digestion that have undergone changes in the platypus genome compared to human

Gene	Protein / Peptide	Status in platypus genome	References
GAST	Gastrin	Absent	[21]
ATP4A	ATPase, H^+/K^+ exchanging, α polypeptide	Absent	[21]
ATP4B	ATPase, H^+/K^+ exchanging, α polypeptide	Pseudogene	[21]
CTSE	Cathepsin E	Pseudogene	[21]
NGN3	Neurogenin 3	Absent	[21]
PGA	Pepsin A	Absent	[21]
PGC	Pepsin C	Absent	[21]
GHRL	Ghrelin	Absent	[49]
MBOAT4	Ghrelin O-acyltransferase	Absent	[49]
GCG	Glucagon-like peptide 1	Changes in key residue	[105]
GIP	Gastric inhibitory polypeptide	Changes in key residue	[105]
SCTR	Secretin	Absent ?	Unpublished

Table 2 Summary of the main differences in the monotreme digestive system compared with other vertebrates.

	Platypus	Echidna
Diet	Invertebrates	Ants and termites
Stomach	Small, thin-walled	
	No pylorus	Elongated pylorus
	Lined completely with stratified squamous epithelium	
	Lack of glands (except for Brunner's glands)	
	Neutral pH	
	Loss of genes involved in gastric functions	N/A
Small intestine	Short	Long
	No villi present	Finger-like villi
	Key changes in incretin hormones	
Pancreas	Diffuse	Discrete
	Small but more abundant islets	
	No PP-lobe observed	PP-lobe with PP-rich islets

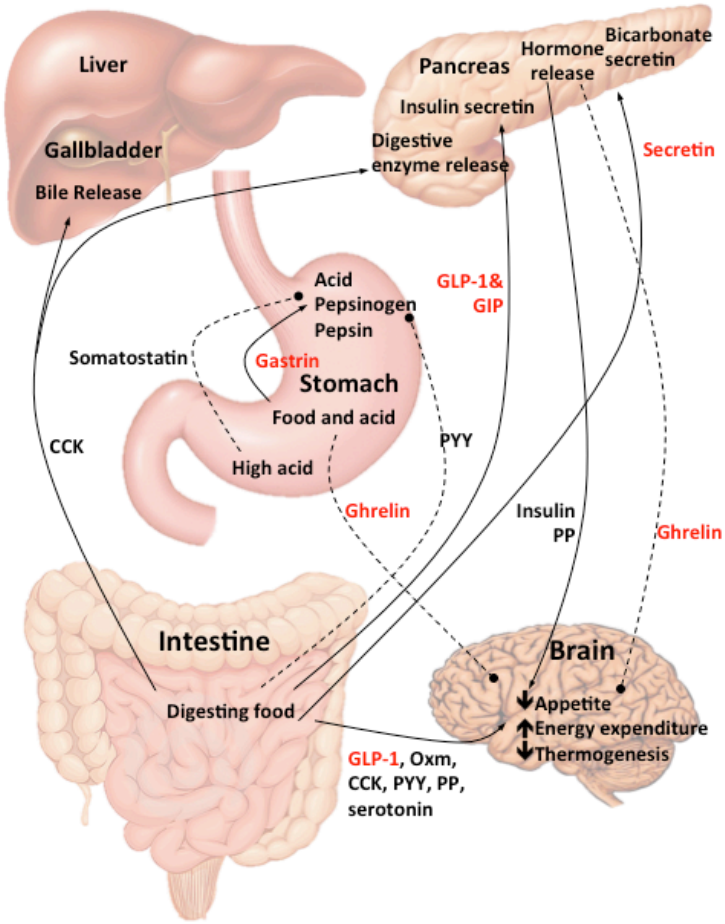


Fig. 1 Key organs and molecules involved in metabolic control in humans. Mammalian metabolic homeostasis involves the peripheral and central control processes of blood glucose, digestion and energy expenditure and is achieved by the brain, liver, GI tract and pancreas. Solid lines with arrows indicate stimulating functions, while dashed lines with solid circles represent repressing effects. Peptides that have undergone changes in the platypus compare to human are highlighted in red. GLP-1, glucagon-like peptide 1; OXM, oxyntomodulin; GIP, gastric inhibitory polypeptide; CCK, cholecystokinin; PYY, peptide YY; PP, pancreatic polypeptide.

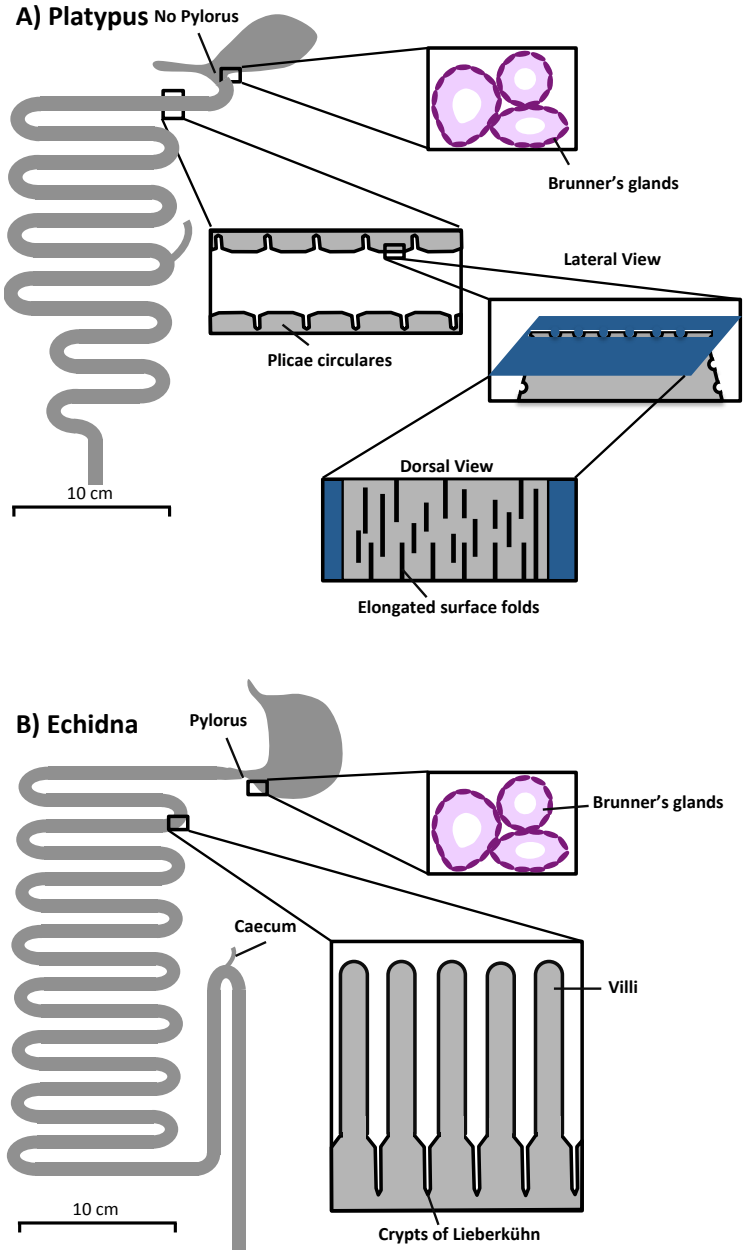


Fig. 2 Schematic comparison between the GI tract of the platypus (A) and echidna (B). A: Platypus has a relatively short GI tract, there is no distinct pylorus in the platypus stomach. The only glands present in the stomach of platypus are the Brunner's glands, which are confined to the submucosa of the distal stomach near its junction with the intestine. Large plicae circulares were found throughout the duodenum. No finger-like villi are present in platypus intestinal mucosa, but numerous transverse elongated surface folds. The small and large intestine is not separated by a sphincter or constriction, but rather by a vermiform caecum. B: Echidna has a longer GI tract. Its stomach has a pylorus, Brunner's gland are confined to the submucosa of the distal stomach near pylorus. Its small intestine is covered by finger-like villi and exhibit

crypts of Lieberkühn as found in the intestine of other vertebrates. The small and large intestine are not separated by a sphincter or constriction, but rather by a vermiform caecum.

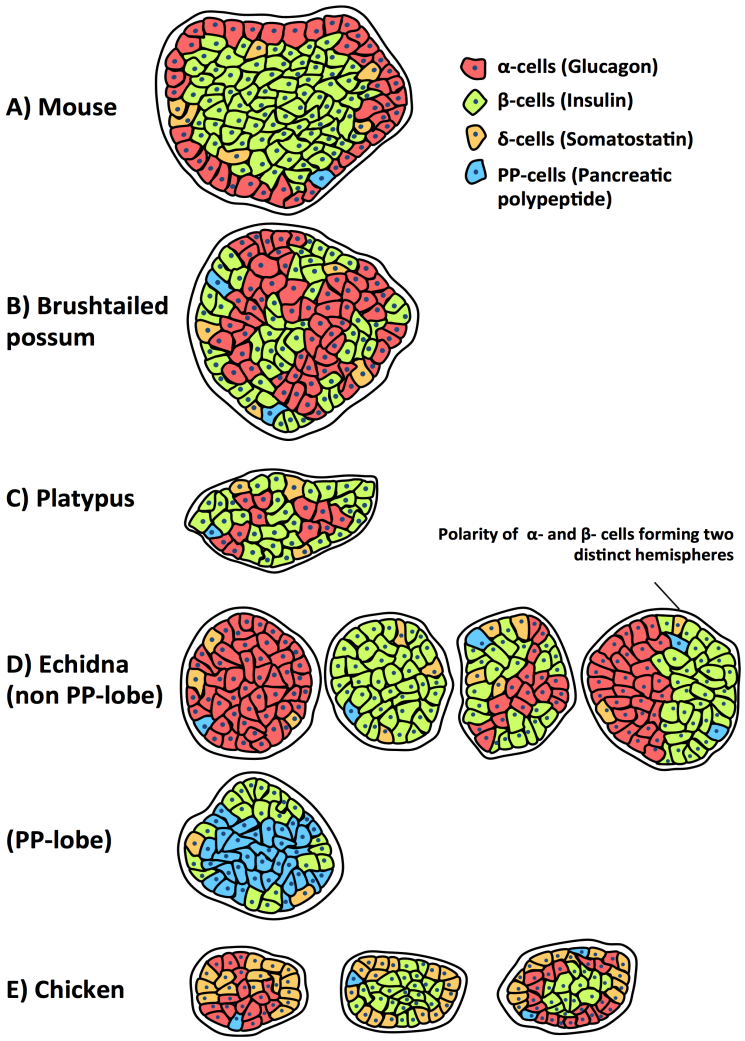
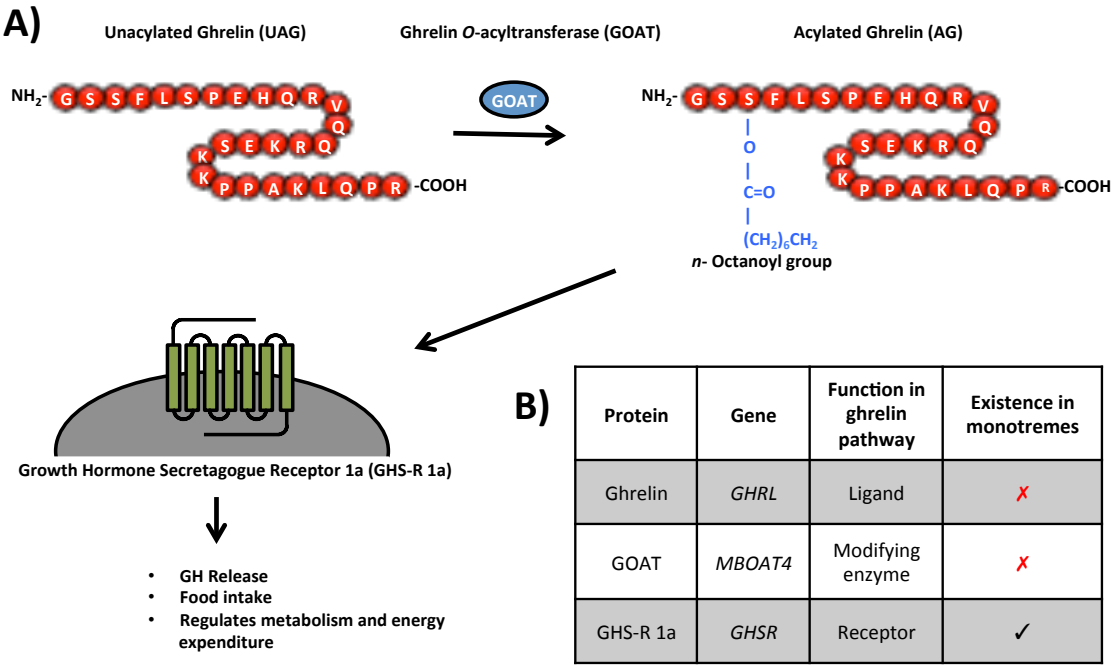


Fig. 3 Comparison of islet structure and composition of mouse (*Mus musculus*, A), brushtailed possum (*Trichosurus vulpecula* ,B), platypus (*Ornithorhynchus anatinus*, C), echidna (*Tachyglossus aculeatus ssp*, D) and chicken (*Gallus gallus*, E). Depicted islets are schematics of actual islets based on immunohistochemical images composed of α-cells (red), β-cells (green), δ-cells (orange) and PP-cells (blue).



Protein	Gene	Function in ghrelin pathway	Existence in monotremes
Ghrelin	*GHRL*	Ligand	✗
GOAT	*MBOAT4*	Modifying enzyme	✗
GHS-R 1a	*GHSR*	Receptor	✓

Fig. 4 Ghrelin pathways in human (A) and status of ghrelin pathway genes in monotremes (B). A: Ghrelin is a 28-amino acid circulating peptide secreted by the stomach, pancreas and a wide variety of cells and tissues. The acylation of unacylated ghrelin (UAG) on the Serine in position 3, into its active form acylated ghrelin (AG) is catalyzed by ghrelin *O*-acylTransferase (GOAT) and is essential for binding to growth hormone (GH) secretagogue receptor 1a (GHS-R1a). Activation of GHS-R 1a stimulates GH release, food intake and regulates metabolism and energy expenditure. B: Genes encoding ghrelin and GOAT are missing in monotremes. *Ghsr* is present and expressed in the brain, pancreas and intestine in platypus.

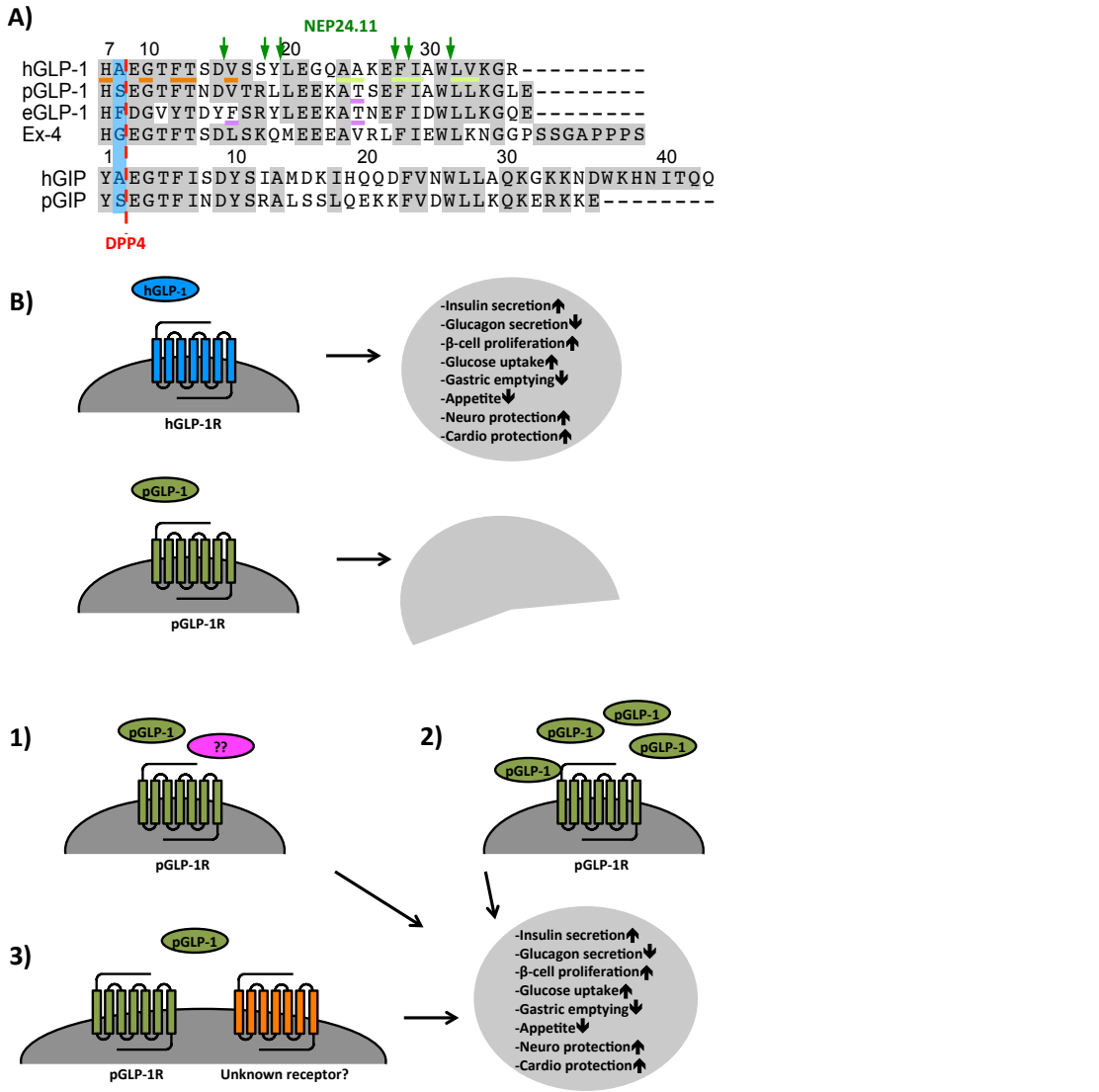


Fig. 5 A: Alignments of GLP-1 and GIP sequences. Identical or conserved residues (>50% consensus) are boxed (grey). DPP-4 cleavage site is depicted with the red dashed line. The penultimate residue is important for DPP-4 cleavage and is shaded in blue. NEP24.11 cleavage sites are depicted by green arrows. hGLP-1 residues important for binding to hGLP-1R core domain: His^{7}, Gly^{10}, Phe^{12}, Thr^{13} and Asp^{15} are underlined in orange. Residues known to interact with nGLP-1R in the C-terminal part of hGLP-1, Ala^{24}, Ala^{25}, Phe^{28}, Ile^{29}, Leu^{32} and Val^{33} are underlined in green. Residues that have undergone radical changes and may affect receptor binding affinities are underlined in purple in pGLP-1 and eGLP-1.

B: hGLP-1 binds and activates hGLP-1R and has effects on various tissues. In comparison pGLP-1 has lower affinity and potency to pGLP-1R when compared with hGLP-1 to hGLP-1R. This indicates that pGLP-1 pathways may have evolved a different mechanism. There are three possible scenarios, (1) a different ligand other

than pGLP-1 can bind to pGLP-1R; (2) physiological concentration of pGLP-1 is higher compared to human; (3) pGLP-1 acts via a different receptor.

Project hypothesis and aims

The small and glandless stomach of platypus and echidna is one of the most striking anatomical differences between the monotreme lineage and other mammalian species [7]. Consistent with the anatomical changes, many genes required for gastric functions have been lost or are non-functional in the platypus [20]. This raises questions about fundamental aspects of metabolic control in monotremes.

Hypothesis

The central hypothesis underlying this work is that the anatomical and molecular changes in the monotreme digestion system have led to major changes in morphology and functions of other organs (such as pancreas), as well as to the molecular basis of metabolic homoeostasis.

Aims

1. To investigate genes in the appetite regulating ghrelin pathway.

Ghrelin is a growth hormone releasing and appetite regulating hormone predominately released from the oxyntic gland of the stomach. The glandless gut of the monotremes may suggest the differences in the expression and regulation of ghrelin pathway genes.

2. To characterise the structure and distribution of pancreatic islets and endocrine cells in monotreme pancreas.

The pancreas is an essential component of both the digestive and endocrine systems. The morphology of pancreas may be different in monotreme in order to compensate the digestive functions of stomach.

3. To investigate monotreme GLP-1: susceptibility to proteolytic degradation, ability to activate the GLP-1 receptor as well as biological functions.

Previous work identified the *GCG* gene encoding GLP-1 in the platypus genome database. The sequence encoding the pGLP-1 differs from hGLP-1 (Tsend-Ayush, Myers and Grutzner personal communication), raising fundamental questions about the stability and potency of monotreme GLP-1. Moreover, the unique expression of pGLP-1 in venom gland suggests pGLP-1 may have gained different biological functions.

Chapter 2

Research Paper

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

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Contribution to the Paper		Performed analysis on all samples, interpreted data, wrote manuscript.	
Signature		Date	20/02/15

Name of Co-Author		Enkhjargal Tsend-Ayush	
Contribution to the Paper		Supervised development of work	
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Name of Co-Author		Mark Myers	
Contribution to the Paper		Supervised development of work, helped in data interpretation and helped to edit the manuscript.	
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Contribution to the Paper		Supervised development of work, helped in data interpretation and helped to edit the manuscript.	
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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).

1X PCR reaction buffer (Promega) and final concentrations of dNTPs (0.1 mM), each primer (0.4 μM) and Taq polymerase (0.012 U/μl). Reactions were carried out on a PTC-200 DNA Engine thermal cycler (MJ Research) using the following parameters: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C (except for primer pairs 9&10 where annealing was done at 45 °C) for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were checked on a 1.5% agarose gel. The identity of all PCR products was confirmed by DNA sequencing.

RNA extraction

Platypus and echidna tissues were obtained from an adult platypus (Animal ethics permits AEEC R.CG.07.03 and AEC S-49-200 to F.G). Total RNA was extracted from snap frozen platypus tissues (brain (frontal cortex), pancreas, liver, lung, intestine (proximal small intestine), stomach, heart and kidney) using TRIzol reagent (Invitrogen, USA) according to the manufacture's instructions. RNA was resuspended in nuclease free water and stored at −80 °C.

cDNA synthesis

cDNA was synthesized from 3 μg RNA with Superscript III Reverse Transcriptase (Invitrogen) following the manufacture's instructions. Briefly, RNAs were treated with DNase I (Roche) to remove genomic DNA, incubated with 50 ng of random hexamers and 0.5 μl of 10 mM dNTPs for 5 min at 65 °C. After incubation, 2 μl of 5X First-strand RT buffer, 0.5 μl of 0.1 M dithiothreitol (DTT), 0.5 μl of RNaseOUT™ (40 U/μl), and 0.5 μl SuperScript III Reverse Transcriptase (200 U/μl) were added and incubated at 25 °C for 10 min, and then 50 °C for 50 min, followed by the final termination at 85 °C for 5 min. Finally, 0.2 μl of RNase H (Biolabs, 5 U/μl) were added to each tube and incubated at 37 °C for 20 min. cDNAs were stored at −20 °C.

RT-PCR

RT-PCR was performed to determine the presence of *GHSR* mRNA in different platypus tissues. Two gene-specific primers (No. 11&12 in Table 1) were designed based on the known cds of platypus *GHSR*. Amplification cycles were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were resolved on 1.5% agarose gels and then visualized with ethidium bromide. The identity of all PCR products were determined by DNA sequencing.

Degenerate RT-PCR

RT-PCR was performed to acquire missing 5′ sequence of platypus *GHSR*. Degenerate forward primer (No. 14 in Table 1) is based on the 5′ sequence of human *GHSR* (ENST00000241256). A gene-specific reverse primer (No. 15 in Table 1) was designed according to known platypus *GHSR* cds (ENSOANG00000016050) (Supplementary Fig. 6A). Amplification cycles were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 64 °C for 30 s (annealing), and 72 °C for 1 min (extension), followed by a final extension at 72 °C for 7 min. PCR products were run on 1.5% agarose gels, stained with ethidium bromide and sequenced.

Database searches

Gene and contig information for multiple alignments physical location and synteny analysis were acquired from Ensembl genome browser <http://www.ensembl.org/>. BLAST searching including Trace Archive Nucleotide BLAST was performed by NCBI BLAST <http://blast.ncbi.nlm.nih.gov/>, UCSC <http://genome.ucsc.edu/> and also transcriptome databases ViroBLAST (Deng et al., 2007) and ISA modules (Brawand et al., 2011). Sequence alignments were conducted using the ClustalW algorithm (Thompson et al., 1994). Synteny analysis was done by searching the UCSC genome browser <http://genome.ucsc.edu/index.html> for the following species: human (*H. sapiens*), opossum (*M. domestica*), chicken (*G. gallus*), anole lizard (*Anolis carolinensis*) and platypus (*O. anatinus*). Phylogenetic trees were built using the MacVector v11.0.4 software package (Cary, NC, USA). The ratio (dS/dN) of synonymous (dS) to non-synonymous (dN) substitutions was estimated by using the Nei–Gojobori method <http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html> (Korber, 2000).

Results

Loss of GHRL and MBOAT4 in the platypus genome

Previous research reported wholesale loss of genes required for gastric function (Ordonez et al., 2008), which prompted us to look at a set of genes central to metabolic control involving both stomach and pancreas.

Interestingly, at first glance the ghrelin gene (*GHRL*), encoding a key hormone expressed in stomach and pancreatic ε cells, seemed to be missing in publicly available platypus genome databases (Ensembl, NCBI and UCSC) (Warren et al., 2008). BLAST search with the full opossum *GHRL* sequences in the platypus genome including trace archives also failed to reveal the existence of *GHRL* in the platypus genome. A general NCBI BLASTN (Altschul et al., 1997) search with opossum *GHRL* cDNA sequences produced matches across vertebrate species, including human (*H. sapiens*), mouse (*M. musculus*), Tasmanian devil (*Sarcophilus harrisii*), wild boar (*Sus scrofa*) and other species with the notable exception of platypus. We also searched the transcriptome databases ViroBLAST (Deng et al., 2007) and ISA modules (Brawand et al., 2011) to search for transcript signatures of a monotreme ghrelin gene but no transcripts were detected.

To exclude poor assembly as a reason for the absence of *GHRL* sequences, we investigated the genomic region surrounding the *GHRL*. Synteny analysis of the region containing *GHRL* showed strong conservation among vertebrate species (Fig. 1A). Analysis of eight genes flanking *GHRL* in human (*IRAK2*, *TATDN2*, *SEC13*, *ATP2B2*, *SLC6A11*, *HRH1*, *VGLL4* and *TAMM41*) revealed that these genes are present in platypus but located on different ultra contigs and chromosomes: *IRAK2* lies on Contig31581, *TATDN2* and *TAMM41* on Ultra529, *SEC13* on Ultra602, *ATP2B2, SLC6A11, HRH1* and *VGLL4* is located on chrX1 (Fig. 1A).

In humans *GHRL* is situated between *TATDN2* and *SEC13*. In platypus these two genes are located on different contigs (Fig. 1A). To address the possibility that this region has undergone rearrangements and that *GHRL* has been translocated elsewhere in the genome, we performed a series of degenerate PCR experiments to identify any *GHRL* related sequences in platypus.

Degenerate primers were designed to bind to the most conserved regions identified by cds alignments of *GHRL* in several vertebrate species (see "Materials and methods", Table 1 and Supplementary Fig. 1A). These primers successfully amplified *GHRL* in chicken, opossum and mouse genomic DNA, but no band was observed in platypus (Fig. 2A and Table 2) or echidna (Supplemen-

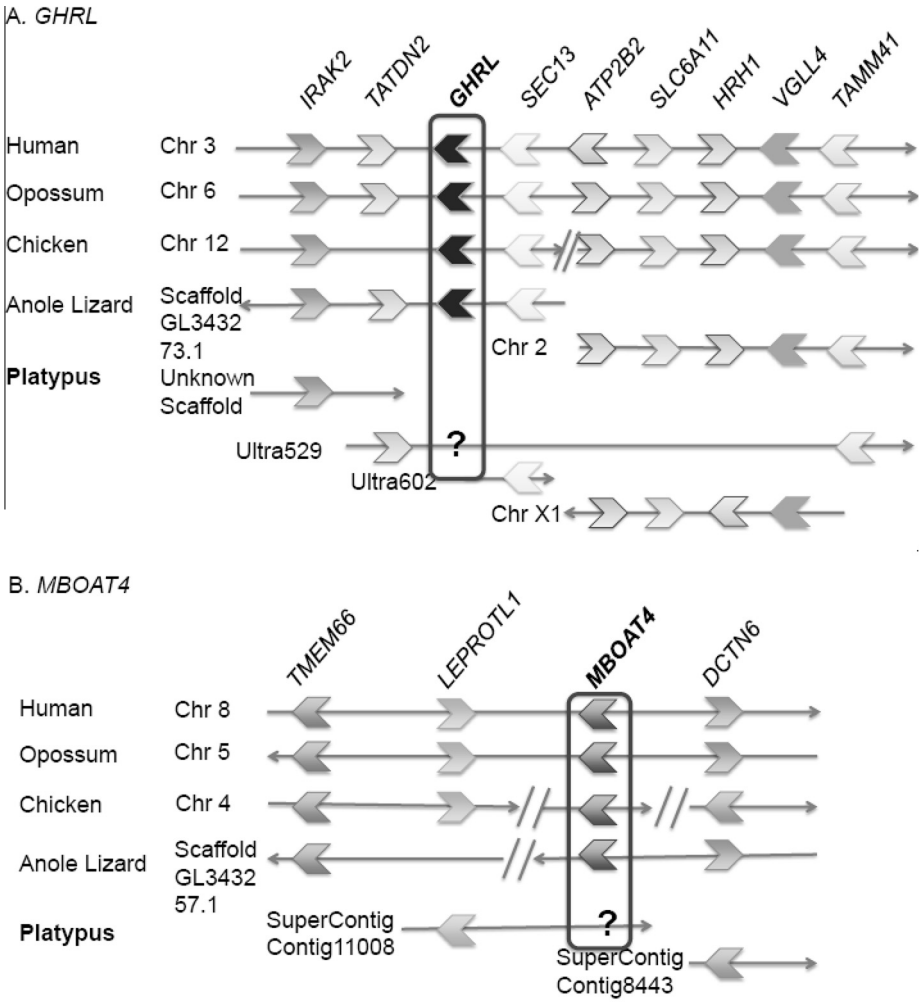

Fig. 1. Synteny analysis of regions containing *GHRL*/*MBOAT4* genes in the platypus genome. Questions marks showing the expected loci of *GHRL*/*MBOAT4*. (A) Schematic map of chromosomal regions containing *GHRL* and flanking genes. The genome sequences are from human (*H. sapiens*, chr3:10,206,563-11,888,352), opossum (*M. domestica*, chr6:242,934,488-245,235,982), chicken (*G. gallus*, chr12:19,418,040-19,444,315 and chr12:3,775,267-4,600,713), anole lizard (*A. carolinensis*, chrUn_GL343273:845,097-936,970 and chr2: 156,896,565-158,270,088) and platypus (*O. anatinus*, Contig31581:1,335-5,319, Ultra529:199,992-241,218, Ultra602:19902-32064 and chrX1:31,069,179-31,851,890). (B) Schematic synteny map of chromosomal region containing *MBOAT4* in vertebrates. The genome sequences are from human (chr8:29,920,631-30,041,155), opossum (chr5:14,151,151-14,249,485), chicken (chr4:48,646,916-48,654,556 and chr4:34,194,466-49,034,980), anole lizard (chrUn_GL343257:1,770,450-1,772,997 and chrUn_GL343257: 754382-776298) and platypus (Contig11008:6,985-10,946 and Contig8443:11,417-18,658).

tary Fig. 2). This suggests that the *GHRL* has been lost or mutated beyond recognition in monotremes.

The only known function of GOAT is to catalyze the *O*-n-octanoylation of ghrelin, which led us to speculate that the loss of functional ghrelin removed the need for GOAT activity and, therefore, rendered the gene encoding GOAT (*MBOAT4*) obsolete. Hence, we investigated the presence of *MBOAT4* in the platypus genome following the same strategy as used for *GHRL*.

Again searches, as described above, did not reveal any sequence in the platypus genome that matched *MBOAT4* in other species. A general NCBI BLASTN (Altschul et al., 1997) search with opossum *MBOAT4* cDNA sequences produced matches across vertebrate species, including human (*H. sapiens*), mouse (*M. musculus*), Tasmanian devil (*S. harrisii*), wild boar (*S. scrofa*) and other species but not platypus.

We next investigated the region surrounding the *MBOAT4* in other mammals. Synteny analysis of the region harboring *MBOAT4* showed conservation among vertebrate species (Fig. 1B). Analysis of three genes flanking *MBOAT4* in human, namely, *TMEM66*, *LEPROTL1* and *DCTN6* showed that these genes are located on two different super contigs in the platypus genome database (Fig. 1B).

We then used degenerate PCRs to identify any *MBOAT4* like sequences in platypus. Degenerate primers were designed based on multiple cds alignments of *MBOAT4* in several vertebrate species (see "Materials and methods", Table 1 and Supplementary Fig. 1). Again, primers amplified from genomic DNA of opossum and mouse but not platypus or echidna (Fig. 2B). This suggests that *MBOAT4* along with *GHRL* has been lost or substantially changed in monotremes.

Identification and characterization of platypus ghrelin receptor GHSR

The *GHSR* has two exons and one intron in eutherian mammals. We identified partial sequence of this gene in the platypus genome database. The platypus genomic and predicted protein coding sequences available in ENSEMBL (Transcript ID: ENSOANG00000016050) and NCBI (Gene ID: 100092085) contains gaps at the 5′ end of the first exon and lacks the entire second exon of platypus *GHSR*. Partial exon1 sequences are present on contig 104977. In order to acquire the 5′ missing sequence of exon 1, degenerate RT-PCR was performed. Primers 14&15 amplified a 300 bp PCR product with platypus brain and kidney cDNA (see

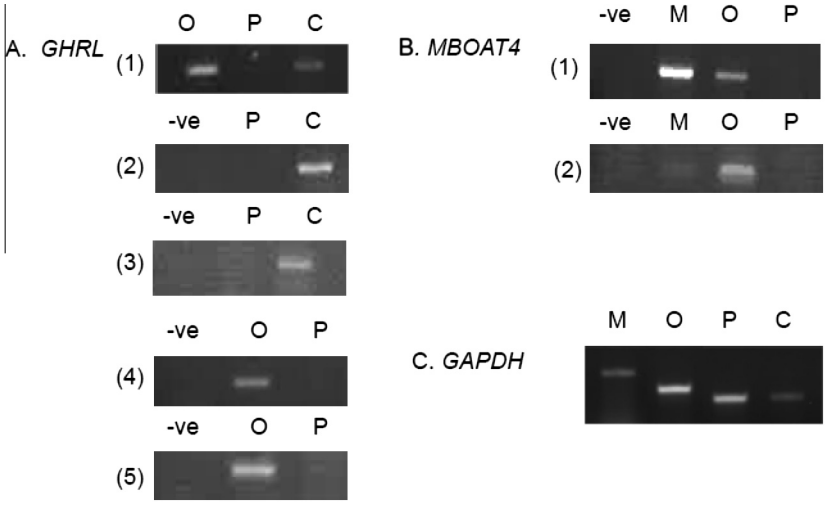

Fig. 2. Degenerate PCR of *GHRL* and *MBOAT4* using platypus (P) genomic DNA fails to amplify a product but bands of expected size are observed in control species including mouse (M), opossum (O) and chicken (C). (A) Degenerate PCR for *GHRL*, (1) Primer set 1&4, band size ~370 bp; (2) Primer set 6&7, band size ~630 bp; (3) Primer set 2&5, band size ~480 bp; (4) Primer set 2&3, band size ~350 bp; (5) Primer set 1&3, band size ~300 bp. (B) Degenerate PCR for *MBOAT4*. Primer set (1) 8&10 and (2) 9&10 generated bands in both mouse and opossum at ~480 bp and ~250 bp respectively, but did not amplify any product in platypus. (C) Positive control with housekeeping gene *GAPDH* (expected band size, M: ~410 bp, O: ~350 bp, P&C: ~300 bp). −ve means negative control.

Table 2
Summary of results from degenerate PCR (genomic DNA) of *GHRL*.

Primer pairs	Species			PCR product size (bp)
	Opossum	Chicken	Platypus	
1&4	Y	Y	N	370
6&7	-	Y	N	630
2&5	-	Y	N	480
2&3	Y	-	N	350
1&3	Y	-	N	300

"Y"means one band was observed in this species; "N" means no band was observed in this species; "-" means no PCR was performed.

"Materials and methods", Table 1 and Supplementary Fig. 6B), which is the size of the equivalent sequence of human *GHSR* cDNA. Sequencing of this PCR product revealed 167 bp of additional platypus *GHSR* cDNA sequence at 5′ end.

BLAST search using human *GHSR* sequences in UCSC identified sequence homologous to exon 2 on the platypus UltraContig 395:458,994-459,299. The same contig also harbors the flanking genes (*FNDC3B* Ultra395:12,310-364,361 and *TNFSF10* Ultra395:425299-444108) (Supplementary Fig. 3). In order to investigate whether the two contigs containing *GHSR* sequence are linked, we performed genomic PCR with primer sets spanning exon 1 and exon 2. This amplified a 4.5 kb product showing that platypus has an intact ghrelin receptor gene (Supplementary Fig. 4). Multiple alignment of platypus *GHSR* with other species shows a high level of sequence conservation and no sign of positive selection (averages of all pairwise comparisons of synonymous (ds) vs nonsynonymous (dn) substitutions: ds = 1.1177, dn = 0.2748, ds/dn = 7.8032, ps/pn = 4.5716 (Korber, 2000)). Next we investigated the expression pattern of this receptor in platypus. RT-PCR revealed strong expression of platypus *GHSR* in brain, intestine and kidney, with weaker expression in the heart and stomach (Fig. 3). This expression pattern is similar to that seen in other mammals, which predominantly express *GHSR* in brain, but also in peripheral organs, including heart, lung, liver, kidney, pancreas, stomach and intestine (Gnanapavan et al., 2002; Guan et al., 1997).

Alignment of the partial amino acid sequences of platypus GHS-R 1a with other amniote species shows that this gene is conserved through evolution including platypus (Fig. 4B). Sequence comparison between human and platypus showed a high level of conservation with 81% amino acid identity. Moreover, residues that are important for ghrelin to bind and exert biological activities are: Cys[116], Gln[120], Glu[124], Glu[187], Cys[198], Trp[276], Phe[279], Arg[283], Phe[286] and Asn[305] in human GHS-R 1a (Holst et al., 2006, 2009; Ueda et al., 2011). These residues are identical in platypus GHS-R 1a (Fig. 4A), implying that although ghrelin and GOAT have been lost in platypus, the *GHSR* remains intact, and it encodes GHS-R 1a with a conserved ghrelin binding site.

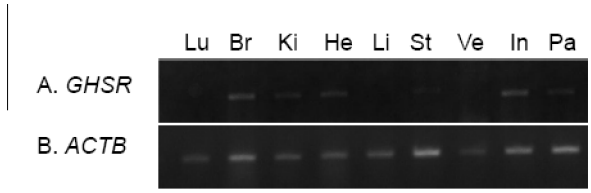

Fig. 3. Expression pattern of platypus *GHSR*. (A) RT-PCR with primer sets 11&12 amplified *GHSR* showing a strong bands in brain, intestine and kidney and faint bands in the pancreas, heart and stomach of expected size at ~600 bp. (B) beta actin positive control. Li: liver; Pa: pancreas; In: intestine; Ki: kidney; He: heart; Br: brain; St: stomach; Lu: lung; Ve: venom.

Alternative splicing of human *GHSR* generates two isoforms: GHS-R 1a is produced by the splicing of exon 1 and exon 2 in the primary transcript, in contrast, GHS-R 1b mRNA is created by the termination of transcription at part of intron 1 (Howard et al., 1996) (Fig. 5A). We tried to investigate whether these isoforms exist in platypus. PCR with primer pairs spanning exon 1 and the 5′ end of intron 1 (Fig. 5B and Supplementary Fig. 5A) produced a 470 bp band in platypus genomic DNA, but not in platypus cDNA from different tissues (Supplementary Fig. 5B). It is therefore likely that the GHS-R 1b splice variant does not exist in platypus.

Discussion

The ghrelin pathway is evolutionarily highly conserved among vertebrate species, but little is known about this pathway in egg-laying mammals (platypus and echidna). Monotremes are a fascinating group of mammals to study the evolution of metabolic

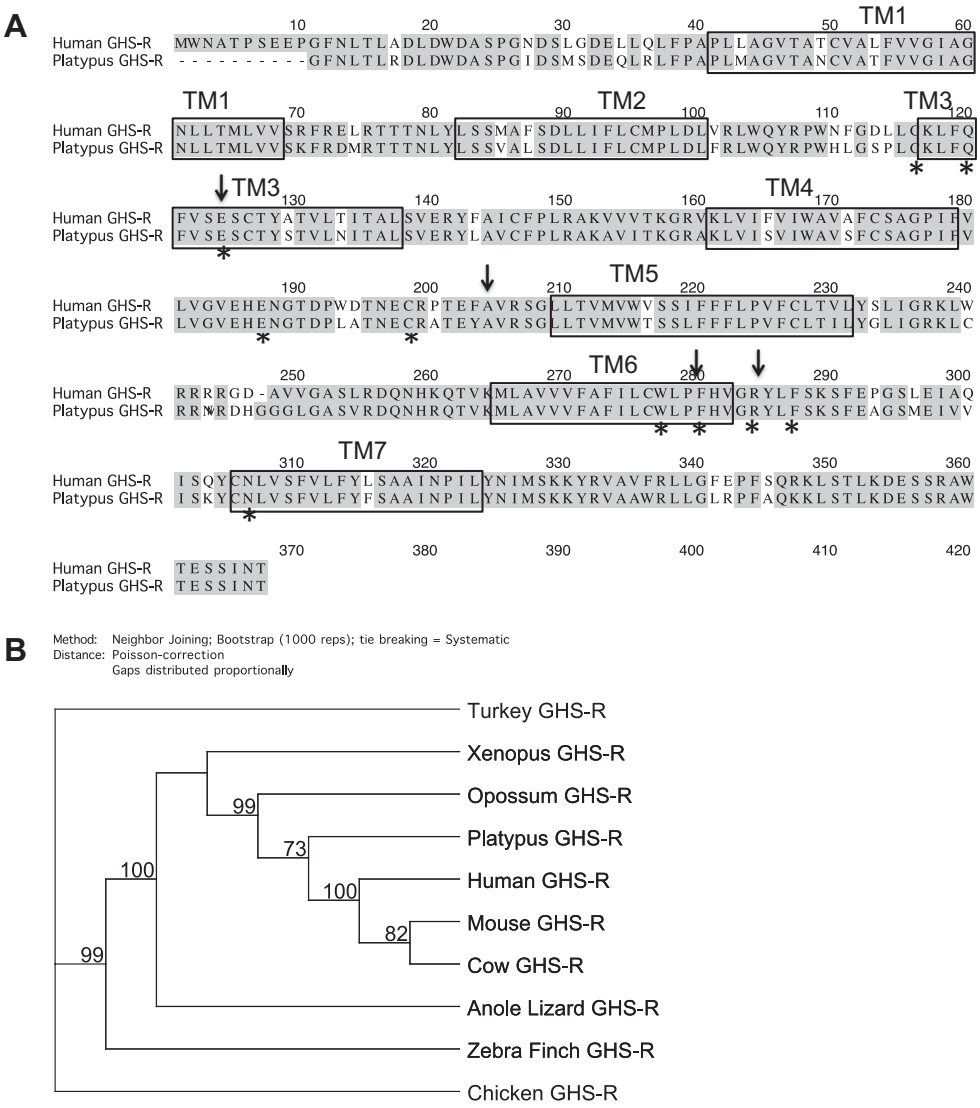

Fig. 4. Comparison of platypus GHS-R 1a. (A) Amino acid alignment of platypus GHS-R 1a with human GHS-R 1a (ENSP00000241256). Boxes highlight the 7 transmembrane domains (TM1-TM7). Asterisks indicate residues vital to ligand binding: Cys^{116}, Gln^{120}, Glu^{124}, Glu^{187}, Cys^{198}, Trp^{276}, Phe^{279}, Arg^{283}, Phe^{286} and Asn^{305}. Arrows indicate residues significant for the constitutive activities of GHS-R 1a: Glu^{124}, Ala^{204}, Phe^{279} and Arg^{283}. These important residues are conserved in platypus GHS-R 1a. (B) Evolutionary comparison of platypus GHS-R 1a with orthologs in other vertebrate species. The phylogenetic tree was constructed based on multiple GHS-R 1a amino acid sequence alignments of xenopus (ENSXETP00000050945), human (ENSP00000241256), opossum (ENSMODP00000026651), mouse (ENSMUSP00000061153), cow (ENSBTAP00000014446), anole lizard (ENSACAP00000016116), zebra finch (ENSMGAP00000010379), chicken (ENSGALP00000014948) and turkey (ENSTGUP00000011153) using the ClustalW algorithm (Thompson et al., 1994). The neighbor-joining algorithm with bootstrap analysis with 1000 replicates was conducted using the MacVector v11.0.4 software package.

control as they have a dramatically different gastric system compared to other mammalian species and have lost many digestion-related genes (Ordonez et al., 2008). Here we investigated three genes, *GHRL*, *MBOAT4* and *GHSR* of the brain-gut axis ghrelin pathway in monotremes.

Surprisingly our experiments suggest that in monotremes genes encoding *GHRL* and *MBOAT4* have been lost or substantially mutated whereas *GHSR* is present, conserved and expressed as in other mammals.

Ghrelin acylation appears to be catalyzed exclusively by the enzyme GOAT and no other substrates of GOAT are known (Yang et al., 2008b; Ohgusu et al., 2009). In this context the attrition of the *MBOAT4* may have been the result of the loss of ghrelin.

These results raise the possibility that the lineage specific loss of ghrelin and GOAT in monotremes may be related to the loss of gastric function or has been replaced by redundant ligands acting through the ghrelin receptor.

Is the loss of ghrelin related to the monotreme specific changes in the gastrointestinal tract?

Ghrelin is mainly released by the P/D_1 cell in humans and X/A-like cell in rodents in the oxyntic gland of the gastric mucosa (Date et al., 2000). The platypus stomach is anatomically and physiologically different from other mammalian species. Many genes involved in digestion and stomach function have been lost or are inactivated (Ordonez et al., 2008). The loss of ghrelin raises questions about the existence of the ghrelin producing X/A-like cells in platypus. The platypus stomach is generally described as glandless, with exception of Brunner's glands that have identified in the submucosa of the distal part of the stomach (Griffiths, 1978; Krause, 1971).

Another gene that is typically expressed in X/A-like cells, *NUCB2* encoding nesfatin-1 (Stengel et al., 2009), is present in the platypus genome. Whether X/A-like cells exist or if *NUCB2* may function in other cell types in the platypus is unclear at present.

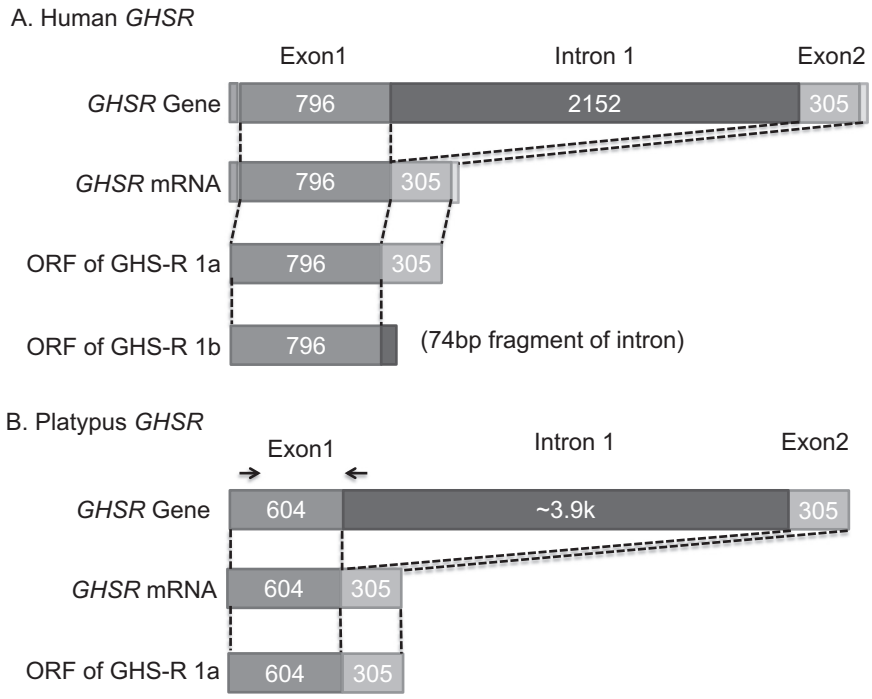

Fig. 5. Schematic representation of *GHSR* open reading frame (ORF) in human and platypus. (A) GHS-R 1a is produced by the splicing of exon 1 and exon 2 in the primary transcript. In contrast, GHS-R 1b mRNA is created by the termination of transcription within intron 1; (B) Only GHS-R 1a but not 1b exists in platypus. Arrows indicate the position of primer pair 11&13 (sequences refer to Table 1). Numbers indicate the length of different exons/introns, for example 796 means the exon 1 of human *GHSR* is 796 bp in length.

Ghrelin has also been described as a marker gene for the newly discovered ε cells in the human pancreas (Wierup et al., 2004), but there is some controversy over whether ghrelin is exclusively expressed in ε cells in the human pancreas as its expression has also been reported in human and rat pancreatic α and β cells (Date et al., 2002; Volante et al., 2002). In contrast to the stomach, the monotreme pancreas anatomy is similar to other mammals. Previous histological work (Yamada et al., 1990; Tsend-Ayush et al. unpublished observation) has identified endocrine islets of Langerhans containing α, β, δ and PP cells in echidna and platypus, but it is unknown if pancreatic ε cells exist in monotremes.

The loss of ghrelin raises questions about metabolic control in monotremes

The effects of ghrelin deletion and overexpression have been investigated in a number of vertebrates but this is the first time the evolutionary loss of this gene has been reported. Ghrelin was initially discovered as a GH releasing and appetite regulating hormone, however the effects of ghrelin vary among species. Ghrelin enhances GH release in most mammals, birds, frog and fish (reviewed by Kaiya et al., 2013). However, the effects after loss of ghrelin on development differ between species. For example, while zebrafish lacking ghrelin show impaired growth (Li et al., 2009), *Ghrl* KO mice have a normal growth rate, body composition and size (De Smet et al., 2006; Sun et al., 2003, 2004, 2008). The effects of ghrelin on appetite regulation vary as well. In humans and rat the presence of ghrelin stimulates appetite, whereas there is no effect on other mammals (for example, mice and pigs) and in chicken feeding is inhibited (reviewed by Kaiya et al., 2013). In mice the lack of ghrelin leads to metabolic changes including an enhanced insulin response and faster clearance rate of glucose when fed on a high fat diet (Dezaki et al., 2006). Also, these mice tend to have a lower body fat mass and reduced respiratory quotient when fed on a high-fat diet, indicating increased fat oxidation (Wortley et al., 2004). At this stage we can only speculate as to the effect of lack of ghrelin on metabolism on monotreme metabolism but it is possible that the communication mechanism between hunger signaling and insulin responsiveness through ghrelin may be have been lost as a consequence of the loss of peptic digestion.

Is there another ligand(s) acting via GHS-R 1a in monotremes?

In contrast to the loss of *GHRL* and *MBOAT4* we did identify the platypus ortholog *GHSR*. This raises the possibility of ghrelin independent signaling through this receptor in monotremes. The human GHS-R 1a appears to have a high degree of ghrelin-independent constitutive signaling activity (around 50% of its maximum activity) (Holst et al., 2003). Early studies have suggested an endogenous inverse agonist that regulates the receptor by decreasing the constitutive activity (Holst et al., 2003). GHS-R 1a residues involved in constitutive activation: Glu^{124}, Ala^{204}, Phe^{279} and Arg^{281} are conserved in platypus, indicating that the platypus ghrelin receptor may also exhibit a degree of constitutive activity.

Ghrelin, GOAT and GHS-R 1a knockout mice have revealed numerous roles for this signaling system (reviewed by Kang et al., 2011; Albarran-Zeckler et al., 2011) including neuroprotection, learning and memory, modulation of dopamine signaling, and thymopoeisis. Presumably a loss of such wide-ranging activities would be detrimental to monotremes, which raises the possibility that other ligands may be acting via GHS-R 1a in monotremes. The existence of other ligands is also directly indicated by several studies (Furness et al., 2011; Holst et al., 2003; Pfluger et al., 2008). In eutheria, although GHS-R 1a is present and has conserved functions in the CNS, there is no ghrelin in the spinal cord or most other parts of the CNS, and ghrelin released from stomach cannot cross the blood–brain barrier to enter the spinal cord. Therefore, similar to the situation in monotremes, the conserved function and location of spinal cord GHS-R 1a, in

the absence of ghrelin, suggests the presence of another yet unknown ligand in eutheria (Furness et al., 2011; Sakata et al., 2009a). The discovery of ghrelin, was through the construction of a cell line stably expressing GHS-R 1a to monitor the intercellular Ca^{2+} level that were induced by rat tissue extracts. The highest activity was observed in the stomach extracts containing ghrelin (Kojima et al., 1999), leaving the possibility that other ligands with lower activity may have been overlooked.

Inconsistent and unexpectedly mild phenotypes of ghrelin pathway gene knockouts in animal models (De Smet et al., 2006; Sun et al., 2003, 2004, 2008; Kaiya et al., 2013) further supports redundancy in this pathway. Interestingly, simultaneous KO of both *Ghrl* and its receptor enhanced the phenotype of single gene-deficient mice with decreased body weight and fat, increased energy expenditure and locomotor activity on a standard diet.

In conclusion, we report data suggesting for the first time the loss or attrition of *GHRL* and *MBOAT4* in monotremes. It is currently unknown what the consequences are in terms of growth, appetite, metabolic control and glucose regulation in monotremes. The presence of the ghrelin receptor in monotremes and mild and variable phenotype in animal models may indicate that the ghrelin receptor exerts its function independently of ghrelin or possibly via activation by another ligand. Monotremes are a unique system to further study the action of the ghrelin receptor in the absence of functional ghrelin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.06.003.

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Chapter 3

Research Paper

Immunohistochemical analysis of pancreatic islets of platypus (*Ornithorhynchus anatinus*) and echidna (*Tachyglossus aculeatus ssp*)

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Abstract

Monotremes have undergone remarkable changes to their digestive and metabolic control system, however the monotreme pancreas remains poorly characterised. Previous work in echidna demonstrated the presence of pancreatic islets but no information is available for platypus and the fine structure has not been described for either monotreme. Based on our recent finding that monotremes lack the ghrelin gene, which is expressed in mouse and human pancreatic islets, we investigated the structure of monotreme islets in more detail. Generally, as in birds, the islets of monotremes were smaller but greater in number compared to mouse. β-cells were the most abundant endocrine cell population in platypus islets and were located peripherally, while α-cells were observed both in the interior and periphery of the islets. δ- and PP-cells were mainly found in the islet periphery. Distinct PP-rich (PP-lobe) and PP-poor areas (non PP-lobe) are present in therian mammals and we identified these areas in echidna but not platypus pancreas. Interestingly, in some of the echidna islets, α- and β-cells tended to form two poles within the islets, which to our knowledge is the first time this has been observed in any species. Overall, monotreme pancreata share the feature of consisting of distinct PP-poor and PP-rich islets with other mammals. A higher number of islets and α- or β-cell only islets are shared between monotremes and birds. The islets of monotremes were larger than those of birds but smaller compared to therian mammals. This may indicate a trend of having fewer larger islets comprising several endocrine cell types during mammalian evolution.

Keywords

Monotremes, Platypus, Echidna, Pancreas, Islets of Langerhans

Introduction

The egg-laying monotremes diverged prior to marsupial and eutherian lineages and hence are of great importance for our understanding of mammalian evolution. Monotremes have undergone striking physiological, anatomical and genetic changes in regards to their digestive system. Previous work reported an extraordinarily small stomach, the lack of glands (the only glands present are the Brunner's glands), and a neutral pH (Griffiths, 1978, Krause, 1971). Subsequent work showed wholesale loss or inactivation of genes required for the formation of gastric juice and gastric function such as gastrin and pepsin (Ordonez, et al., 2008). Our previous studies have shown the loss of another key metabolic gene in the monotreme genome, *Ghrl,* that encodes ghrelin, a hormone known to be released from stomach and pancreatic ε-cells in eutherian mammals (He, et al., 2013, Wierup, et al., 2002).

The pancreas is an essential component of both the digestive and endocrine systems. It is divided into two components, the exocrine pancreas that produces digestive enzymes and bicarbonate that are released into the small intestine, and the endocrine pancreatic islets of Langerhans which in humans and mice consist of five main endocrine cell types. These five major hormone releasing cell types are α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), PP-cells (pancreatic-polypeptide) and ε-cells (ghrelin) (Wierup, et al., 2002). Islets play important roles in metabolic control, in particular in regulating blood glucose homeostasis, as well as influencing digestion through effects of endocrine hormones on exocrine pancreas secretions (Henderson, et al., 1981, Youngs, 1972). The hormone secreting islets of Langerhans, evolved hundreds of millions of years ago in early vertebrates and appear as multiple discrete entities within the exocrine pancreas in vertebrates (Bonner-Weir and Weir, 1979). Despite this

conservation of islets, their architecture varies among species. For instance, the thoroughly studied rodent islets have a well-defined core-mantle structure, with β-cells clustered in the central core surrounded by non β-cells in the periphery (Elayat, et al., 1995), whilst human islets have a more scattered distribution of α- and β-cells (Bosco, et al., 2010, Cabrera, et al., 2006).

Very little is known about the anatomical structure and function of the platypus pancreas. Previous work in echidna pancreas found distinctive endocrine and exocrine parts and two different categories of islets, the β-islets composing mostly β-cells with α-, δ- and PP-cells fewer in number, and PP-islets, containing predominantly PP-cells with few or no other endocrine cell types present (Yamada, et al., 1990). Several studies revealed the relation between PP levels and diseases. For instance, plasma PP levels were elevated in diabetic patients (Floyd, et al., 1976) as well as patients with pancreatic endocrine tumors (Larsson, et al., 1976). A recent study also showed a diet-induced β-cell function improvement in Type 2 Diabetes patients was associated with decreased PP release (Kahleova, et al., 2012).

The aim of this study was to characterise the structure and distribution of pancreatic islets and endocrine cells in monotreme pancreas in more detail. This revealed overall therian like islets in monotremes, with some notable similarities with birds and also monotreme specific characteristics.

Materials and Methods

Sample collection

Platypus and echidna specimens were captured by netting (Animal ethics permits AEEC R.CG.07.03 and AEC S-49-200 to F.G) at the Upper Barnard River (New South Wales, Australia) during breeding season. Specimens were euthanized with an intraperitoneal injection of pentobarbitone sodium (Nembutal) or pentobarbital (Lethabarb) at a dose of 0.1 mg/g. Tissue samples including pancreatic tissue were snap frozen or fixed in formalin and processed as describe below.

Antibodies

Sequence comparison between platypus and human glucagon, insulin and somatostatin has been done to compare the conservation of antibody epitopes across species (Table 1, Electronic Supplementary Material, Fig. S4). Amino acid sequence of platypus PP is not available in the genome database (http://asia.ensembl.org/Ornithorhynchus_anatinus/Info/Index). All the primary antibodies utilized in the present study are listed in Table 1.

Immunohistochemistry

Pancreas tissue was fixed in formalin and processed in butanol before embedding in low melting paraffin. All pancreas samples were sectioned at 5 μm, and 10 consecutive sections were obtained with the first and last section stained with haemotoxylin and eosin (H&E) following standard methods.

Before immunofluorescence, sections were deparaffinized and dehydrated with ethanol. Antigen retrieval process was done by 20 minutes treatment in 37 °C with proteinase K (1:1000, Roche, cat. no. 03115887001) for glucagon and insulin staining or incubation

in sodium citrate (0.01M, pH 6.0) buffer for 20 minutes for somatostatin and PP staining. 10% normal horse serum in antibody diluent [NaCl, NaH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, 10% NaN_3 in distilled water (pH 7.1)] was used as blocking agent. Primary antibodies (Table 1) were diluted in 10% normal horse serum. Antigens were visualized using appropriate secondary antibodies, Alexa 488-conjugated goat anti-guinea pig (1:100, Invitrogen, cat. no. A11073), Alexa 568-conjugated goat anti-rabbit (1:100, Invitrogen, cat. no. A11011) and Alexa 488-conjugated donkey anti-goat (1:100, Invitrogen, cat. no. A11055). Primary antibodies were incubated on tissue sections overnight at 4 °C followed by 4 × 5 minute 1× PBS washes. Secondary antibodies were incubated at room temperature for 2 hours with 4 × 5 minute 1× PBS washes before mounting with ProLong® Gold antifade reagent with DAPI (Life technologies).

Image acquisition and quantification

For imaging, a Zeiss (Jena, Germany) AxioImager Z1 microscope was used equipped with a ×10 ocular and ×20 and ×63 objective lenses. Fluorescent tags were visualised using three filters for DAPI (blue), green (Alexa 568) and red (Alexa 488). Images were taken with an Axiocam charge-coupled device camera and image analysis was performed using Zeiss Axiovision software.

Statistical Analysis

Data are expressed as mean values with standard deviation (SD). Statistical analyses were performed using paired Student's *t* test. Differences were considered to be significant at $P<0.05$

Results

Histology of monotreme pancreatic tissue

Anatomically the pancreas of the platypus appeared diffuse and was difficult to discriminate from surrounding connective and adipose tissue, while in the echidna the pancreas was a more discrete organ readily distinguished from surrounding tissue (observed by F. Grützner and A. Casey during dissection). The overall pancreatic histology and cytology of platypus (Fig. 1 c, d) and echidna (Fig. 1 e, f) was similar to mouse (Fig. 1 a, b) and other mammals with well-structured exocrine acini, ducts, blood vessels and endocrine islets of Langerhans. H&E staining of monotreme pancreas shows numerous lobules of acinar glands consisting of basophilic cells with apical granules. The lumen of the acinus is the origin of the secretory duct and contains centroacinar cells, which are pale staining and smaller in size than the acinar cells (Fig. 1 b, d, f).

The eosinophilic endocrine islets of Langerhans were scattered throughout the exocrine tissue. The size of islets varied considerably, from ten or fewer cells to over 100 cells per islet. Generally, platypus (mean diameter 46 μm) and echidna islets (mean diameter 41 μm) were similar in size, but were generally smaller than mouse (mean diameter 88 μm) (Fig. 2) or human islets (mean diameter 140 μm) (Hellman and Hellerström, 1969, Kim, et al., 2009, Takei, et al., 1994). Also, there appeared to be a lot of endocrine cells scattered either singly or in small groups of 2-3 through the exocrine region in the pancreas of both platypus and echidna. The shape of most islets in both species was round or oval, but some showed a more irregular shape (Electronic Supplementary Material, Fig. S5 a). No distinct pattern of distribution was identified in terms of accumulation of islets in certain areas. Most islets were surrounded by exocrine tissue,

but some were located within connective tissue next to blood vessels, ducts or between fat cells. The outlines of small islets are not always clearly delineated, whereas large islets tended to have more clear boundaries (Electronic Supplementary Material, Fig. S5 b, c).

Immunolocalization of endocrine hormones

The mouse islets of Langerhans had a well-defined core-mantle structure, with β-cells accounting for 72.8% (Table 2) of all endocrine cells, clustering in the central core surrounded by non β-cells, mainly α-cells (12.4%), but also δ- cells (10.8%) and PP-cells (4.0%) in the periphery (Fig. 3 a-d, Fig. 4 a-c, Table 2).

Glucagon and insulin immunoreactive cells were identified in platypus pancreas (Fig. 3 e-h, Fig. 4 d). In most islets, β-cells were the predominant cell type (64.4%) and were distributed peripherally, whereas α-cells were located both in the centre and periphery of the islets (Table 2). Somatostatin and PP immunoreactive cells were fewer in number, and were observed in the islet periphery. In some islets, PP-cells were also found in the core (Fig. 4 f).

The pancreatic tissue of echidna could be grouped into two categories based on the predominant cell type in the endocrine islets: the PP-lobe and the non PP-lobe. In the PP-lobe, PP-cells predominate (55.1%) and were located in the core (Fig. 4 i). Insulin (Fig. 4 g) and somatostatin (Fig. 4 b) positive cells were also observed but mainly in the periphery. α-cells were rare in the PP-lobe. In contrast, in the non PP-lobe, while some islets consisted predominantly of α-cells, others were predominantly made up of β-cells (Fig. 3 i-l). Interestingly, 17.6% of the islets (Fig. 3 l, Electronic Supplementary

Material, Fig. S2) had a unique architecture where α- and β-cells clustered at opposite ends of the one islet. The overall proportion between α- and β-cells across the whole non PP-lobe was around 1:1 (36.3 : 37.7%, Table 2). δ- and PP-cells were identified in fewer numbers than α- and β-cells in the periphery, accounting for 14.6% and 11.4% of total islet cells, respectively.

In the exocrine region of both platypus and echidna pancreas, immunoreactive cells of all four types were observed to be scattered either singly or in small groups of 2-3 cells throughout.

Discussion

The structure and histology of exocrine pancreas is overall conserved between mammals and birds (Mobini, 2013, Motta, et al., 1997). We demonstrated that conservation of the general anatomy of the exocrine pancreas is also maintained in monotremes, in spite of the large-scale physiological, anatomical and genomic changes in their digestive system. In contrast, endocrine pancreas cytoarchitecture can be quite different between species. This may reflect evolutionary adaptations to different diets or other environmental conditions (Cabrera, et al., 2006). Consistent with their phylogenetic position, the histology of monotreme endocrine pancreas showed greater resemblance to that of birds and marsupials than to eutherian mammals (reviewed by Heller, 2010, Steiner, et al., 2010).

The overall anatomy of the platypus pancreas appeared more diffuse in platypus compared to the distinct pancreas of echidna (Grützner and Casey personal communication). Such differences are also observed in therian mammals: rodents and lagomorphs also feature a diffuse pancreas (Dimitrov, et al., 2013), while they are more defined in carnivores (Griffith, 1989). How this might relate to dietary or feeding behavior is currently unclear.

In terms of islet anatomy we found that generally, the monotreme islets are smaller than those of other mammalian species (Fig. 2), but larger than those of birds (Kim, et al., 2009, Steiner, et al., 2010). Overall this indicates a trend towards fewer islets with enlarged size during mammalian evolution.

Immunolocalization of glucagon, insulin, somatostatin and PP in the pancreas identified most main cell types within the islets. The arrangement of mouse pancreatic islets described here is consistent with numerous other studies of rodent islets, with a well-defined core-mantle structure of central β-cells surrounded by non β-cells fewer in number in the periphery (Heller, 2010, Steiner, et al., 2010). Unlike this characteristic distribution of rodent islet cells, platypus pancreas had an arrangement where β-cells mainly located in the periphery, an arrangement previously observed in horse (Furuoka, et al., 1989, Helmstaedter, et al., 1976). Also the ratio between β- and α-cells was lower than that of mouse (~3:1 in platypus compared to ~6:1 in mouse, Table 2).

The existence of a PP-cell rich region is well established in the pancreas of several therian mammals (Edwin, 1979, Edwin, 1987, Orci, et al., 1978, Reddy, et al., 1986, Wang, et al., 2013). Interestingly, we did not see such an arrangement of PP-cells into a PP-rich lobe in platypus. This may be a result of the diffuse structure of the platypus pancreas, which made dissection and identification of distinct head, body or tail regions difficult. In contrast, a greater proportion of PP-cells has been reported in the head region of echidna pancreas (Edwin, 1987) and our work confirmed the existence of distinct PP- and non PP-lobes in echidna.

In the non PP-lobe of echidna pancreas, islets were either exclusively α-cells or β-cells or a mixture of both α- and β-cells (Fig. 3 l). This arrangement of α- and β-cells is different from those of mouse and human, where most islets contain both α- and β-cells, except for those located in the PP-rich head regions (Steiner et al., 2010, Wang et al., 2013). Islets that consist of exclusively α-cells or β-cells are also seen in several marsupial species and birds (Hazelwood, 1973, Reddy, et al., 1986, White and Harrop,

1975). In birds, pancreatic islets are divided into three different categories: (1) islets consisting of α-cells in the core and several δ-cells in periphery and a few β-cells; (2) islets containing mainly β-cells, surrounded by several δ-cells and a few α-cells and (3) mixed islets consisting of α-, β- and δ-cells (do Prado, et al., 1989, Hazelwood, 1973, Lucini, et al., 1995, Watanabe, et al., 1975). We observed all these three type of islets in echidna. However, we discovered that some of the mixed islets in echidna had a distinctive polarity where α- and β-cells were juxtaposed to form two distinct hemispheres (Fig. 3 l, Electronic Supplementary Material, Fig. S2). This has not been reported in other species and maybe a fourth, possibly monotreme specific category of islets. It is currently unclear what these different islet types may mean for the functioning of the islets. However, several studies suggested that in humans, heterologous contact between α- and β-cells favors insulin release stimulated by glucagon (Huypens, et al., 2000, Wojtusciszyn, et al., 2008). On the other hand, (Bosco, et al., 1989) showed that homologous interaction between β-cells improves insulin secretion in mouse. Whether the arrangement of different cell types in the monotreme islets has a functional significance relating to insulin secretion remains unknown. Moreover, studies of human and mouse pancreas suggested that there is considerable plasticity in the structure of islets influenced by physiological and pathophysiological conditions, such as pregnancy, fat mass or diabetes (Cabrera, et al., 2006, Steiner, et al., 2010). Hence, the differences in general pancreas anatomy as well as the arrangement of islet cells between platypus and echidna may be related to their different metabolic requirements and dietary habits. Platypuses are highly active aquatic animals which feed mainly on invertebrates (Burrell, 1927), whereas echidna are known to have a low metabolic rate and a diet of mainly ants and termites (Augee, et al., 2006).

In summary, the pancreata of monotremes contained both endocrine and exocrine structures and share a similar overall structure with other mammals. However, the endocrine islets of monotremes were smaller compared to therian mammals and larger than those of birds, indicating a trend towards the reduction of numbers whilst enlargement of islets size during mammalian evolution. The cytoarchitecture of the islets in platypus was more similar to those of eutherian species, whilst echidna showed more resemblance to birds and marsupials. We confirmed the existence of PP-lobe and non PP-lobe in echidna. In the echidna non PP-lobe, which is similar to that in birds, there are three different islet types: islets containing exclusively α-cells or β-cells or a mixture of both α- and β-cells. Interestingly, the arrangement of α- and β-cells in some of the mixed islets, where α- and β-cells formed two poles of one islet is unique and to our knowledge has not been observed in other species. Overall, this detailed histological analysis shows that the monotreme pancreas is largely mammal-like with some similarity to birds. Importantly the conservation of the islet structure contrasts the dramatic changes observed in other parts of the gastrointestinal system in monotremes.

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Table 1 Monoclonal (mAb) and polyclonal (pAb) antibodies used in the present study

Antibodies	Host	Epitope	Source	Optimal dilution	Cat. no.
Anti-glucagon mAb	Rabbit	N-terminal (HSQGTFTSDYSKYLDSR)	Abcam, Cambridge, UK	1:1000	ab92517
Anti-insulin pAb	Guinea pig	Unknown	Dako, Carpitneria, CA	1:50	A0564
Anti-somatostatin-14 pAb	Rabbit	C-terminal (AGCKNFFWKTFTSC)	Abcam, Cambridge, UK	1:100	ab64053
Anti-PP pAb	Goat	C-terminal (TRPRYGKRHKEDT)	Sigma-Aldrich	1:25	SAB2500747

Table 2 Cellular compositions of the islets of Langerhans in mouse, platypus, echidna, possum and chicken.

Species		No. of animals	No. of islets	α-cells	β-cells	δ-cells	PP-cells	Reference
Mouse		3	53	Periphery 12.4%	Core 72.8%	Periphery 10.8%	Periphery 4.0%	
Platypus		3	135	Core / Periphery 21.6%	Periphery 64.4%	Periphery 10.6%	Core / Periphery 3.4%	
Echidna	PP-lobe	2	74	Periphery 1.1%	Periphery 24.8%	Periphery 19.0%	Core 55.1%	
	Non PP-lobe	2	90	Core 36.3%	Core 37.7%	Periphery 14.6%	Periphery 11.4%	
Possum				Core / Periphery highest % (up to 70%)	Core / Periphery	Core (few)/ Periphery	Periphery / Scattered	(Reddy, et al., 1986, White and Harrop, 1975)
Chicken	A-islets			Core	Few	Periphery 22%	Periphery 11%	(do Prado, et al., 1989)
	B-islets			Few	Core			
	Mixed islets			Periphery	Core			

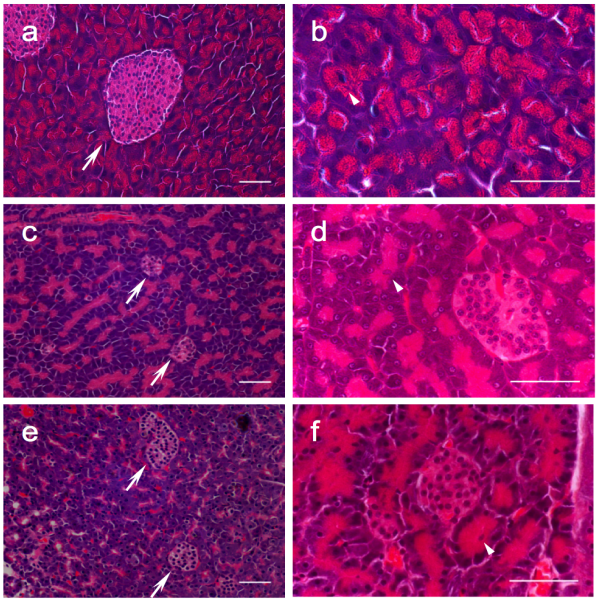
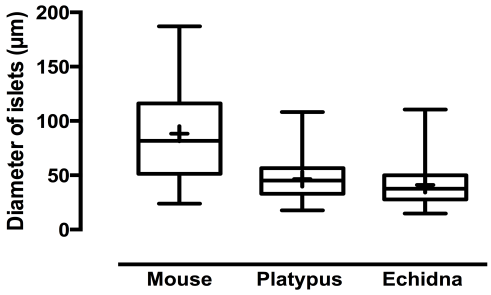


Fig. 1 Histology of pancreatic tissue from mice, platypus and echidna. H&E (haematoxylin and eosin) stained sections of mouse pancreas (a, b), platypus pancreas (c, d), and echidna pancreas (e, f). Higher magnification images of b, d and f can be found in Electronic Supplementary Material, Fig. S1. Arrows point to the islets of Langerhans in a, c, and e. Arrow heads show the centroacninar cells of the exocrine acinus in b, d and f. The apex of acinar cells is filled with eosinophilic zymogen granules. Scale bar, 50µm.

a



b

Species	No. of animals	No. of islets	Median diameter (µm)	Average diameter (µm)
Mouse	3	67	82	88 ± 43
Platypus	4	111	45	46 ± 17 *
Echidna	4	115	38	41 ± 18 *

Fig. 2 Size of the islets of Langerhans in mouse, platypus and echidna. Islet size was described as an effective diameter of a circle that depicts an area corresponding to a measured islet area. Means are shown as "+" in the box and whiskers graph (a). Values shown in table (b) are Mean ± SD per animals. Tissue measured is H&E stained. *$P <$ 0.05 compared with mouse

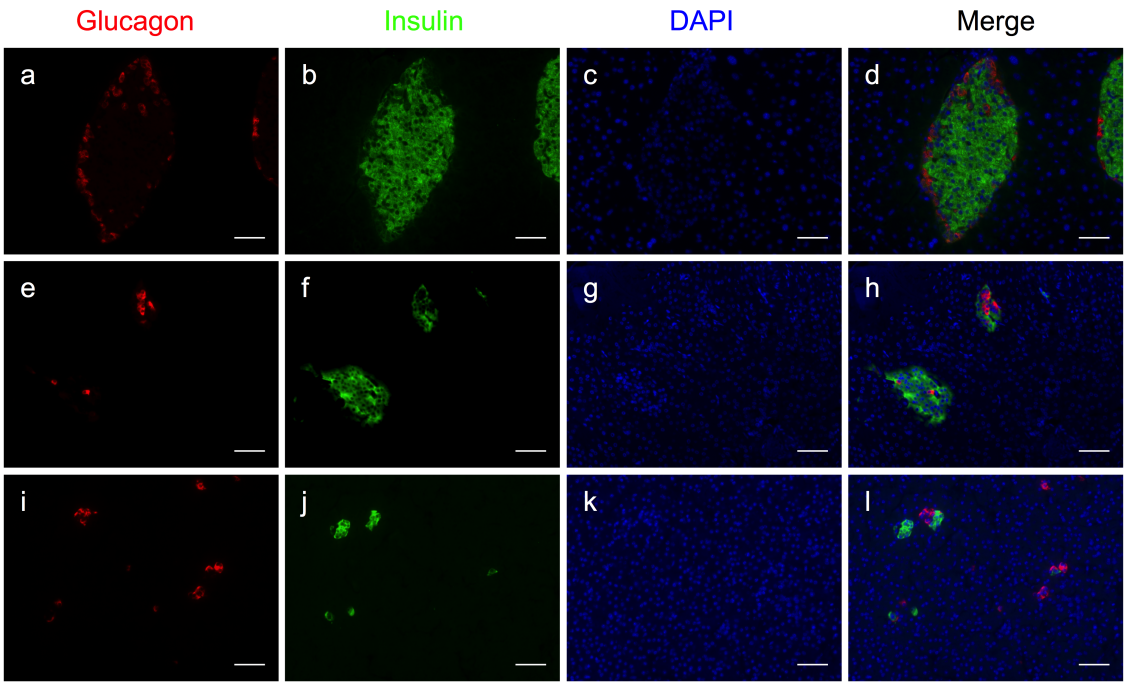


Fig. 3 Immunohistochemical localization of glucagon (red), insulin (green) and nuclei (blue) in the endocrine pancreas of mouse (a-d), platypus (e-h) and echidna (i-l). Scanned with a Zeiss AxioImager 2.1 microscope. Scale bar, 50 µm.

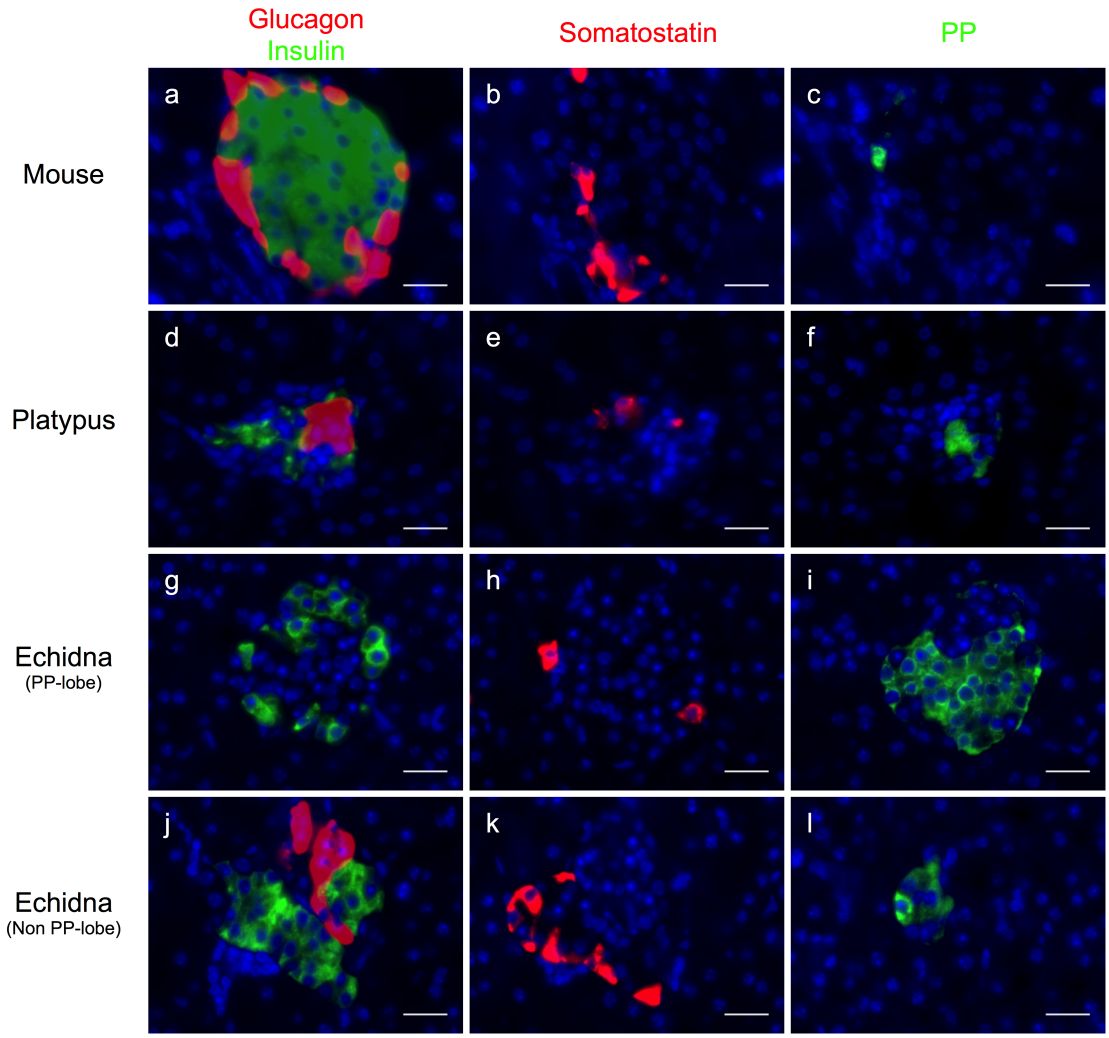


Fig. 4 Immunohistochemical localization of glucagon, insulin, somatostatin and pancreatic polypeptide in the endocrine pancreas of mouse (a-c), platypus (d-f) and echidna (g-l). a-c, Consecutive mouse pancreatic sections incubated with the anti-glucagon (a, red), anti-insulin (a, green), anti-somatostatin (b) and anti-pancreatic polypeptide (c) antibodies. d-f, Consecutive platypus pancreatic sections incubated with the anti-glucagon (d, red), anti-insulin (d, green), anti-somatostatin (e) and anti-pancreatic polypeptide (f) antibodies. g-l, Consecutive echidna pancreatic sections incubated with the anti-glucagon (g, j red), anti-insulin (g, j green), anti-somatostatin (h, k) and anti-pancreatic polypeptide (i, l) antibodies. In all three species, glucagon, insulin, somatostatin and pancreatic polypeptide immunoreactivities were present on different cell populations. Scale bar, 20 µm.

Supplementary materials

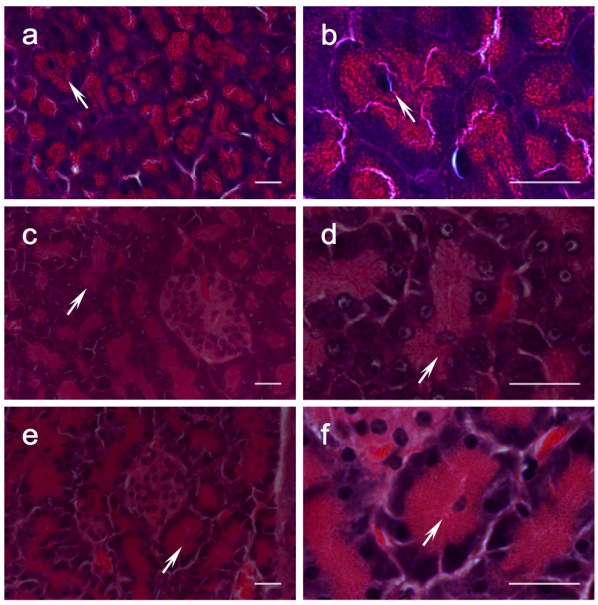


Fig. S1 Histology of pancreatic tissue from mice, platypus and echidna. H&E stained sections of mouse pancreas (a, b), platypus pancreas (c, d), and echidna pancreas (e, f). The images shown in b, d and f are a higher magnification of a region in a, c and e respectively. Centroacninar cells are arrowed. The apical region of acinar cells is filled with eosinophilic zymogen granules. Scale bar, 20µm.

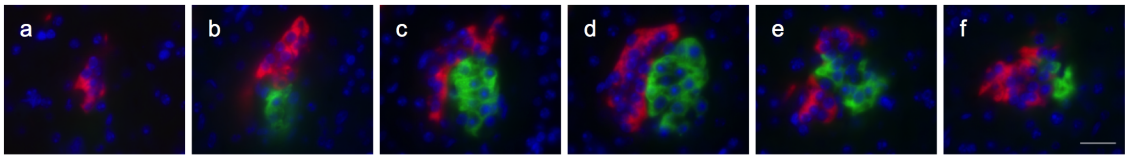


Fig. S2 Immunohistochemical localization of glucagon (red), insulin (green) and nuclei (blue) in the six consecutive echidna endocrine pancreas sections (a-f, 5μm apart), showing the unique cytoarchitecture of α- and β-cells clustering to two opposite hemispheres of the given islet. Scale bar, 20 μm.

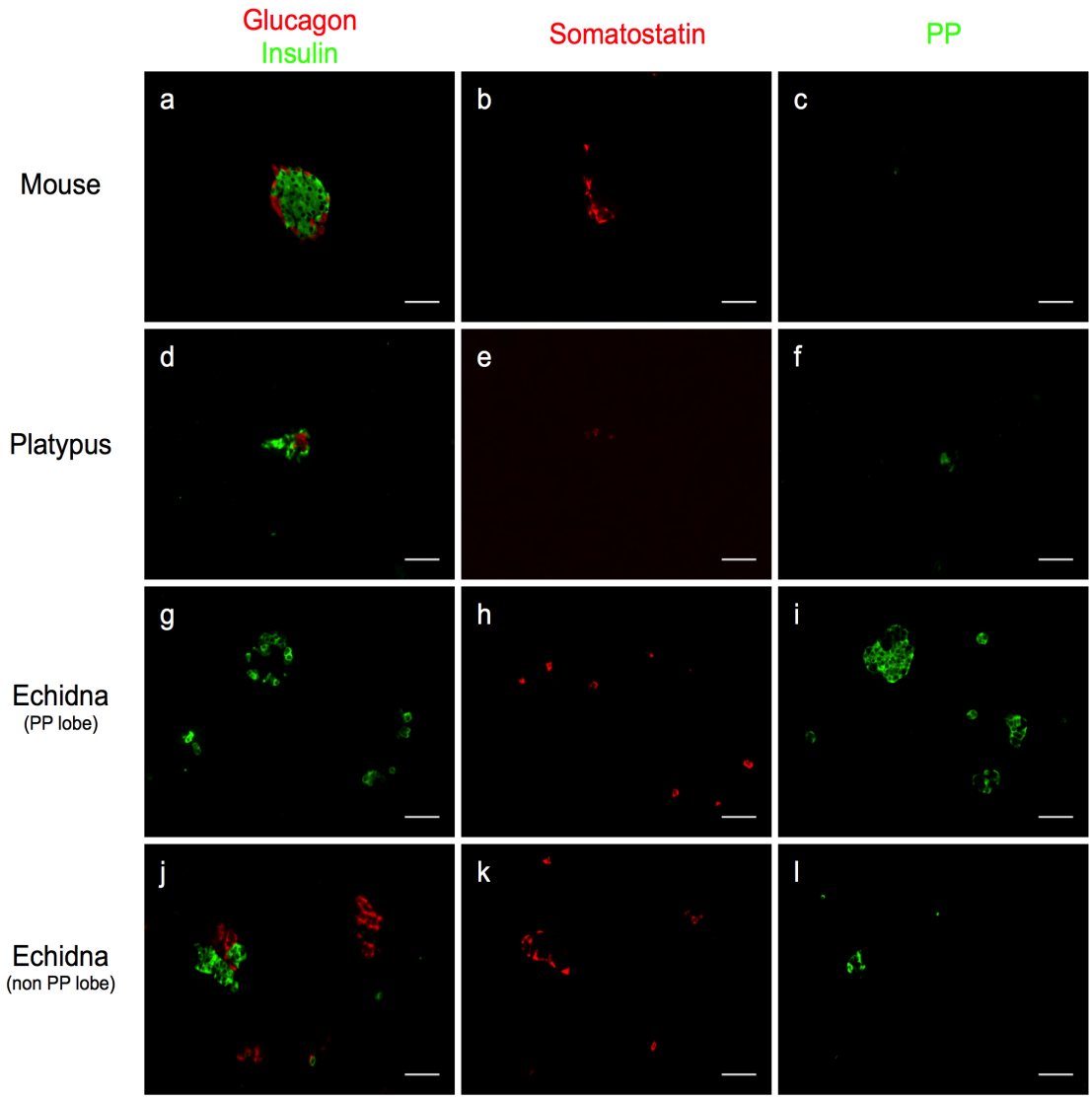


Fig. S3 Immunohistochemical localization of glucagon, insulin, somatostatin and pancreatic polypeptide in the endocrine pancreas of mouse (a-c), platypus (d-f) and echidna (g-l). a-c, Consecutive mouse pancreatic sections incubated with the anti-glucagon (a, red), anti-insulin (a, green), anti-somatostatin (b) and anti-pancreatic polypeptide (c) antibodies. d-f, Consecutive platypus pancreatic sections incubated with the anti-glucagon (d, red), anti-insulin (d, green), anti-somatostatin (e) and anti-pancreatic polypeptide (f) antibodies. g-l, Consecutive echidna pancreatic sections incubated with the anti-glucagon (g, j red), anti-insulin (g, j green), anti-somatostatin (h, k) and anti-pancreatic polypeptide (i, l) antibodies. In all three species, glucagon, insulin, somatostatin and pancreatic polypeptide immunoreactivity is present on different cell populations. Scanned with a Zeiss AxioImager 2.1 microscope. Scale bar, 50 µm.

```
hGlucagon     1 HSQGTFTSDYSKYLDSRRAQDFVQWLMNT 29
pGlucagon     1 HSQGTFTSDYSKHLDAIRAKQFVDWLMNY 29

hInsulin A chain  1 GIVEQCCTSICSLYQLENYCN 21
pInsulin A chain  1 GIVEECCKGVCSMYQLENYCN 21

hInsulin B chain  1 FVNQHLCGSHLVEALYLVCGERGFFYTPKT 30
pInsulin B chain  1 FPNQHLCGSHLVEALYLVCGEKGFYYIPRM 30

hSomatostatin  1 AGCKNFFWKTFTSC 14
pSomatostatin  1 AGCKTFFWKTFTSC 14

hPPY    1 APLEPVYPGDNATPEQMAQYAADLRRYINM 30
       31 LTRPRYGKRHKEDTLAFSEWGSPHAAVPR 59
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Fig. S4 Sequence comparison between human and platypus pancreatic endocrine hormones. Epitope of each antibody has been underlined where known. All sequences were acquired from Ensembl (http://www.ensembl.org/index.html).

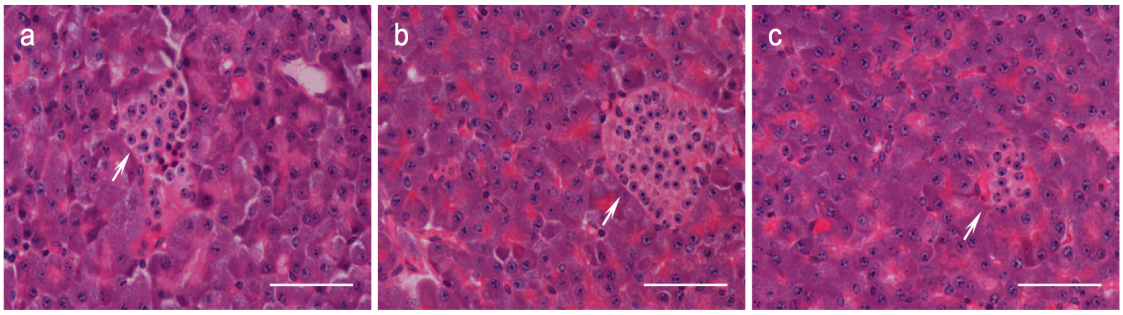

Fig. S5 Histology of pancreatic tissue from echidna. H&E stained sections of echidna pancreas showing islets with irregular shape (a), big islets with clear boundary (b), and small islets without a clear boundary (c). The islets of Langerhans are arrowed. Scale bar, 50 μm.

Chapter 4

Research Paper

In monotremes glucagon-like peptide 1 (GLP-1) is dipeptidyl peptidase 4 (DPP-4) resistant and expressed in venom

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Statement of Authorship

Title of Paper	In monotremes glucagon-like peptide 1 (GLP-1) is dipeptidyl peptidase 4 (DPP-4) resistant and expressed in venom
Publication Status	Publication style
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis

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Contribution to the Paper		Performed experiments, interpreted data, contributed in development of work.	
Signature		Date	25/7/2014

Name of Principal Author (co-first author, candidate)		Chuan He	
Contribution to the Paper		Performed experiments, interpreted data, contributed in development of work, prepared figures for manuscript and helped to write and edit the manuscript.	
Signature		Date	20/02/15

Name of Co-Author		Mark Myers	
Contribution to the Paper		Supervised development of work, helped in data interpretation.	
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Name of Co-Author		Sof Andrikopoulos	
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Contribution to the Paper		Performed insulin release part of experiments helped in data interpretation.	
Signature		Date	16/7/14

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Contribution to the Paper		Provide research materials and facilities for receptor studies and helped in data interpretation.	
Signature		Date	July 16th 2014

Name of Co-Author		Denise Wootten	
Contribution to the Paper		Contributed to experimental supervision for receptor studies and helped in data analysis and interpretation.	
Signature		Date	28.7.14

Name of Co-Author		Briony Forbes	
Contribution to the Paper		Supervised development of work, helped in data interpretation and helped to edit the manuscript. Acted as corresponding author.	
Signature		Date	30-07-2014

Name of Co-Author		Frank Grützner	
Contribution to the Paper		Supervised development of work, helped in data interpretation and wrote manuscript. Acted as corresponding author.	
Signature		Date	

Abstract

Insulin is a key regulator of glucose homeostasis in animals. Glucagon-like peptide 1 (GLP-1), a hormone released after food intake that triggers insulin release from the pancreas, is rapidly degraded by dipeptidyl peptidase-4 (DPP-4)[1-3]. This pathway is targeted by different venomous species and has been crucial for treatment options of type 2 Diabetes Mellitus (T2DM)[3-5]. Platypus and echidna represent the basal mammalian lineage of egg-laying mammals (monotremes) that evolved a sophisticated venom delivery system[6] and a radically different digestive system that lacks key metabolic control genes[7-9]. Here we show that monotreme *Gcg*, *Dpp-4* and *Glp-1r* expression in tissues including gut and venom gland is associated with the evolution of fundamental changes that affect GLP-1 degradation, receptor affinity and signalling bias. Platypus and echidna GLP-1 are resistant to DDP-4 cleavage in human and mouse sera but surprisingly are degraded by a DPP-4 independent mechanism in platypus and echidna. Monotreme GLP-1 peptides bind both the human and platypus GLP-1 receptor with decreased affinities and with a bias towards ERK signalling activation compared to human GLP-1. Both stimulate insulin release in cultured rodent islets. This shows that co-option of genes encoding DPP-4 resistant GLP-1 like peptides into venom occurred independently in monotremes, arthropods and reptiles[4,10]. The dual role of monotreme GLP-1 in venom and pancreas explains the evolution of DPP-4 independent GLP-1 degradation and decreased GLP-1R affinity and activation. This is the first report of a DPP-4 resistant mammalian GLP-1 that provides novel evolutionary insights into the control of insulin release.

Main

Insulin release from pancreatic β-cells is usually initiated by food intake and the resulting influx of glucose, but insulin release and action are compromised in patients with type 2 diabetes. The incretin effect discovered in 1902 by Bayliss *et al.* revealed that ingested glucose leads to enhanced insulin release compared to intravenous glucose administration demonstrating the existence of insulin release factors in the gut[1]. Gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are the only known insulin stimulating incretin hormones released from intestinal cells after food intake[11-14]. Both GIP and GLP-1 peptides are rapidly degraded by the peptidase dipeptidyl peptidase-4 (DPP-4)[15]. During vertebrate evolution the incretin system is highly conserved and *GIP*, *GCG, GLP-1R* and *DPP-4* orthologs have been identified in a range of vertebrate species, (reviewed in[16,17]). The rapid and debilitating effects of low blood glucose is used by venomous species on their prey. Examples of the evolution of venom components affecting glucose homeostasis include a *Glp-1* like gene (exendin-4) expressed in the lizard *Heloderma suspectum* (Gila monster) venom[18] and a fish insulin mimetic in cone snails[19].

Incretin hormones have attracted much interest from pharmaceutical companies with DPP-4 resistant versions of the peptides, and DPP-4 inhibitors becoming key treatment options to improve insulin release in patients with T2DM[3]. One of the key drugs used in restoring glycaemic control in T2DM patients derives from a *Glucagon*-like gene, the aforementioned exendin-4 (Ex-4), which was isolated from venom of the lizard *Heloderma suspectum*[4]. *Exendin* genes share sequence similarity with vasoactive intestinal peptide (*VIP*) and *GCG* genes, which have been identified in a number of non-mammalian vertebrate species. The evolutionary origin of *exendin* genes (*exendin*

1-4) has not been fully resolved but it seems likely that exendins evolved by duplication from a glucagon-like peptide gene precursor[18].

We investigated the incretin hormone system in monotremes because of their phylogenetic position as the most basal lineage amongst living mammals. In addition monotremes feature extraordinary changes in their digestive system, which has led to loss of genes involved in protein degradation and metabolic control[8,9], suggesting unique mechanisms of metabolic homeostasis. Monotremes also possess an elaborate venom delivery system (platypus in particular), which delivers venom during the breeding season, presumably used in competition for mating[20,21].

Gcg, *Glp-1r*, *Gip* and *Dpp-4* sequences were identified in the platypus genome database (Fig. 1a-c and Extended Data Fig. S1). While therian mammal *GCG* genes encode identical GLP-1 protein sequences, there are significant changes in the platypus *Gcg* ortholog. Importantly the sequence encoding the platypus GLP-1 peptide (pGLP-1) has changed in 11 of the 30 amino acids (37%) compared to human GLP-1 (hGLP-1, Fig. 1a). The amino acid changes lie in structurally and functionally significant regions of the protein, including those involved in interactions with the N-terminal domain and core of the GLP-1 receptor (Fig. 1a)[2,22,23]. Notably we also discovered specific changes in the DPP-4 cleavage site in pGLP-1 (Ala^8 to Ser) (Fig 1a) and in the platypus GIP peptide (Extended Data Fig. S2, Ala^2 to Ser). To investigate if this change is also present in the echidna GLP-1 (eGLP-1) we cloned the echidna *Gcg* (*eGcg*) transcript and found a different amino acid at residue eight (Ala^8 to Phe) in the eGLP-1 DPP-4 cleavage site (Fig. 1a), as well as a total of 17 differences from hGLP-1 of the 30 amino acids (57% changed). Remarkably, peptide sequence comparisons revealed a total of 12 differences between both monotreme sequences (i.e. 40% of the sequence), suggesting

not only the divergence from other mammalian *GCG* genes but between both monotreme *Gcg* genes (Fig. 1c).

Expression analysis showed that *Gcg*, *Glp-1r* and *Dpp-4* are expressed in platypus and echidna tissues similar to other mammals (Fig. 1b, Extended Data Fig. S3)[24,25]. Surprisingly, both *Gcg* and *Dpp-4* genes are also expressed in platypus and echidna venom (Fig. 1b, Extended Data Fig. S3). Insulinotropic effects associated with GLP-1-like peptides have been reported in a range of venomous species including arthropods, reptiles[10,26-29], and recently in cone snail venom, which induces severe hypoglycemic shock in its fish prey[19]. In contrast to the evolution of *Glp-1* like genes and insulin mimetics in other species, monotremes are the first example of a species that has recruited the endogenous GLP-1 system into venom that is used during intraspecific conflict. The fact that the venom is used in the same species may have favored the use of the endogenous gene as the most effective venom component. These results raise fundamental questions about stability and potency of monotreme GLP-1.

To test if the specific amino acid changes at the DPP-4 cleavage site of pGlp-1 and eGlp-1 result in resistance to degradation we compared their cleavage to that of hGLP-1 and the DPP-4 resistant Gila monster exendin-4 (Ex-4) (Fig. 2). Incubation of the peptides with purified human DPP-4 resulted in rapid degradation of hGLP-1 (50% reduction of intact peptide within 1 hour) but not Ex-4. Significantly, both echidna and platypus GLP-1 were not degraded by human DPP-4 (Fig. 2a). Next we investigated stability in human serum. In albumin-depleted human serum platypus and echidna GLP-1 and Ex-4 remained stable whereas hGLP-1 was again rapidly degraded (Fig. 2b). Surprisingly degradation of platypus and echidna GLP-1 was observed when incubated in platypus and echidna serum (Fig. 2c,d). Degradation was slower than

hGLP-1 but clearly measurable, with less than 50% uncleaved pGLP-1 and eGLP-1 remaining after 11 hours of incubation (Table 1). Interestingly, Ex-4 was cleaved slowly in echidna serum but remained intact in platypus and human sera.

DPP-4 is not the only enzyme that can degrade GLP-1. Human neural endopeptidase (NEP24.11) for example also cleaves GLP-1 but utilizes different target sites within the peptide[30]. In order to test if monotremes evolved a DPP-4 independent pathway to degrade GLP-1 we used a DPP-4 inhibitor in platypus and echidna sera and synthetic DPP-4 peptide or hGLP-1 as substrates and Ex-4 as a control substrate that is not normally cleaved by DPP-4. Firstly, inhibition of DPP-4 activity in human and monotreme sera was confirmed using the synthetic DPP-4 substrate (Extended Data Fig. S4). When degradation of GLP-1 and Ex-4 was then measured with inhibitor, monotreme GLP-1 and Ex-4 were degraded despite inhibition of DPP-4 (Extended Data Fig. S5). To further test our prediction that in monotremes a DPP-4 independent GLP-1 degradation mechanism is present we used mass spectrometry to analyse the pGLP-1 and eGLP-1 cleavage products. We saw cleavage products indicating trypsin or chymotrypsin-like activity with cleavage after basic residues (Extended Data Fig. S6), as well as products indicating cleavage after hydrophobic residues. Together this supports the idea that in monotremes a DPP-4 independent system has evolved to degrade GLP-1. While the DPP-4 resistance is likely a change to enhance the insulinotropic effect the changes in the degradation system may have evolved as countermeasure to GLP-1 in venom.

We then asked how changes in pGLP-1 and eGLP-1 affect binding and activation of the GLP-1 receptor (GLP-1R). All of the known key hGLP-1 residues (underlined in Fig. 1a) involved in binding to the human GLP-1R (hGLP-1R) core and four of the six C-

terminal residues (excepting Ala^{25} to Thr and Val^{33} to Leu) involved in binding to the hGLP-1R N-terminal domain are conserved in pGLP-1. In echidna GLP-1 there seems less conservation of the receptor binding residues with additional changes at the N-terminal receptor binding residues Phe^{12} (conservatively substituted to Tyr) and Asp^{15} (changed to Glu). The GLP-1R amino acid sequence is conserved in platypus (76% identity compared to hGLP-1R), including the residues important for ligand binding. The pattern of pGLP-1R expression (Extended Data Fig. S3) is also similar to other mammals[31]. Receptor binding assays on hGLP-1R overexpressing cells showed that compared to hGLP-1 and Ex-4 both platypus and echidna GLP-1 peptides have lower affinity for the human receptor (Fig. 3a, Table 2). hGLP-1 has an almost identical affinity for the platypus GLP-1R (pGLP-1R) and the human receptor, but unexpectedly both monotreme GLP-1 peptides had a significantly lower affinity than hGLP-1 for the pGLP-1R (Fig. 3b, Table 2). For both receptors monotreme GLP-1 peptides were equipotent with the GLP-1R agonist oxyntomodulin (OXM). It may be that the decreased affinity for the receptor has also evolved in response to the use of GLP-1 in venom.

We then investigated if this difference in affinities translates into a difference in activation of the GLP-1 receptor. As expected monotreme GLP-1 peptides showed significantly less potency than hGLP-1 in assays measuring cAMP accumulation, Ca^{2+} mobilization and ERK1/2 phosphorylation acting through both human and platypus receptors (Fig. 3 c-e). eGLP-1 showed a markedly lower potency at both human and platypus receptors that was even lower than OXM. We were interested if differences in the structure of monotreme GLP-1 peptides compared to hGLP-1 could account for the lower affinity for the receptor. Circular dichroism spectroscopy (CD) on Ex-4 and hGLP-1 yielded results similar to previous published data[32] and showed that all

peptides utilized were folded correctly (Extended Data Fig. S7). All retained significant helical content, although pGLP-1 had more and eGLP-1 had slightly less helical content compared with hGLP-1. As has been seen with Ex-4[32], a difference in helical content can result in a different mode of interaction with the GLP-1R.

Closer examination of potencies in receptor activation revealed differential signalling bias for monotreme GLP-1 peptides in comparison to that elicited by hGLP-1. Distinct signalling bias arising through activation of the GLP-1R by different ligands (including OXM) has recently been established and may, at least in part, underlie differences in the physiological profile of naturally occurring ligands of the GLP-1R[33]. Indicators used to determine the signalling profile of peptides include cAMP and intracellular Ca^{2+} mobilisation, which are involved in promotion of insulin release, and pERK1/2 that is part of the mitogenic signalling pathways activated via the GLP-1R[33]. Intriguingly, both the platypus and echidna GLP-1 peptides displayed a distinct pattern of signalling in comparison to hGLP-1, and the clinically approved mimetic exendin-4, which was apparent at both the human and platypus GLP-1 receptors (Fig. 3g, Fig. S8-9). The signalling profile of the monotreme GLP-1 peptides closely mimicked that of oxyntomodulin with a bias towards pERK1/2, and to a lesser extent iCa^{2+}, relative to cAMP (Fig. 3g, Fig. S8-9), although the bias towards calcium mobilisation was less apparent for the pGLP-1 at the human receptors (Fig. 3g). These observations suggest that perhaps monotreme GLP-1 peptides have gained new as yet undefined functions.

Ultimately, the signal cascade triggered by incretins results in the release of insulin from pancreatic islet cells. We investigated the ability of pGLP-1 to stimulate insulin release from isolated mouse islets. This shows that 100nM pGLP-1 can stimulate insulin release *in vitro* similar to hGLP-1 (Fig. 3f). It appears at least in mice that

pGLP-1 would act with classical incretin function to promote insulin release although whether this is the primary function in the platypus remains to be proven.

To our knowledge this is the first example where endogenous GLP-1 has been recruited into venom and independently evolved changes that affect DPP-4 cleavage. It is likely that the use of the venom in intraspecific conflict has favoured use of the "natural" platypus GLP-1. In monotremes an arms race between the function of GLP-1 in gut and in venom can explain the changes observed. Evolution of DPP-4 independent GLP-1 degradation and decreased receptor activation maybe have evolved in response to GLP-1 in venom. The independent evolution of these components affecting glucose homeostasis and insulin release highlights the importance of metabolic control as a target for venomous species. Venom components have been developed as drugs for chronic pain and autoimmune functions. Exendin-4, a GLP-1 like peptide isolated from reptile, has already been developed successfully as a T2DM treatment[5]. Our finding of a naturally occurring DPP-4 resistant mammalian GLP-1 ortholog with unique functional properties may further the development of novel or improved treatment options for T2DM in the future.

Methods

Methods are found in the supplementary section.

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serum, Ms Carlie Delaine for echidna *Gcg* PCR analysis, Dr Tasman Daish and Mr Aaron Casey for tissue collection, and the China Scholarships Council and Adelaide University for supporting CH. FG is supported by an ARC fellowship.

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Table 1 Percentage of intact peptides remaining after 11h incubation in each serum.

	Intact peptide remaining (%)		
Peptides	Human Serum	Platypus Serum	Echidna Serum
hGLP-1	9.4±2.3	11.4±2.8	10.8±2.7
pGLP-1	96.4±2.0	55.1±3.5*	49.4±2.9*
eGLP-1	99.3±1.8	48.3±3.5*	42.4±4.3*
Ex-4	98.1±1.0	100.5±1.2	70.5±3.1*

Data were derived by determining the area under the curve following rHPLC analysis. *Statistically significant, P < 0.05 compared with human serum group All values are means ± S.E.M. of three experiments conducted in triplicate.

Table 2 Characterisation of the binding to human and platypus GLP-1R.

	pIC50	
Peptides	hGLP-1R CHO	pGLP-1R CHO
hGLP-1	9.1±0.1	8.9±0.1
pGLP-1	7.9±0.1*	7.4±0.1*,$^{\&}$
eGLP-1	7.6±0.1*,$^{\#}$	7.4±0.1*
Ex-4	9.2±0.1	9.2±0.1
OXM	7.4±0.1*	7.5±0.1*

Data were analysed with a three-parameter logistic equation. pIC_{50} values represents the negative logarithm of the concentration of agonist that inhibits binding of half the total concentration of radiolabelled agonist ^{125}I-hGLP-1 (7-36). All values are means ± S.E.M. of two or three experiments conducted in triplicate. *Statistically significant, $P < 0.05$ compared with hGLP-1, $^{\#}$Statistically significant, $P < 0.05$ when comparing eGLP-1 with pGLP-1, $^{\&}$Statistically significant, $P < 0.05$ when compared with binding to hGLP-1R (paired *t* test).

Table 3 Characterisation of activation of human and platypus GLP-1R.

	pEC_{50}						
	hGLP-1R CHO			pGLP-1R CHO			INS-1 (832/13)
Peptides	cAMP accumulation	Ca^{2+} mobilization	pERK1/2	cAMP accumulation	Ca^{2+} mobilization	pERK1/2	cAMP accumulation
hGLP-1	10.5±0.1	7.8±0.1	8.8±0.1	10.7±0.1	8.3±0.1[&]	8.9±0.1	9.6±0.3
pGLP-1	8.7±0.1*	6.5±0.1*	7.9±0.1*	8.8±0.1*	7.0±0.1*,[&]	8.4±0.1[&]	7.6±0.3*
eGLP-1	6.9±0.1*,[#]	5.0±0.2*,[#]	6.1±0.1*,[#]	6.8±0.1*,[#]	5.0±0.2*,[#]	6.3±0.1*,[#]	6.1±0.3*,[#]
Ex-4	11.1±0.1*	8.4±0.1*	9.0±0.1	11.1±0.1	8.1±0.1	9.2±0.1	10.3±0.2
OXM	8.0±0.1*	6.4±0.1*	7.8±0.1*	7.8±0.1*	6.4±0.1*	8.1±0.1*	8.6±0.3

Data were analysed with a three-parameter logistic equation as defined in [34-36], where pEC50 values represent the negative logarithm of the concentration of agonist that produces half the maximal response. All values are means ± S.E.M. of at least three experiments conducted in triplicate. *Statistically significant, $P < 0.05$ compared with hGLP-1; [#]Statistically significant, $P < 0.05$ when comparing eGLP-1 with pGLP-1. [&]Statistically significant, $P < 0.05$ when compared with data on the hGLP-1R (paired *t* test).

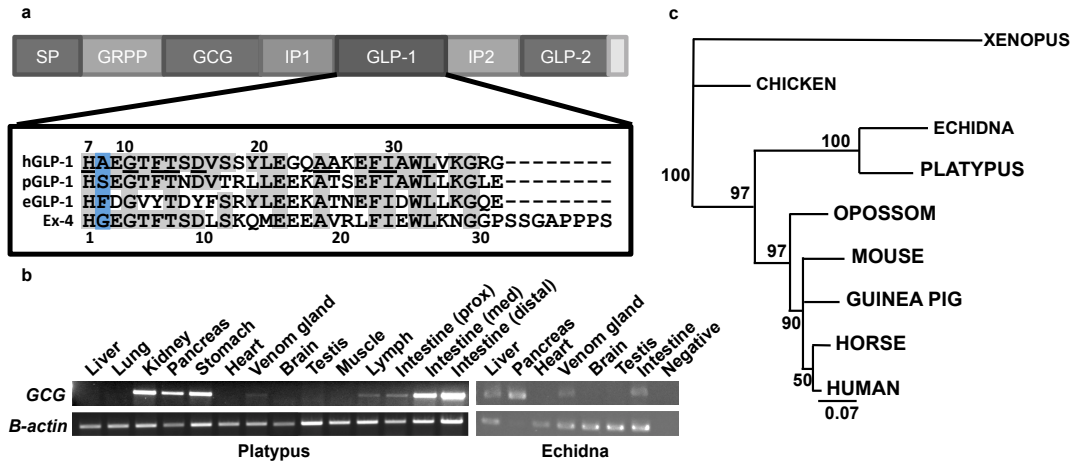


Fig. 1 Identification and characterisation of monotreme *Gcg* genes and GLP-1 peptides. (a) The proglucagon peptide (schematic above) includes a signal peptide (SP), glicentin-related polypeptide (GRPP), glucagon (GCG), intermediate peptides-1 and -2 (IP-1 and IP-2), glucagon like peptides 1 and 2 (GLP-1 and GLP-2) and is proteolytically processed into the mature glucagon-like peptides. Alignment of human, platypus, echidna and Gila monster (Ex-4) GLP-1 sequences highlights hGLP-1 residues involved in hGLP-1R binding (underlined), including residues 24, 25, 28, 29, 32-33 within the helix that interacts with the GLP-1R extracellular N-terminal domain[32]. hGLP-1 Gly[22], involved in kinking of the helix, is a Glu in the extended EX-4 helix, leading to different modes of interaction with the GLP-1R[2]. N-terminal underlined residues interact with the GLP-1R core[37,38]. The DPP-4 target site is highlighted in blue. Residues identical to those of hGLP-1are boxed (grey). (b) Expression of platypus and echidna *Gcg* in different tissues assessed by RT-PCR. (c) Phylogenetic reconstruction (MrBayes) based on GCG AS sequences.

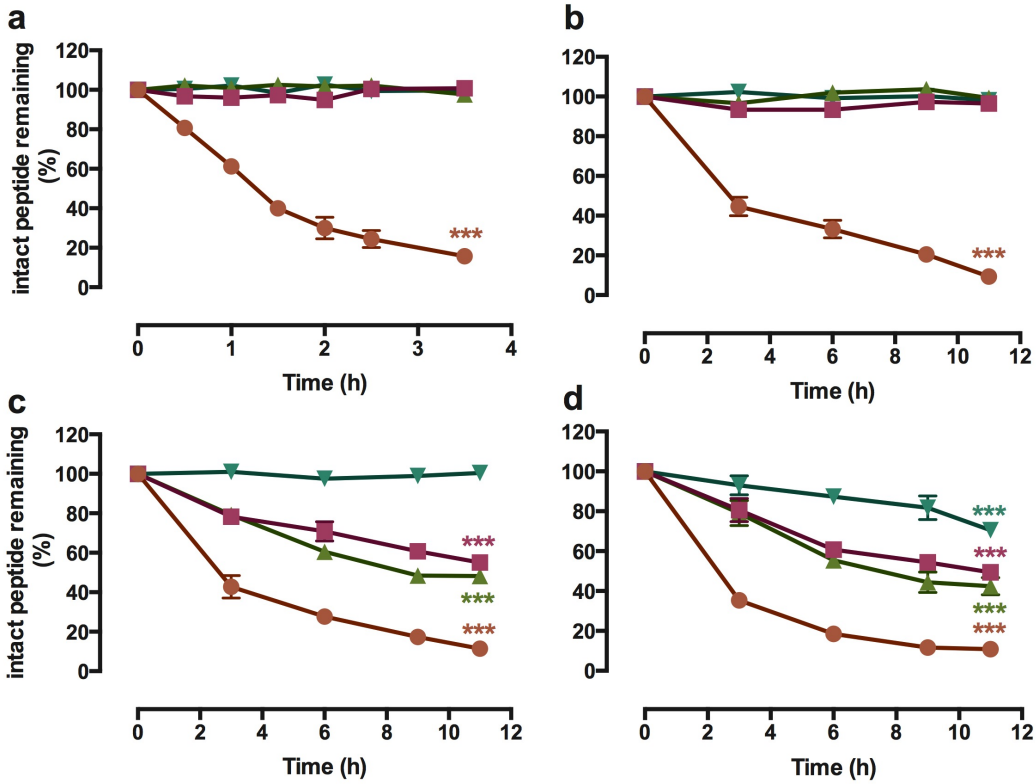


Fig. 2 Degradation of hGLP-1 (orange circles), pGLP-1 (pink squares), eGLP-1 (green triangles) and Ex-4 (turquoise inverted triangles) at different incubation times by purified human DPP-4 enzyme (a), human serum (b), platypus serum (c) and echidna serum (d) determined by measuring the area under the curve of the intact peptide following rHPLC analysis. All values represent means ± S.E.M. (n= 3). ***Statistically significant, $P < 0.001$ peptide remaining at last time point compared with starting peptide concentration.

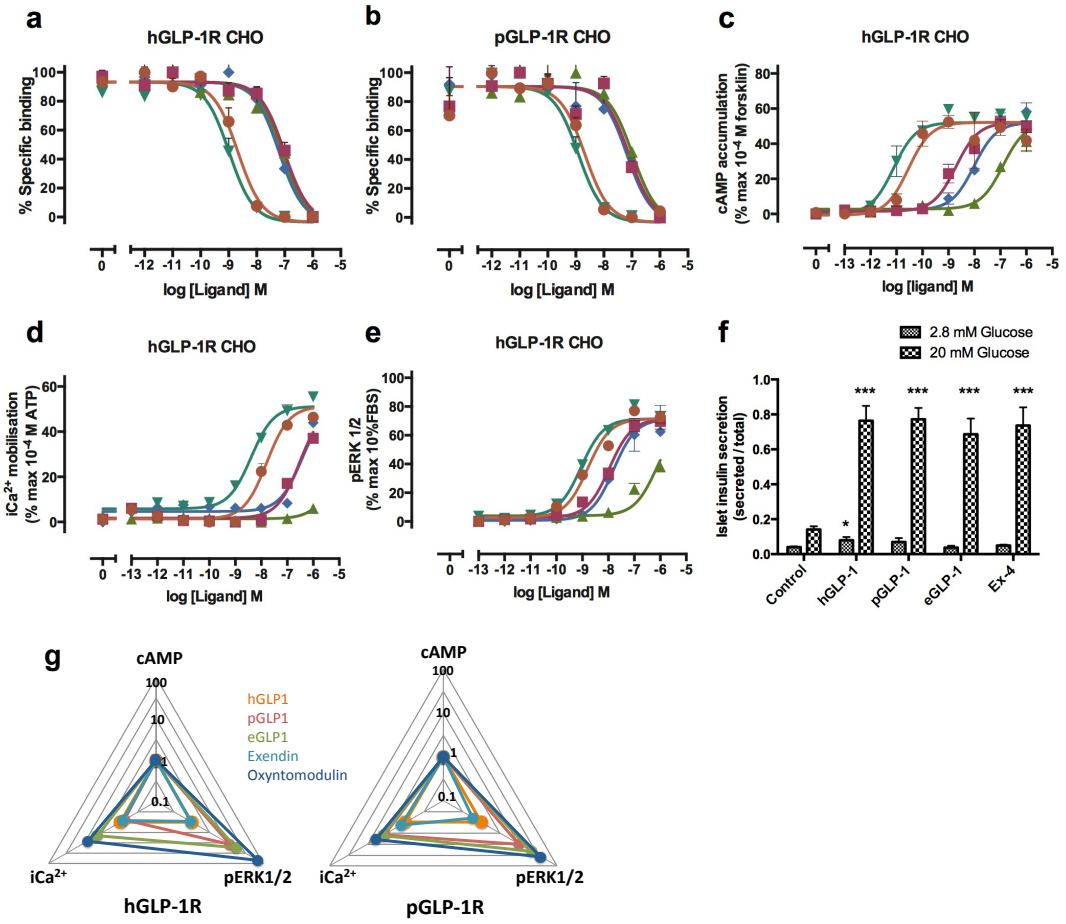


Fig. 3 hGLP-1R and pGLP-1R binding and hGLP-1R signalling. Characterisation of the binding of hGLP-1 (orange circles), pGLP-1 (pink squares), eGLP-1 (green triangles), Ex-4 (turquoise inverted triangles) and OXM (blue diamonds) in competition with radiolabeled ^{125}I-hGLP-1 (7-36) in FlpInCHO cells stably expressing hGLP-1R (a) or pGLP-1R (b). Data are normalized to the maximum ^{125}I-hGLP-1 (7-36) of each data set.

cAMP accumulation (c), Ca^{2+} mobilization (d) and ERK1/2 phosphorylation (e) was measured using FlpInCHO cells stably expressing hGLP-1R. Data were normalized to the maximal response induced by 100µM forsklin (cAMP), 100µM ATP (Ca^{2+}) or 10% FBS (ERK1/2). Data were analysed with a three-parameter logistic equation as described previously [34]. (f) Glucose-induced insulin release stimulated with or without 100 nM each peptide in the presence of either 2.8 mM or 20 mM of glucose. (g) Webs of bias were generated to quantify and compare signalling bias. Quantification of signal bias was performed using pharmacologically derived parameters of agonist affinity (Ka) and efficacy (tau) for each ligand in each of the three signaling pathways. The transduction ratio (tau/Ka) was extracted from standard concentration-response data

that was analysed with the operational model of agonism (Kenakin & Christopoulos 2012). This value was used to calculate ΔΔ(tau/Ka) values through normalization of the transduction coefficient (tau/Ka) for each ligand in each signalling pathway to the reference ligand (hGLP1 in black) and the reference signalling pathway (cAMP). Data are presented on a log scale. All values are the means ± S.E.M from at least three independent experiments performed in triplicate. *Statistically significant at $P < 0.05$ versus negative control group without peptide, ***Statistically significant at $P < 0.001$ versus negative control group without peptide.

Supplementary materials

Table S1 Primers used in this study.

		Primer sequences	Fragment size (bp)
pGcg	sense	5'-TGG TCC GAT TAC CAA ACG TCA-3'	350
	anti-sense	5'-TCA CCT CTC CTC AAC TTC GG-3'	
eGcg	sense	5'-TGG CTG GAT TGT TCG TGA TG-3'	360
	anti-sense	5'-GCT GCC GCT ACC TCT CTT GA-3'	
pGlp-1r	sense	5'-GAC GAC TAC GCC TGT TGG-3'	404
	anti-sense	5'-GAG CAC GGT GTC CTT GAT GA-3'	
Dpp-4	sense	5'-GCA AAC CAG GAA ATA GGC AGT G-3'	402
	anti-sense	5'-TCT GAA CTC GCA AGG GAG GTA G-3'	
β-actin	sense	5'-GCC CAT CTA CGA AGG TTA CGC-3'	348
	anti- sense	5'-AAG GTC GTT TCG TGG ATA CCA C-3'	

Table S2 Characterisation of the agonist signalling in FlpInCHO cells stably expressing pGLP-1R and INS-1(832/13).

	pEC50			
	INS-1 (832/13)	pGLP-1R CHO		
Peptides	cAMP accumulation	cAMP accumulation	Ca^{2+} mobilization	pERK1/2
hGLP-1	9.6±0.3	10.7±0.1	8.3±0.1$^{\&}$	8.9±0.1
pGLP-1	7.6±0.3*	8.8±0.1*	7.0±0.1$^{*,\&}$	8.4±0.1$^{\&}$
eGLP-1	6.1±0.3$^{*,\#}$	6.8±0.1$^{*,\#}$	5.0±0.2$^{*,\#}$	6.3±0.1$^{*,\#}$
Ex-4	10.3±0.2	11.1±0.1	8.1±0.1	9.2±0.1
OXM	8.6±0.3	7.8±0.1*	6.4±0.1*	8.1±0.1*

Data were analysed with a three-parameter logistic equation as defined in1-3, where pEC50 values are the negative logarithm of the concentration of agonist that produces half the maximal response. All values are means ± S.E.M. of at least three experiments conducted in triplicate. *Statistically significant, $P < 0.05$ compared with hGLP-1; #Statistically significant, $P < 0.05$ when comparing eGLP-1 with pGLP-1; &Statistically significant, $P < 0.05$ when compared with Table 3.

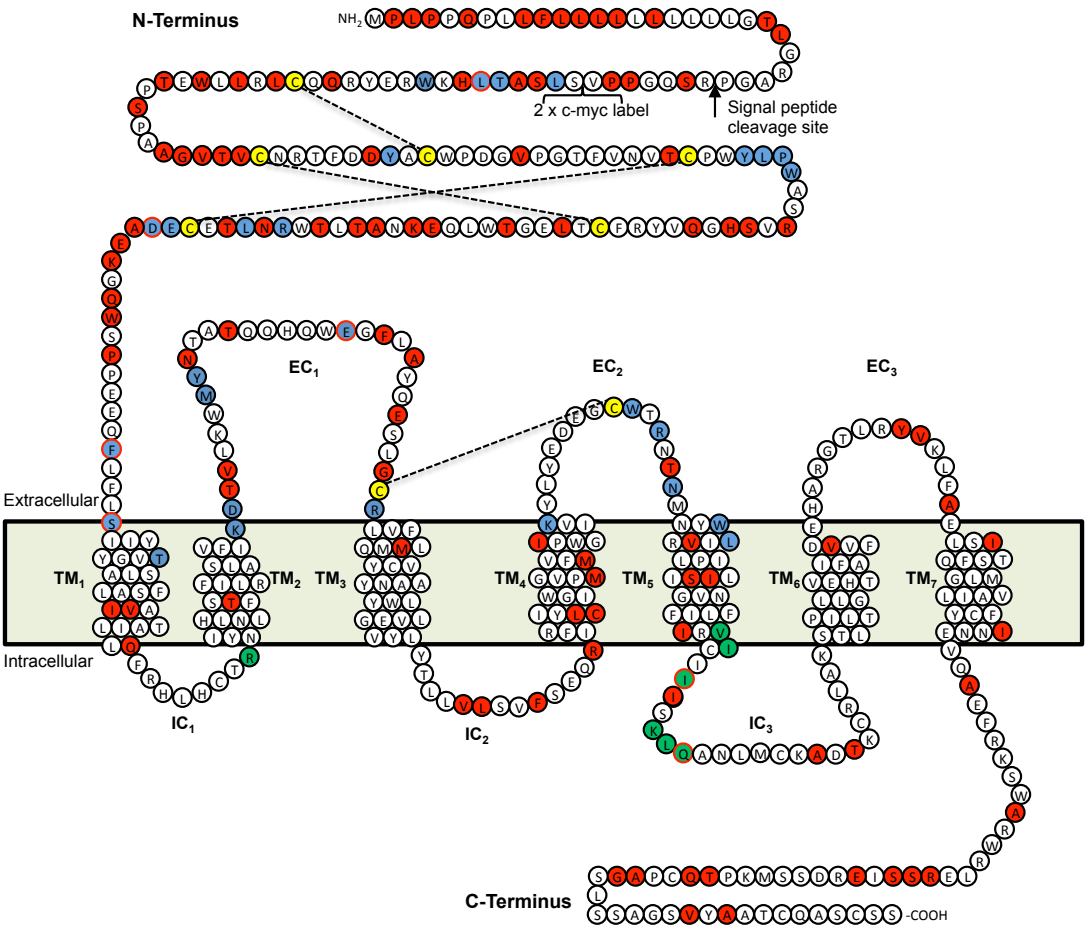


Fig. S1 Schematic diagram of the pGLP-1R highlighting residues equivalent to hGLP-1R important for structure and function. Cysteine residues involved in disulphide bonds, denoted by dashed lines, are highlighted in yellow. Residues vital for ligand recognition and binding are highlighted in blue[4-6]. Residues important for receptor signalling through interaction with Gs-proteins are highlighted in green[5]. Residues different between hGLP-1R and pGLP-1R are highlighted in red. Where a residue is marked blue or green but is different to hGLP-1 it is highlighted by a red circle. The putative signal peptide cleavage site is depicted with a black arrow.

Substrate Category	Names and Amino Acid Sequences Comparisons	Similarities (%)
Gastrointestinal hormone	hGLP-1 HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG pGLP-1 HSEGTFTNDVTRLLEEKATSEFIAWLLKGLE	77.4
	hGIP YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ pGIP YSEGTFINDYSRALSSLQEKKFVDWLLKQKERKKE-------	61.9
	hGLP-2 HADGSFSDEMNTILDNLAARDFINWLIQTKITD pGLP-2 HADGTFSHELSGVLDQLATKDFFNWLLLPEVEE	75.8
	hPHM-27 HADGVFTSDFSKLLGQLSAKKYLESLM pPHM-27 HADGLFTSGFSRLLGQLSAKKYLESLI	96.3
	hVIP HSDAVFTDNYTRLRKQMAVKKYLNSILN pVIP HSDAVFTDNYTRFRKQMAVKKYLNSVLA	92.9
	hGlucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNT pGlucagon HSQGTFTSDYSKHLDAIRAKQFVDWLMNY	82.8
	hPACAP-38 HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK pPACAP-38 HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	100
Neuropeptide	hNPY YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY pNPY YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY	100
	hPYY YPIKPEAPREDASPEELNRYYASLRHYLNLVTRQRY pPYY YPVKPQPPPDNATPEELAQYFASLRHYLNLVTRQRY	65.2
Chemokine	hCCL22 GPYGANMEDSVCCRDYVRYRLPLRVVKHFYWTSDSCPRPGVVLLTFRDKEICADPRVPWVKMILNKLSQ pCCL22 VPYATNLEDSICCTEFVKWPVRFRYLTEFYFTSLSCRRRGVVLKTVKNLEICADPQIPWVKKAIDLLKL	88.9
	hCXCL11 FPMFKRGRCLCIGPGVKAVKVADIEKASIMYPSNNCDKIEVIITLKENKGQRCLNPKSKQARLIIKK-VERKNF pCXCL11 VPRFRGSRCLCIGPRVNSVTPMQIKSISVFLPTSTCDRKEVIVTLKKGKGQRCLNLDSKQAQLILKRIVEIKKV	67.6
	hCXCL12 KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNKRFKM pCXCL12 KPISLSYRCPCRFSESNVAKANIKQLKILNTPNCALQIVARLKNS-RQVCIDPKLKWIQEYLEKALNKRFKM	95.8

Fig. S2 Amino acid sequence comparisons of a range of DPP-4 substrates (including peptide hormones, neuropeptides and chemokines) in human and platypus. Identical residues are highlighted in grey boxes. The red dashed line indicates DPP-4 cleavage sites. The percentage of identity between platypus and human homologs is shown. For high cleavage efficiency, hydrophobic and basic residues (e.g., His and Tyr) at N-terminus and Ala or Pro in the penultimate position are preferred (REF). Some peptides with Gly, Ser (e.g., glucagon[7]), Val or Leu in the penultimate P1' position are cleaved by DPP-4 at very low rates (REF, Mentlein). The different penultimate residue (Ala rather than Ser as in humans) of pGLP-1 and pGIP renders these two peptides DPP-4 resistant.

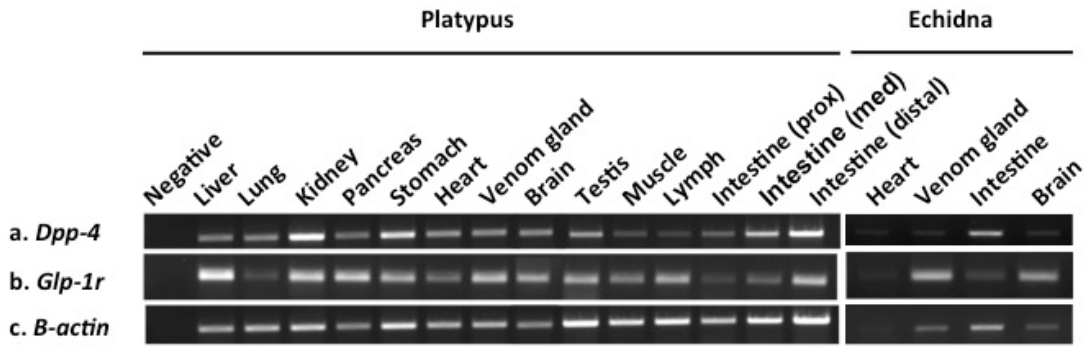


Fig. S3 Expression pattern of platypus *Dpp-4* and *Glp-1r*. (a) RT-PCR amplified *Dpp-4* showing strong bands in platypus intestine and kidney and echidna intestine, and faint bands in all the other tissues examined (expected size at ~ 400bp). (b) RT-PCR amplified *Glp-1r* resulted in strong bands in platypus liver, kidney, pancreas, brain and venom gland and echidna venom gland and brain, and faint bands in all the other tissues (expected size at ~ 400bp). (c) Beta actin as positive control (expected size at ~350bp).

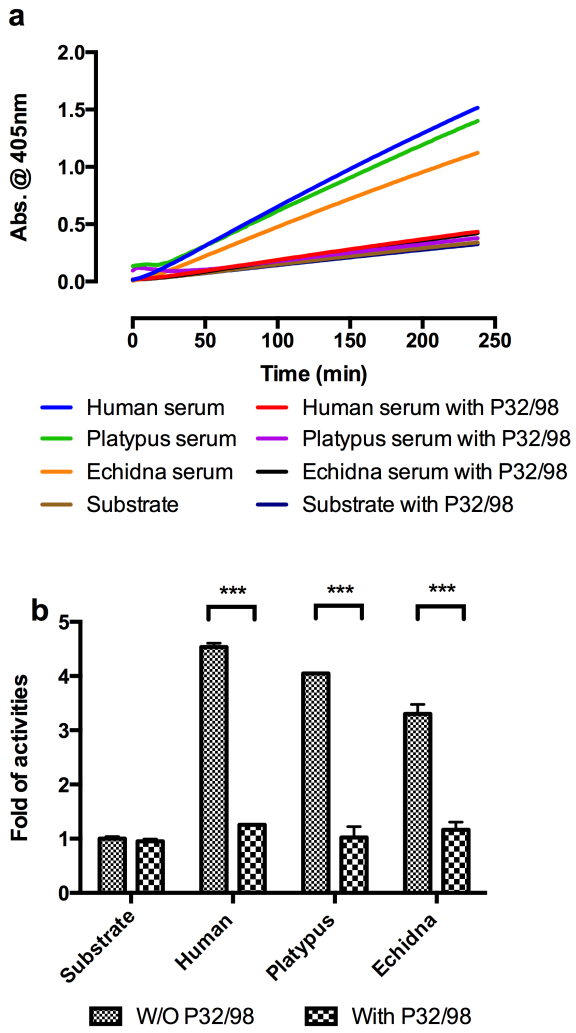


Fig. S4 Inhibition of DPP-4 activity in human, platypus and echidna serum by the DPP-4 inhibitor P32/98 monitored during the enzyme assay. (a) 12.5% human, platypus or echidna serum was incubated with H-Ala-Pro-*p*-nitroanilide as a substrate in the presence/absence of 100µM DPP-4 inhibitor P32/98 at 37 °C. The absorbance of the released *p*-nitroanilide was measured every two minutes over four hours at 405 nm and is directly related to DPP-4 cleavage activity. (b) DPP-4 activity in each serum with or without inhibitors is shown as fold change in absorbance per minute normalized to the substrate only group. *** Statistically significant, $P < 0.001$ versus groups without inhibitor.

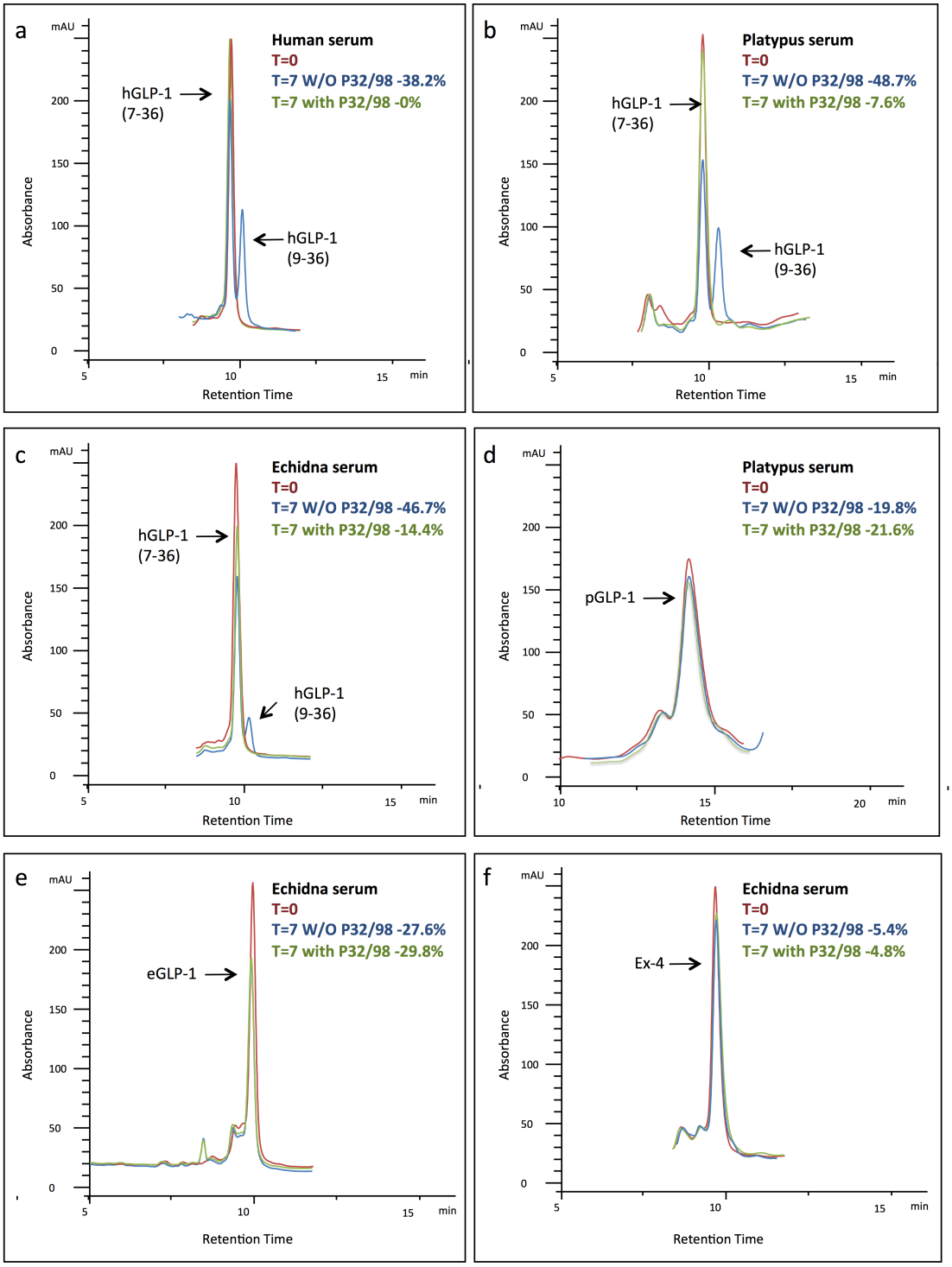


Fig. S5 Degradation of GLP-1 and Ex-4 by human, platypus or echidna serum monitored by RP-HPLC in the absence and present of DPP-4 inhibitor (P32/98). Degradation of hGLP-1 by human (a), platypus (b) or echidna serum (c) monitored by RP-HPLC in the absence (blue line) and presence (green line) of a DPP-4 inhibitor (P32/98). In human serum, hGLP-1 remained intact (0% degraded) in the presence of DPP-4 inhibitor, whereas in monotreme sera the inhibitor failed to completely protect the hGLP-1 DPP-4 degradation, indicating the possibility of existence of both DPP-4

and additional enzymes that can degrade hGLP-1 in monotreme sera. This was particularly evident in echidna serum (c), where 14% of the hGLP-1 was degraded despite the presence of inhibitor. Degradation by platypus serum of pGLP-1 (d), eGLP-1 (e) and Ex-4 (f) in echidna serum was monitored by RP-HPLC in the absence (blue line) and presence (green line) of DPP-4 inhibitor (P32/98). The presence of DPP-4 inhibitor in serum did not alter the cleavage pattern, indicating enzymes other than DPP-4 may be responsible for pGLP-1, eGLP-1 and Ex-4 degradation in monotreme serum.

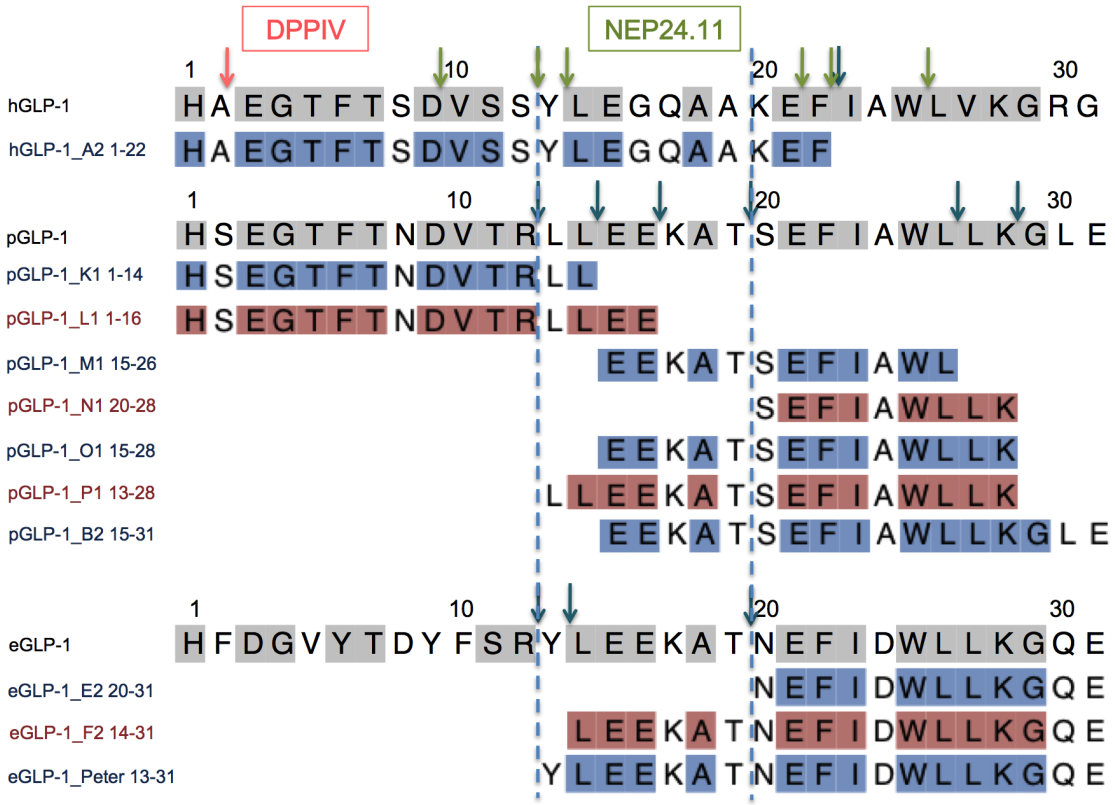
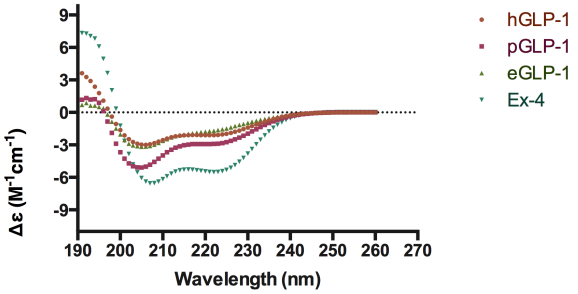


Fig. S6 Amino acid sequences of cleavage products predicted by MALDI-Mass spectrometry (spectra are shown in Appendix II, done by Adelaide Proteomics Centre). Degradation of hGLP-1, pGLP-1 and eGLP-1 in echidna serum for seven hours were monitored by RP-HPLC, fragments were collected and analysed by Mass Spectometry. Known cleavage sites of DPP-4 and NEP24.11 are shown in red and green arrows, respectively. Predicted cleavage sites are depicted by blue arrows.



	Helix (%)	Strand (%)
hGLP-1	23.8	21.4
pGLP-1	36.4	6.2
eGLP-1	19.3	25.2
Ex-4	55.9	3.1

Fig. S7 CD spectroscopy of hGLP-1 and analogs (75µM). hGLP-1 (orange circles), pGLP-1 (pink squares), eGLP-1 (green triangle) and Ex-4 (turquoise inverted triangles). The secondary structure content of each peptide was estimated using the CONTIN algorithm[8,9] .

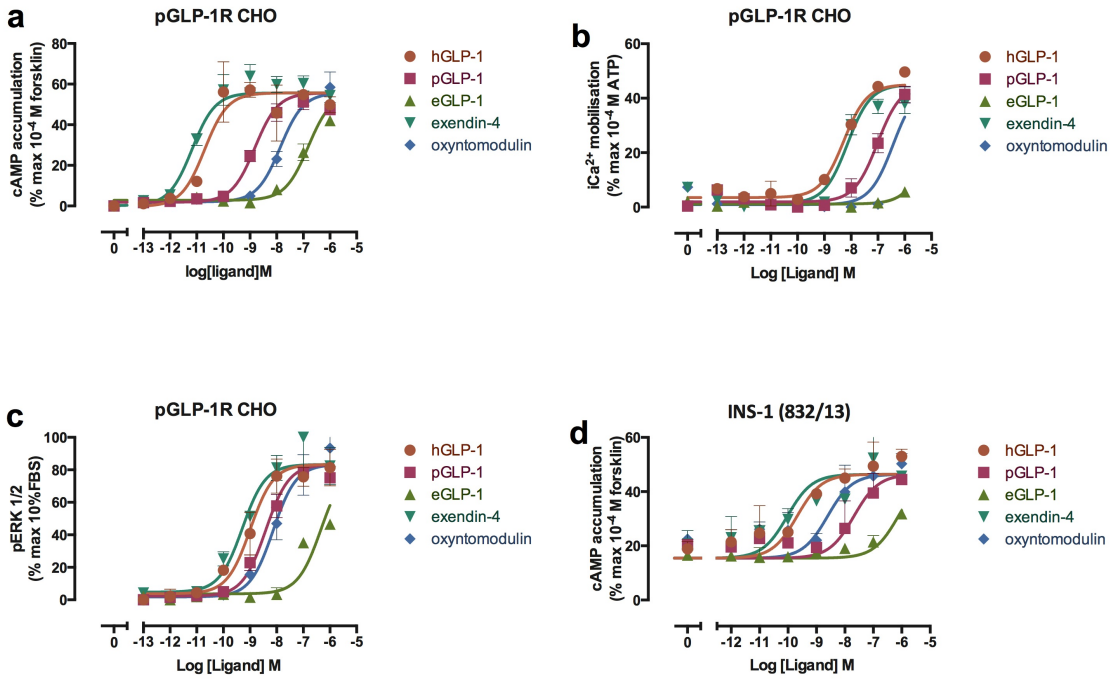
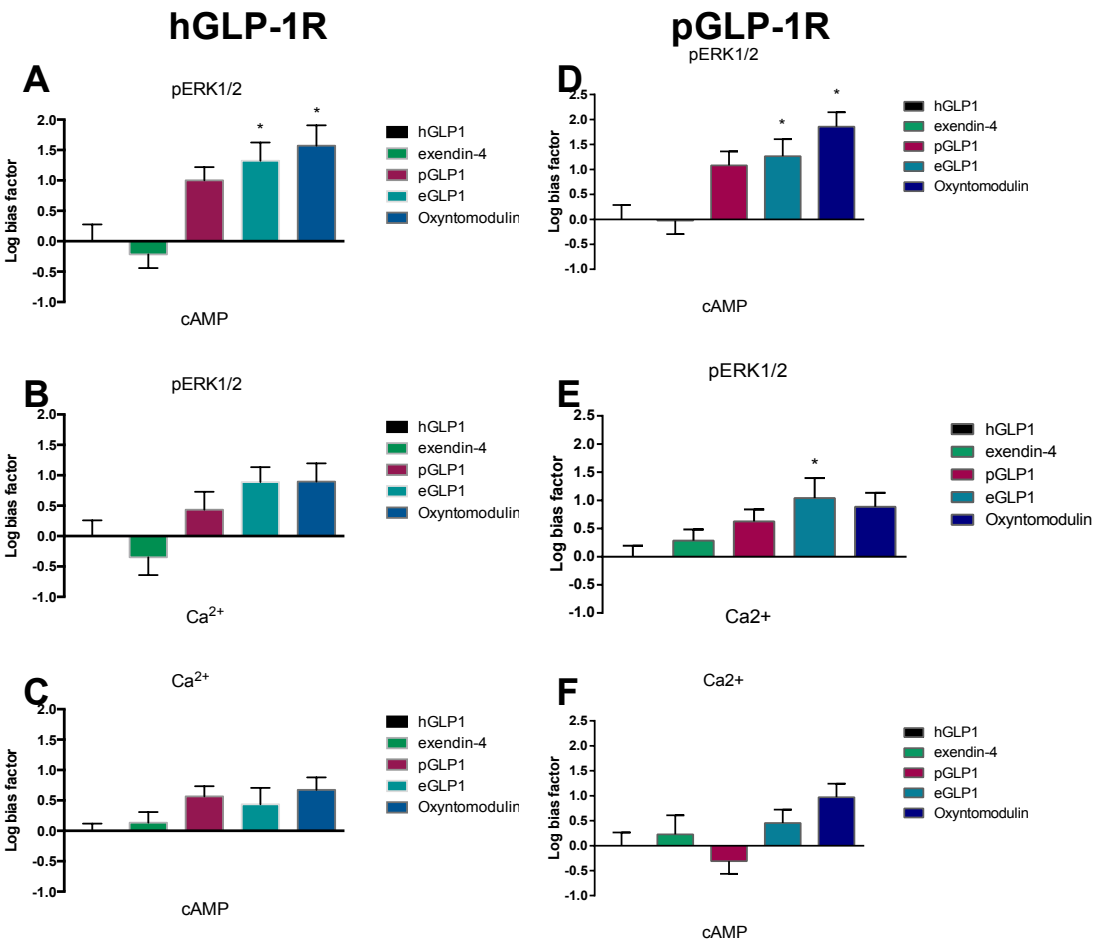


Fig. S8 Characterisation of the signalling outcomes stimulated by hGLP-1, pGLP-1, eGLP-1, Ex-4 and OXM in FlpInCHO cells stably expressing pGLP-1R via cAMP accumulation (a), Ca^{2+} mobilization (b) and ERK1/2 phosphorylation (c) and in INS-1 (832/13) cells via cAMP accumulation (d). Data were normalized to the maximal response induced by 100µM forsklin (cAMP), 100µM ATP (Ca^{2+}) or 10% FBS (ERK1/2). Data were analysed with a three-parameter logistic equation as defined in [1,3]. All values are means ± S.E.M. of at least three experiments conducted in triplicate.



hGLP-1R

Ligand	ΔlogRn (Bias factor)		
	pERK:cAMP	pERK:Ca^{2+}	Ca^{2+}:cAMP
hGLP1	0 ± 0.27 (1.0)	0 ± 0.26 (1.0)	0 ± 0.12 (1.0)
Exendin	-0.2 ± 0.22 (0.61)	-0.35 ± 0.29 (0.45)	0.13 ± 0.18 (1.3)
pGLP1	1.0 ± 0.22 (10)	0.44 ± 0.29(2.7)	0.57 ± 0.17 (3.7)
eGLP1	1.32 ± 0.29 (21)*	0.89 ± 0.25 (7.7)	0.44 ± 0.30 (2.7)
Oxyntomodulin	1.57 ± 0.33 (37)*	0.90 ± 0.29 (7.9)	0.67 ± 0.21 (4.7)

pGLP-1R

Ligand	ΔlogRn (Bias factor)		
	pERK:cAMP	pERK:Ca^{2+}	Ca^{2+}:cAMP
hGLP1	0 ± 0.29 (1.0)	0 ± 0.19 (1.0)	0 ± 0.27 (1.0)
Exendin	-0.03 ± 0.27 (1.0)	0.29 ± 0.20 (1.9)	-0.31 ± 0.26 (0.49)
pGLP1	1.08 ± 0.28 (12)	0.63 ± 0.21(4.3)	0.46 ± 0.27 (2.9)
eGLP1	1.27 ± 0.34 (18)*	1.04 ± 0.36 (11)*	0.23 ± 0.38 (1.7)
Oxyntomodulin	1.86 ± 0.21 (72)*	0.89 ± 0.25 (7.7)	0.97 ± 0.27 (9.4)

Fig. S9 Stimulation bias of monotreme GLP-1 peptides at the hGLP-1R and pGLP-1R compared to the reference ligand hGLP-1: Data were analyzed using an operational model of agonism as previously [10] to estimate log tc/KA ratios. Changes in log tc/KA ratios were calculated to provide a measure of the degree of stimulus bias (ΔlogRn (Bias factor) exhibited between different signaling pathways relative to that of the reference agonist hGLP-1. Values are expressed as means ± S.E.M. of at least three

independent experiments conducted in triplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test. *Statistically significant at $P < 0.05$ versus negative control group without peptide.

Materials and Methods

Materials

For tissue culture Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, hygromycin-B, and Fluo-4 acetoxymethyl ester were used (Invitrogen Carlsbad, CA). AlphaScreen reagents, ^{125}I-Ex(9–39), ^{125}I-hGLP-1 and 384-well ProxiPlates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). hGLP-1, pGLP-1, eGLP-1 and Ex-4 were purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or BDH Merck (Melbourne, VIC, Australia) and were of an analytical grade. The parental INS-1 (832/13) cell line was kindly provided by Chris Newgard [11].

RNA extraction

Platypus tissues were obtained from a male adult platypus (Animal ethics permits AEEC R.CG.07.03 and AEC S-49-200 to F.G). Total RNA was extracted from snap frozen platypus tissues (frontal cortex, pancreas, liver, lung, small intestine, stomach, heart, venom gland, testis, muscle, lymph and kidney) and echidna tissue (small intestine, pancreas, liver, venom gland, heart and brain) using TRIzol (Invitrogen, USA) according to the manufacture's instructions. RNA was resuspended in nuclease free water and stored at -80 °C.

cDNA synthesis

cDNA was synthesized from 3 μg RNA with Superscript III Reverse Transcriptase (Invitrogen) following the manufacture's instructions. Briefly, RNAs were treated with DNase I (Roche) to remove genomic DNA, incubated with 50 ng of random hexamers and 0.5 μl of 10 mM dNTPs for 5 min at 65 °C. After incubation, 2 μl of 5 × First-

strand RT buffer, 0.5 µl of 0.1 M dithiothreitol (DTT), 0.5 µl of RNaseOUT™ (40 U/µl), and 0.5 µl SuperScript III Reverse Transcriptase (200 U/µl) were added and incubated at 25 °C for 10 min, and then 50 °C for 50 min, followed by the final termination at 85 °C for 5 min. Finally, 0.2 µl of RNase H (Biolabs, 5 U/µl) were added to each tube and incubated at 37 °C for 20 min. cDNAs were stored at -20 °C.

RT-PCR

RT-PCR was performed to detect the presence of *Dpp-4* and *Glp-1r* mRNA in different platypus tissues. Gene-specific primers (pGLP1R sense and anti-sense, DPP-4 sense and anti-sense in Table S1) were designed based on the known cds of *pGlp-1r* or *Dpp-4*. Amplification cycles were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were run on 1.5 % agarose gels and then visualized with ethidium bromide. The identity of PCR products were determined by DNA sequencing.

DPP-4 enzyme assays

The enzyme assay was performed in flat-bottom 96- well microplates, where each reaction consisted of 100 µL of either 12.5 % human, platypus or echidna serum, 0.5 mM of H-Ala-Pro-*p*-nitroanilide (Bachem) as a substrate in the presence/absence of 100 µM of DPP-4 inhibitor P32/98 (Santa Cruz Biotech) in 100 mM sodium phosphate buffer (PH 7.4) supplemented with 150 mM NaCl and 0.2 % Tween-20 with a total volume of 200 µM. Reactions were performed at 37 °C. The absorbance of the released *p*-nitroanilide was measured every 2 min over 4h at 405 nm using a Thermo Scientific Multiskan Ascent microplate reader. Enzyme activity was shown as fold changes in absorbance per minute normalized to substrates only group.

DPP-4 cleavage of GLP-1

hGLP-1, pGLP-1, eGLP-1 and Ex-4 (30μM) were incubated with DPP-4 (3 U/L) in sodium phosphate buffer (0.1 M, pH 7.4) for 0, 30, 60, 90 and 120 min in 37 °C incubator, respectively, when trifluoroacetic acid (TFA, 0.1 %) was added to terminate the reactions. Samples were analysed by RP-HPLC with solvent A (0.1 % TFA in water) and B (80 % acetonitrile in 0.1 % aqueous TFA) at a flow rate of 0.5 ml/min. The peptides were eluted with a linear gradient of solvent B from 35 % to 50 % for 30 min. Elution of the peptides was detected by measuring UV absorption at 215.8 nm.

Stability of GLP-1 in human, platypus and echidna serum

Human (or platypus or echidna) serum was purified by DEAE Affi-Gel Blue Cartridges (Bio-Rad). hGLP-1, pGLP-1, eGLP-1 and Ex-4 (7μM) were incubated with purified human serum in 37 °C incubator in the presence/absence of 100μM of DPP-4 inhibitor P32/98 (Santa Cruz Biotech), and aliquots of the reaction solutions were extracted after 0, 3, 6, 9 and 11h incubation respectively, when TFA (0.1 %) was added to terminate the reactions. Aliquots were analysed by RP-HPLC.

MALDI mass spectrometry

1 μl of fragments collected from RP-HPLC was sported onto an 800 μm Anchor Chip target plate (Bruke Daltonics, Bremen, Germany) separately and air dried. 1 μl of matrix [alpha-Cyano-4-hydroxycinnamic acid, 0.5 mg/mL in water/acetonitrile/TFA 10/90/0.1] was spotted subsequently and air dried. Mass spectra were acquired on an ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) operating in reflective positive mode. Instrument settings were set in flexControl software (Version 3.4, Bruker Daltonik GmbH). Sample *m/z* range was set to 300-4000 Da. 1000 shots were collected for the external calibration and sample measurement. External

calibration was performed using a 1:20 dilution of peptide calibration standard (Bruker Daltonics). Laser intensity and detector gain were manually adjusted for optimal resolution. The MS spectra obtained were analysed using the FlexAnalysis software (Version 3.3, Bruker Daltonics) employing smoothing, background subtraction and peak detection algorithms.

Circular dichroism spectroscopy (CD)

Far-UV CD spectra were measured on a Jasco J-815 spectropolarimeter (Jasco Inc., Easton, MD) at 20 °C in a 1 mm quartz cuvette. The scanning range was 185-300 nm at a speed of 20 nm/min, the bandwidth was 1nm, and the spectra were accumulated 5 times. The concentration of hGLP-1, pGLP-1, eGLP-1 and Ex-4 was 75μM in 10mM sodium phosphate buffer (pH 7.0). The secondary structure of each peptide was estimated using the CONTIN algorithm [8,9].

Transfections and cell culture

Human and pGLP-1R cDNAs were isogenically integrated into FlpIn-Chinese hamster ovary (FlpInCHO) cells (Invitrogen) and selection of receptor-expressing cells accomplished by treatment with 600μg/ml hygromycin B as described previously [3]. Transfected and parental FlpInCHO cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and incubated in a humidified environment at 37 °C in 5% CO_2. INS-1(832/13) cells were cultured in RPMI-1640 medium supplemented with 10mM HEPES, 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 100 iU/ml penicillin, and 100 g/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

Radioligand binding assay

FlpInCHO-hGLP-1R and FlpInCHO-pGLP-1R cells were seeded at a density of 3×10^4 cells/well and INS-1(832/13) cells at 10^5 cells/well into 96-well culture plates and incubated overnight at 37 °C in 5% CO_2. Growth media was replaced with binding buffer [phenol-free DMEM containing 25 mM HEPES and 0.1% (w/v) BSA] containing 0.7 nM ^{125}I-Ex(9–39) or 0.15 nM ^{125}I-hGLP-1 and increasing concentrations of unlabelled ligand. Cells were then incubated overnight at 4 °C, followed by three washes with ice-cold 1 × PBS to remove unbound radioligand. Cells were then lysed in 0.1 M NaOH, and radioactivity determined by γ-counting as described previously[1].

cAMP accumulation assay

FlpInCHO-hGLP-1R and FlpInCHO-pGLP-1R cells were diluted to the density of 6×10^5 cells/ml and INS-1(832/13) cells to 2×10^6 cells/ml in stimulation buffer [phenol-free DMEM containing 5 mM HEPES, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.1 % (w/v) BSA]. And cAMP detection was carried out as described previously[12]. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data were subsequently normalized to the response of 100 nM forskolin.

ERK1/2 phosphorylation assay

FlpInCHO-hGLP-1R and FlpInCHO-pGLP-1R cells were seeded at a density of 3×10^4 cells/well into 96-well culture plates and incubated overnight at 37 °C in 5% CO_2. Receptor-mediated ERK1/2 phosphorylation was determined as previously described[1].

Intracellular Ca^{2+} mobilization assay

FlpInCHO-hGLP-1R and FlpInCHO-pGLP-1R cells were seeded at a density of 3×10^4 cells/well into 96-well culture plates and incubated overnight at 37 °C in 5% CO_2 and receptor-mediated intracellular Ca^{2+} mobilization determined as described previously [2].

Measurement of insulin

Insulin concentrations were determined by a commercially available radioimmunoassay specific for rodent insulin (Linco Research Immunoassay, St. Charles, MO) as previously described[13].

Data analysis

All data were analysed in Prism 6.0c (GraphPad Software Inc., San Diego, CA). Concentration response signalling data were analysed using a three-parameter logistic equation as described previously [1,3]. Signalling bias was analysed as described[10]. Statistical analysis was by One-way ANOVA (nonparametric) with Dunnett's post test unless otherwise stated in the Figure legends.

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Chapter 5

Conclusion and Future Direction

Conclusion

Monotremes, our most distant relatives amongst living mammals, have provided unique insights into mammalian evolution. The recent analysis of the platypus genome [1] revealed wholesale loss of genes associated with protein degradation and gastric functions [2]. This reflects their radically different digestive tract and raises questions about how fundamental metabolic control and digestion is carried out in monotremes. Combining comparative genetics, biochemical and histological approaches, I tested the hypothesis that these radical changes in stomach function have also affected other aspects of the metabolic control system in monotremes at the histological and molecular level.

First, I investigated the ghrelin pathway, which is evolutionarily highly conserved among vertebrate species, but little is known about this pathway in platypus and echidna. Work presented in Chapter 2 provides a comprehensive analysis of three genes, *Ghrl* (encoding ghrelin), *Mboat4* (encoding ghrelin *O*-acyl transferase, GOAT), and *Ghsr* (encoding growth hormone secretagogue receptor GHS-R 1a) of the brain-gut axis ghrelin pathway in monotremes. Surprisingly, this work suggests that *Ghrl* and *Mboat4* genes have been lost whereas *Ghsr* is present, conserved and expressed as in other mammals. This is the first report suggesting the loss of ghrelin in a mammal. These results raise the possibility that the lineage specific loss of ghrelin and GOAT in monotremes may be related to the loss of gastric function or has been replaced by redundant ligands acting through the ghrelin receptor. It would be fascinating to know what this means in terms of appetite control and suggests changes to brain gut axis functions which involve ghrelin.

Ghrelin is known to be produced in human and mouse pancreatic ε-cells, which have been shown to be the progenitor of some islet endocrine cell populations [3]. Hence the lack of ghrelin in monotremes led me to investigate the morphology and cell composition of the monotreme pancreas in more detail (Chapter 3). Generally, the monotreme pancreata share the basic characteristics of other mammalian pancreata, including both endocrine islets of Langerhans and exocrine acini. Immunohistochemical analysis revealed the detailed cytoarchitecture of the platypus and echidna endocrine islets and confirmed the existence in both animals of four of the five major endocrine cell types: α-, β-, δ- and PP-cells. The arrangement of the islet cells in the platypus pancreas was more similar to those of eutherian species, whilst echidna showed more resemblance to birds and marsupials. Interestingly, we reported a possible echidna specific category of pancreatic islets, where α- and β-cells tended to form two poles within the islets, which to our knowledge is the first time this has been observed in any species.

One of the key functions of the pancreas is to regulate blood glucose levels by releasing insulin upon food intake. Glucagon-like peptide 1 (GLP-1), a peptide released from the small intestine, stimulates insulin secretion via the GLP-1 receptor (GLP-1R) in pancreatic islet β-cells. In humans, GLP-1 is rapidly cleaved by the enzyme dipeptidyl peptidase-4 (DPP-4) resulting in a very short serum half-life (<2min). Searching for long-acting GLP-1 analogs to improve insulin sensitivity has been a key strategy in type 2 diabetes (T2D) treatment. In Chapter 4, we identified GLP-1 pathway genes (*Dpp-4, Glp-1* and *Glp-1r*) in monotremes and confirmed the conserved expression of *Dpp-4* and *Glp-1* in pancreas and intestine, and also surprisingly showed its expression in venom, which is used by males for intraspecific conflict. The key changes in

monotreme GLP-1 amino acid sequences led us to predict that they may be resistant to human DPP-4 degradation. Comprehensive biochemical experiments revealed that both platypus and echidna GLP-1 were more stable than hGLP-1 in human sera, but can be degraded by enzymes (probably trypsin-like) other than DPP-4 in their endogenous sera. It is highly possible that in monotremes, a DPP-4 independent mechanism has evolved to degrade GLP-1. Receptor binding assays on hGLP-1R or pGLP-1R overexpressing cells showed that compared to hGLP-1 and Ex-4 both platypus and echidna GLP-1 peptides have lower affinity for both the human and platypus receptor. The lower affinities further result in the decreased capability of activating GLP-1R signaling pathways.

Evolution of DPP-4 independent GLP-1 degradation mechanism and decreased receptor activation may have evolved in response to GLP-1 in venom. To our knowledge this is the first example of a species where endogenous GLP-1 is recruited into venom.

Ghrelin and GLP-1 are gut hormones that act on the hypothalamus to regulate appetite (stimulation and inhibition respectively). Together with the loss of ghrelin, the altered GLP-1 susceptibility to degradation mechanism and decrease biological potency further suggests that different appetite control mechanisms exist in monotremes.

Overall this work has uncovered fundamental changes in monotreme metabolic control beyond the gastric system. In contrast to the radically changed stomach anatomy the monotreme pancrease is largely mammal-like. Changes in the insulin release pathway are likely a result of the dual function of GLP-1 in gut and venom.

Future directions

Central growth hormone (GH) release and appetite regulation

The loss of ghrelin pathway genes, *Ghrl* and *Mboat4* raises questions about metabolic control in monotremes, especially in terms of GH release and appetite control. Interestingly, growth hormone releasing hormone (GHRH) has been identified as an agonist of the ghrelin receptor, GHS-R 1a [4]. GHRH is important in promoting GH release and then IGF-1 production and hence is conserved among vertebrate species. Surprisingly, preliminary database searches have shown that together with *Ghrl*, *Ghrh* appears to be missing in the platypus genome, whereas partial sequence of growth hormone releasing hormone receptor (GHRHR) was found with NCBI (http://www.ncbi.nlm.nih.gov/, accession No. XP_001505263.1). However, this needs to be further analysed and confirmed by synteny analysis or degenerate PCRs. The simultaneously loss of *Ghrl* and *Ghrh* suggests a different mechanism of the physiological regulation of GH release in monotremes.

The hypothalamus plays vital roles in controlling appetite. The arcuate nucleus (ARC) of the hypothalamus is the major appetite-controlling centre of the human brain. It consists of two appetite controlling cell types: the pro-opiomelanocortin (POMC) appetite-inhibiting neurons and the neuropeptide Y (NPY) and agouti-related peptide (AgRP) appetite-stimulating co-expressing neurons [5,6]. Upon receiving signals from the periphery/gut, the ARC produces neuropeptides that projects on the downstream neurons to control feeding and energy expenditure [7]. In order to have a better understanding of appetite control in monotremes, the existence and expression of appetite regulating peptides other than ghrelin, including leptin, insulin and PYY, and their receptors, remain to be identified or studied in detail.

Despite the lack of ghrelin, ghrelin receptor GHS-R 1a is present in the platypus and is expressed in the brain. This may indicate the existence of another endogenous ligand in the platypus. The existence of additional GHS-R 1a ligands in other species is also directly suggested by several studies [8-10]. In addition, a number of studies also raised the possibility of the existence of an alternative ghrelin receptor [11], the understanding of which will elucidate additional functions of ghrelin, especially its unacylated form.

Digestion and glucose sensing in the small intestine and pancreas

The unique anatomy of the monotreme stomach [12] and lack of key genes involved in digestion [2,13] indicate that novel digestion and nutrient sensing mechanisms have evolved in monotremes. Still, many other key genes involved in metabolic control remain to be identified or studied in detail. This work has highlighted that changes in monotreme metabolic control go beyond the stomach and digestive system. It is likely that this has resulted in many other changes as well which are yet unexplored.

Revolutionary sequence technologies allow us to study whole transcriptomes in specific parts in mammalian digestive tract. For example, transcriptome analysis has recently been used on human and rat pancreas and a database was set up with detailed information about gene expression in human, mouse and rat pancreatic cells [14]. This valuable resource provides the basis for future comparisons across species; however, so far no global gene expression data are available in species representing the other major mammalian lineages (marsupials and monotremes) or in species representing earlier points of divergence in pancreatic evolution (e.g. birds). Comparisons with chicken,

with its existing complete genome sequence [15], have been very powerful in informing us about mammalian specific changes in evolution. Interestingly there are significant differences between chickens and humans in metabolic control (such as insulin release [16]) and their dietary requirements [17]. A comprehensive transcriptome analysis of the monotreme pancreas and gut (e.g. enteroendocrine intestinal L and K cells) as well as chicken pancreas and gut will fill a significant gap in our knowledge as these animals represent major groups of vertebrates and provide important information about the evolution of genes expressed in the mammalian and avian pancreas. This research will reveal important information about gene sets with conserved function in the monotremes and genes that underwent lineage specific adaptions on the genome wide level.

Along with the better characterisation of metabolic control genes, comprehensive immunohistochemical and *in situ* hybridization analysis of proteins and genes (for example, GLP-1 and GIP) known to be important for digestion and nutrient sensing in the small intestine and pancreas are required to reveal a better picture of the structure and function of the gastrointestinal tract in monotremes. For example GLP-1R is expressed by pancreatic β-cells in mouse and human [18]. However, a recent study has shown that in chicken GLP-1R is exclusively expressed in δ-cells, and suggests GLP-1 performs its insulinotroptic activity via a different mechanism in chicken compared to mammals [19]. Similarly, the analysis of GLP-1R expression in monotremes will shed light on the GLP-1 functions in pancreas, and especially its insulin releasing effects.

Prior to this study monotreme pancreatic islets had not been studied in great detail. We reported a possible monotreme specific category of pancreatic islets in echidna where α- and β-cells are juxtaposed to form two distinct hemispheres in one islet. Future work could involve an investigation of how this unique arrangement of α- and β-cells affects the function of the islets. Previous studies of human and mouse pancreas showed considerable plasticity in the structure of islets. Cell composition in the islets can be influenced by both physiological and pathophysiological conditions, such as pregnancy, fat mass or diabetes [20,21]. It would be interesting (but challenging in these wild animals) to analyze the differences in the islets cytoarchitecture, and ultimately to measure insulin and glucagon levels under different conditions in the monotremes. In order to address how the cell composition affects hormone release, the organization of endocrine cells and blood vessels within islets needs to be investigated. Co-localisation may indicate that those cell types release hormones into the blood stream. The immunohistochemical analysis of blood vessels within islets will delineate the role of blood vessels in the maintenance of islet architecture and function. For example, which cell type is prone to locate adjacent to blood vessels and how does this affect hormones release into blood stream.

Defining the mechanisms by which GLP- 1 controls blood glucose homeostasis

We have uncovered an unusual GLP-1 biology in monotremes. We speculate that changes in the GLP-1 system are triggered by dual function as incretin and venom component. This may have resulted in changes in the interaction with the GLP-1 receptor and also changes in the GLP-1 degradation away from a DPP-4 mediated digestion. Future work should aim to identify which protease(s) are responsible for cleavage of pGLP-1.

We were surprised to find that pGLP-1 has a low affinity for its cognate receptor. It would be important to identify which residues of pGLP-1 are responsible for the low affinity for the pGLP-1R. Sequence comparison of human and platypus GLP-1R shows that residues identified to be important for GLP-1 are conserved (reviewed in [22]). We also showed that hGLP-1 is able to bind the pGLP-1R with an affinity similar to binding its cognate hGLP-1R, thus confirming conservation of the determinants for high affinity ligand binding. While a crystal structure of the entire GLP-1/GLP-1R complex has yet to be solved, the extracellular domain of human GLP-1R (nGLP-R) in complex with GLP-1, and the extracellular domain of human GIP-R in complex with GIP has provided the basis for us to identify likely residues of pGLP-1 that maybe inhibiting a high affinity interaction. These lie within the central helical region where there is the most sequence difference from hGLP-1 (residues 17-26). Future work will conduct systematic substitution of pGLP-1 with human residues across this region to determine the contribution of each residue to pGLP-1R binding.

As pGLP-1 has low affinity for the pGLP-1R, it is possible that the endogenous pGLP-1 concentration is higher than that of hGLP-1 for the hGLP-1R, so as to compensate for the low affinity and thereby achieve full potency. This hypothesis needs to be verified by measuring the concentration of pGLP-1 in platypus serum using an ELISA or radioimmunoassay. If no commercially prepared GLP-1 antibodies are suitable for the capture of pGLP-1, monotreme specific antibodies need to be generated. These antibodies could also be useful for immunohistochemical studies of the pGLP-1 in the small intestine, the site of its expression. Through determination of the serum concentration of pGLP-1 we will reveal if GLP-1 is still able to act as an incretin

hormone. If so one would expect higher levels in the serum to compensate for lower affinity to receptor binding.

Both eGLP-1 and pGLP-1 showed bias towards ERK1/2 signalling and away from cAMP at both cognitive pGLP-1R and hGLP-1R relative to hGLP-1. eGLP-1 is also biased towards ERK1/2 and away from Ca^{2+} signalling relative to hGLP-1 at the pGLP-1R. While cAMP and intracellular Ca^{2+} are involved in the promotion of insulin release, ERK1/2 is part of the mitogenic signalling pathways. Hence, the signalling bias of monotreme GLP-1 suggest that these peptides may have gained new yet undefined functions. It would be of interest to explore other functions in the following work.

Other functions of GLP-1 increasingly draw attention. Previous studies have described neuroprotective and cardioprotective effects of hGLP-1. Also, hGLP-1 stimulates β-cell proliferation [22,23]. How changes in GLP-1 sequence affect those functions is currently unclear. It would be interesting to explore the effects of pGLP-1 in neural cell lines. Preliminary experiments using methods described previously [24,25] to analyse the effect of GLP-1 on stimulating β-cell proliferation failed to showed significant difference between GLP-1 treated and non-treated group (Appendix I), however this needs to be repeated and expanded and include other insulin-releasing cell lines, for instance, RIN, HIT and βTC cells.

Overall this research in monotremes has not only revealed new aspects of monotreme metabolic control and pancreas morphology but also highlights how the use of the comparative approaches further our understanding of the evolution of metabolic control

and opens up new avenues for the treatment of metabolic control disorders.Future work will reveal how the changes in the molecular basis of monotreme metabolic control translate into physiological and even behavioural aspects of feeding, blood glucose regulation and pancreatic function.

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Appendix I

Investigating the effect of GLP-1 on β-cell proliferation

Aim: To investigate pGLP-1 function on regulating proliferation of pancreatic INS-1 β-cells

Method: WST-1 assay and Thymidine incorporation assay as described previously (Buteau, et al., 1999, Wang, et al., 2004)

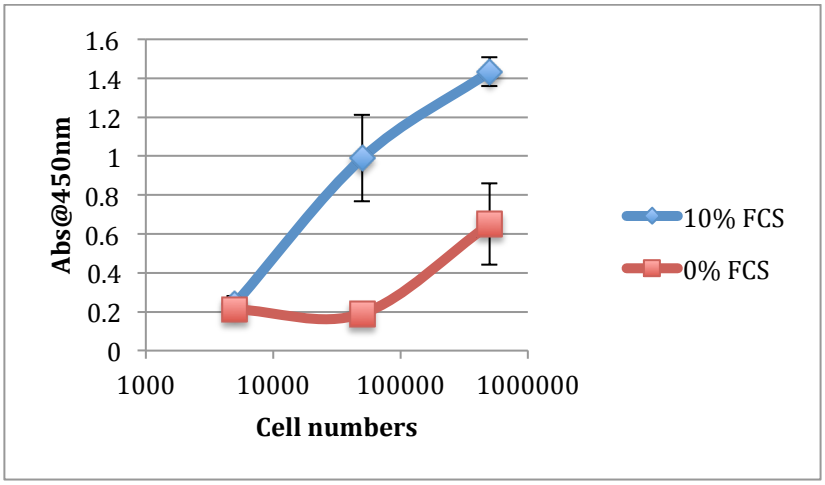
WST-1 assay

Trial #1

Optimize cell numbers of INS-1 cells with fetal calf serum (FCS)

- Day 1 INS-1 cells were seeded at different concentration (5×10^3, 5×10^4,5×10^5 cells per well) in a 96 well plates and cultured in RPMI-1640 medium [supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM *β*-mercaptoethanol, 100 iU/ml penicillin and 100 μg/ml streptomycin, 10 % FCS] @ 37 °C O/N
- Day 2 PBS wash, preincubated cells in serum free media (serum starve) for 24 h
- Day 3 Incubated cells in normal (10 % FCS) or serum-free (0 % FCS) media
- Day 5 10 μl of WST-1 reagent was added to each well and incubated @ 37 °C for 4 h
- Measures absorbance @450nm

Results

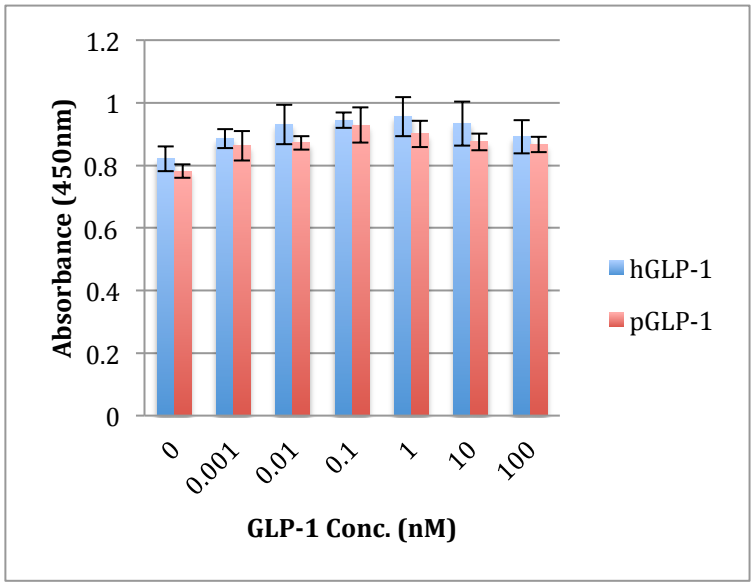


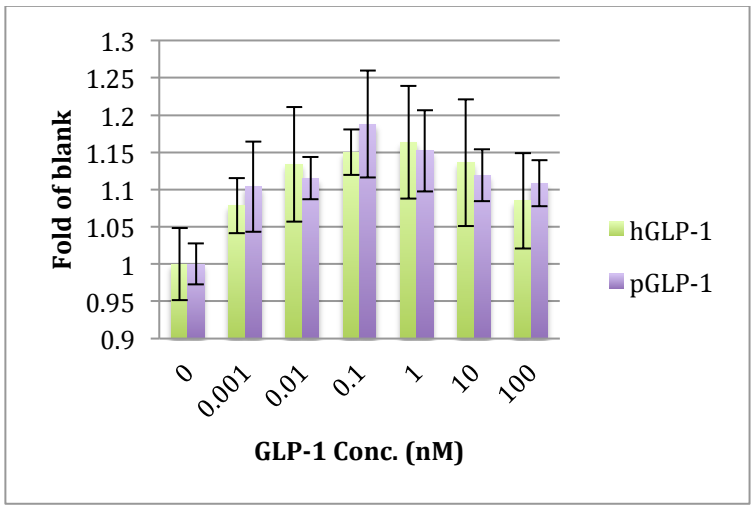
Conclusion

$5x10^4$~$5x10^5$ cells per well is an optimal cell concentration to start with

Trial #2

Start with 5×10^5 cells per well, grow for 24 h, starve for 24 h, treatment with different conc. of hGLP-1 and pGLP-1 for 48 h.





No significant differences between GLP-1 treated and non-treated groups.

Trial #3

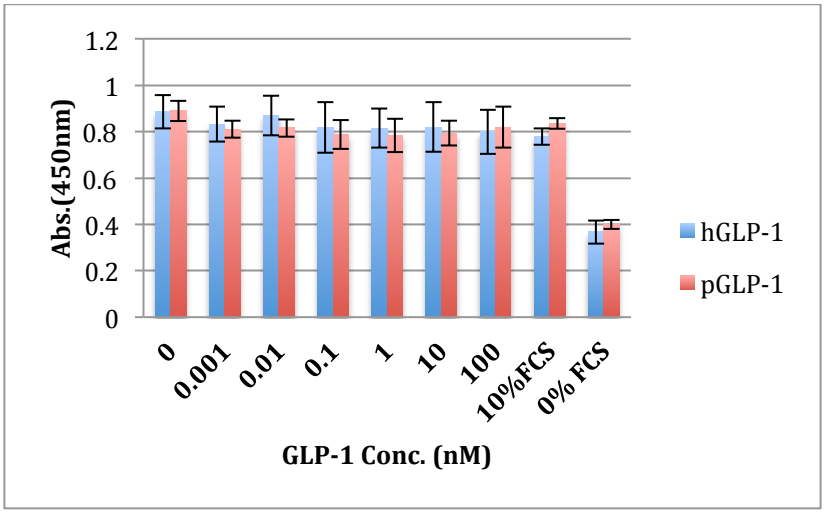
Start with 5×10^4 cells per well, grow for 24 h, starve for 24 h, treatment with different conc. of hGLP-1 and pGLP-1 for 48h.

No significant differences between GLP-1 treated and non-treated groups.

(Data not shown)

Trial#4

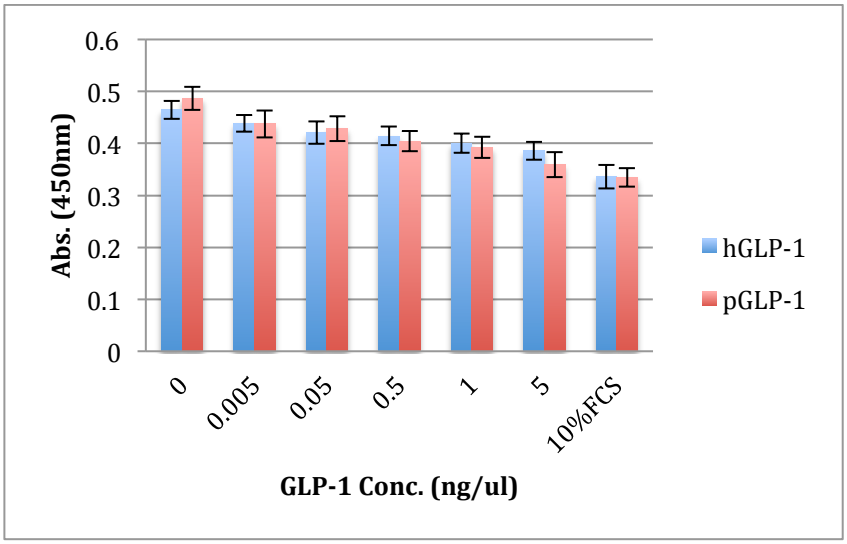
Start with 5×10^4 cells per well, grow for 24 h, starve for 4 h, treatment with 1 ng/µl of insulin and 5 ng/µl of transferrin plus different conc. of hGLP-1 and pGLP-1 and for 48 h.



No significant differences between GLP-1 treated and non-treated groups.

Trial#5

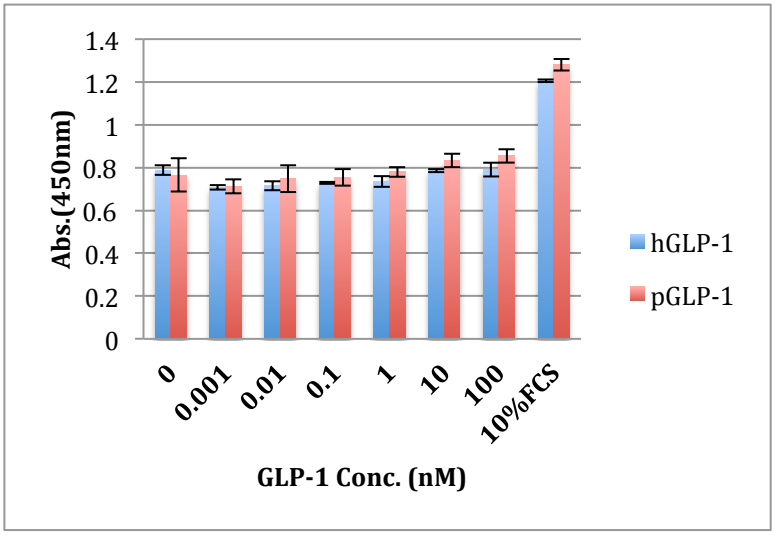
Same as Trail#3, except for using RPMI-1640 medium supplemented with 1 % FCS instead of 10 % FCS



No significant differences between GLP-1 treated and non-treated groups.

Trial #6

Start with 8×10^4 cells per well, grow for 48 h, starve for 24 h, treatment with 3 mM glucose plus different conc. of hGLP-1 and pGLP-1 and for 48h.



No significant differences between GLP-1 treated and non-treated groups.

Thymidine incorporation assay

Trial #1

Protocol

- Day 1 Plate INS-1 cells at $8x10^4$ cells/100µl/well into a 96 well plate. Incubate @ 37 °C O/N
- Day 2 serum starve cells for 2h, then add 100ul of treatment of different conc. of GLP-1/well in triplicate
- Day 3 Add ^{3}H-Thymidine (0.13µCi/well). Incubate @ 37 °C for 4h
  Remove medium by aspiration, add 50µl/well of disrupting buffer [40mM Tris pH 7.5/10mM EDTA/ 150mM NaCl], shake plats for ~2h.
  Measure

Trial #2

Treatment was diluted in serum free medium + 1% BSA +3 mM glucose

Summary

No significant effect of GLP-1 on proliferation of INS-1 cells was observed in either method applied.

I have tried different cultural conditions, including starting cell concentration; medium supplement with insulin, transferrin or glucose; different length of starvation, etc.

INS-1 cell line might not be suitable for such experiment.

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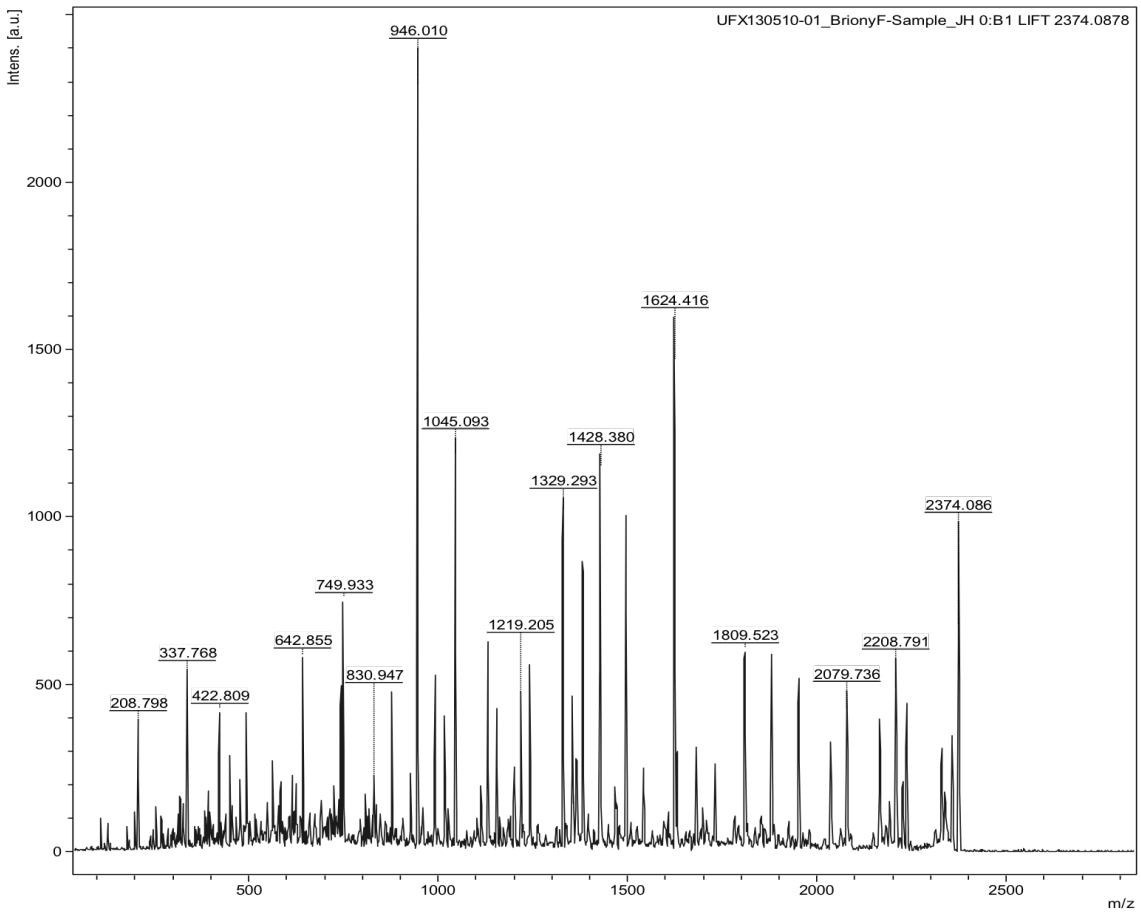
Appendix II

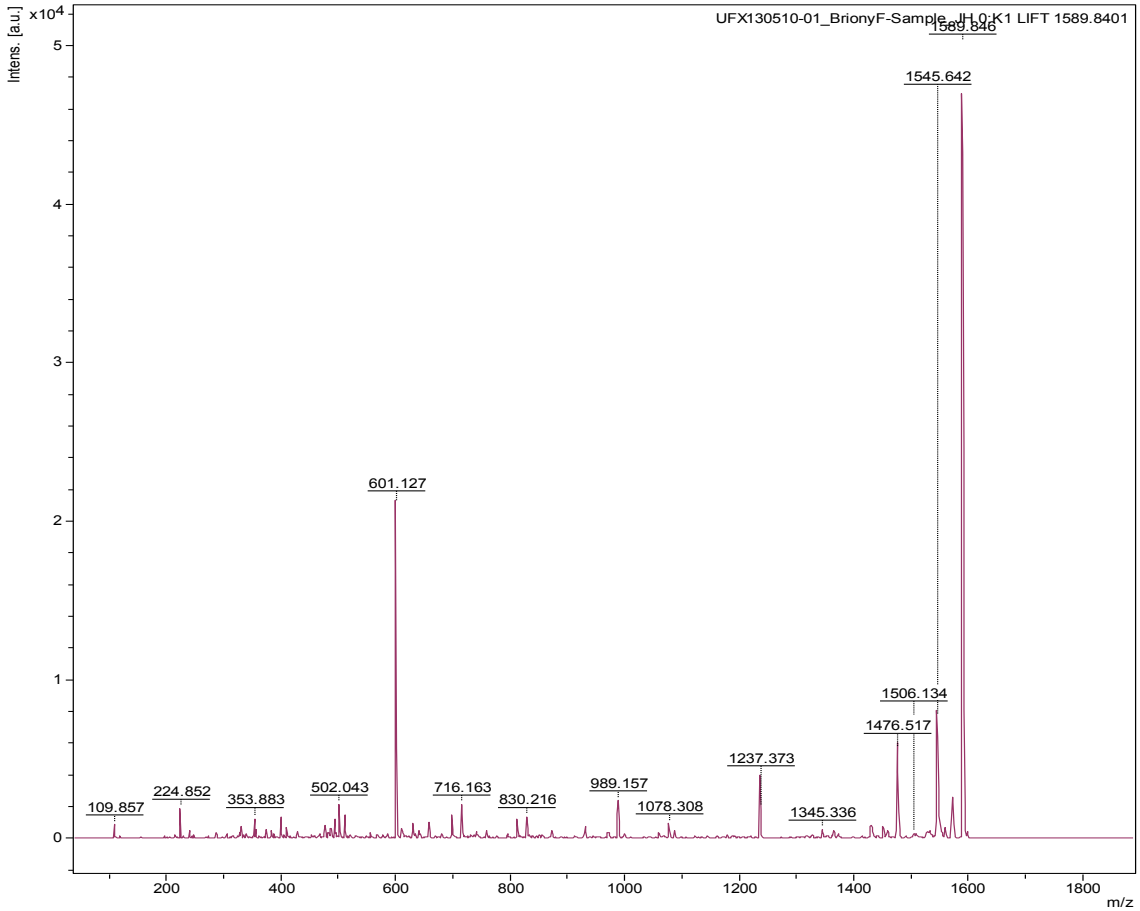
MALDI Mass spectrometry spectrum

Protocols (Done by Adelaide Proteomics Centre):

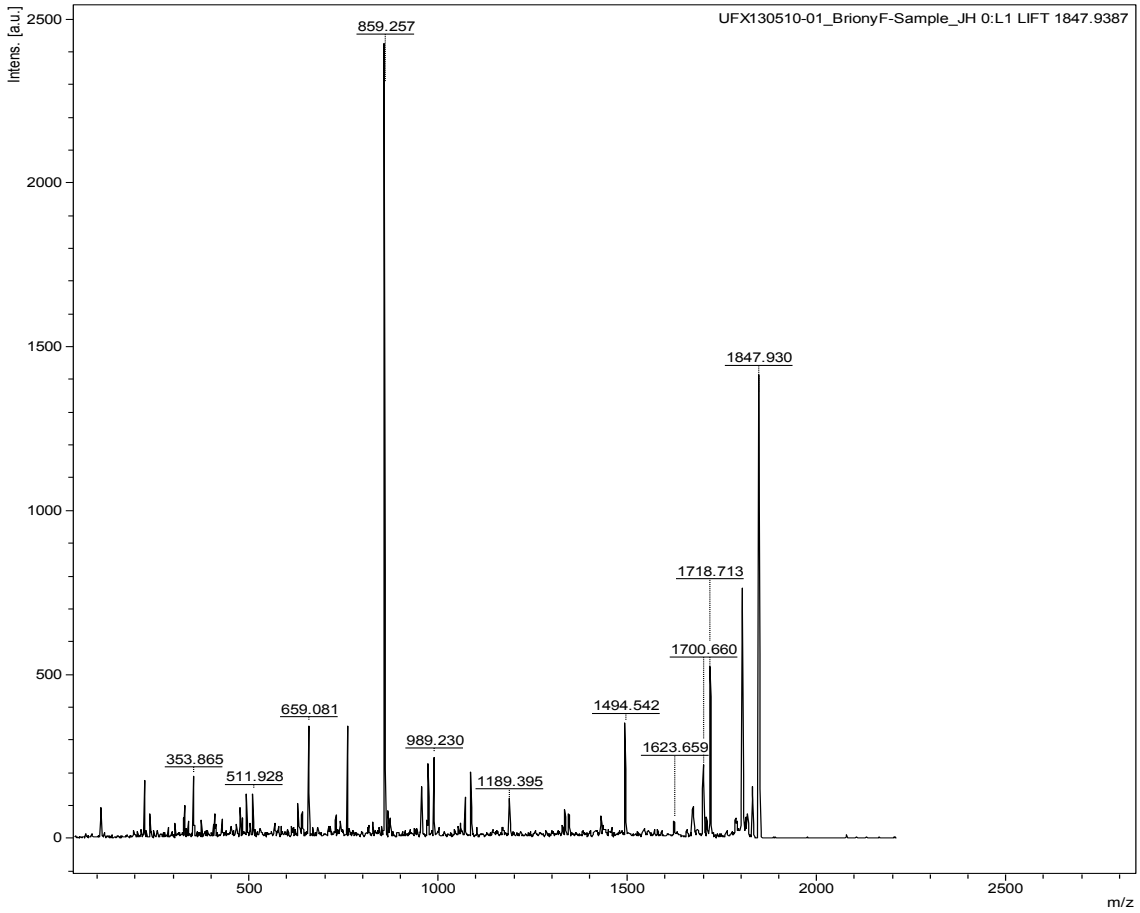
1 µl of fragments collected from RP-HPLC was sported onto an 800 µm Anchor Chip target plate (Bruke Daltonics, Bremen, Germany) separately and air dried. 1 µl of matrix [alpha-Cyano-4-hydroxycinnamic acid, 0.5 mg/mL in water/acetonitrile/TFA 10/90/0.1] was spotted subsequently and air dried. Mass spectra were acquired on an ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) operating in reflective positive mode. Instrument settings were set in flexControl software (Version 3.4, Bruker Daltonik GmbH). Sample *m/z* range was set to 300-4000 Da. 1000 shots were collected for the external calibration and sample measurement. External calibration was performed using a 1:20 dilution of peptide calibration standard (Bruker Daltonics). Laser intensity and detector gain were manually adjusted for optimal resolution. The MS spectra obtained were analysed using the FlexAnalysis software (Version 3.3, Bruker Daltonics) employing smoothing, background subtraction and peak detection algorithms.



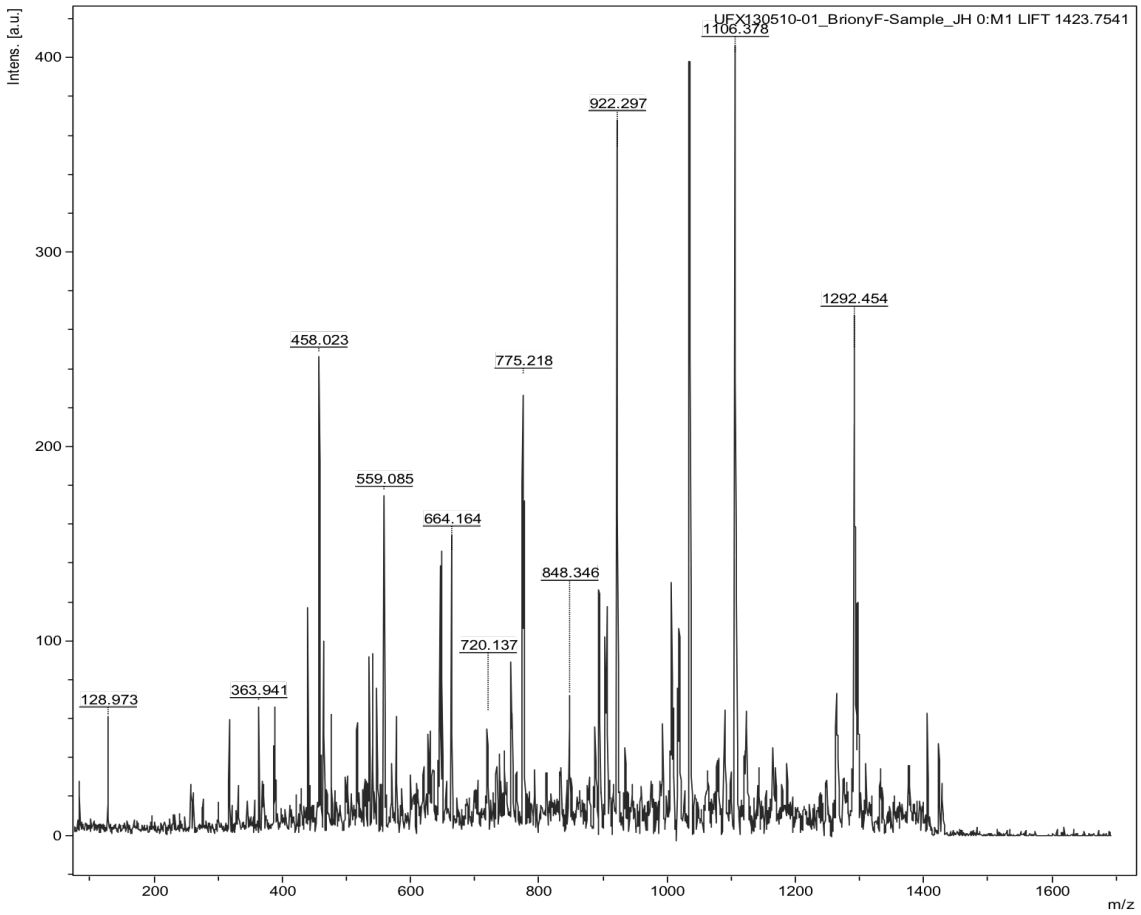
Fragmentation spectrum of hGLP-$1_{1\text{-}22}$
Mass 2374.08
Sequences: HAEGTFTSDVSSYLEGQAAKEF



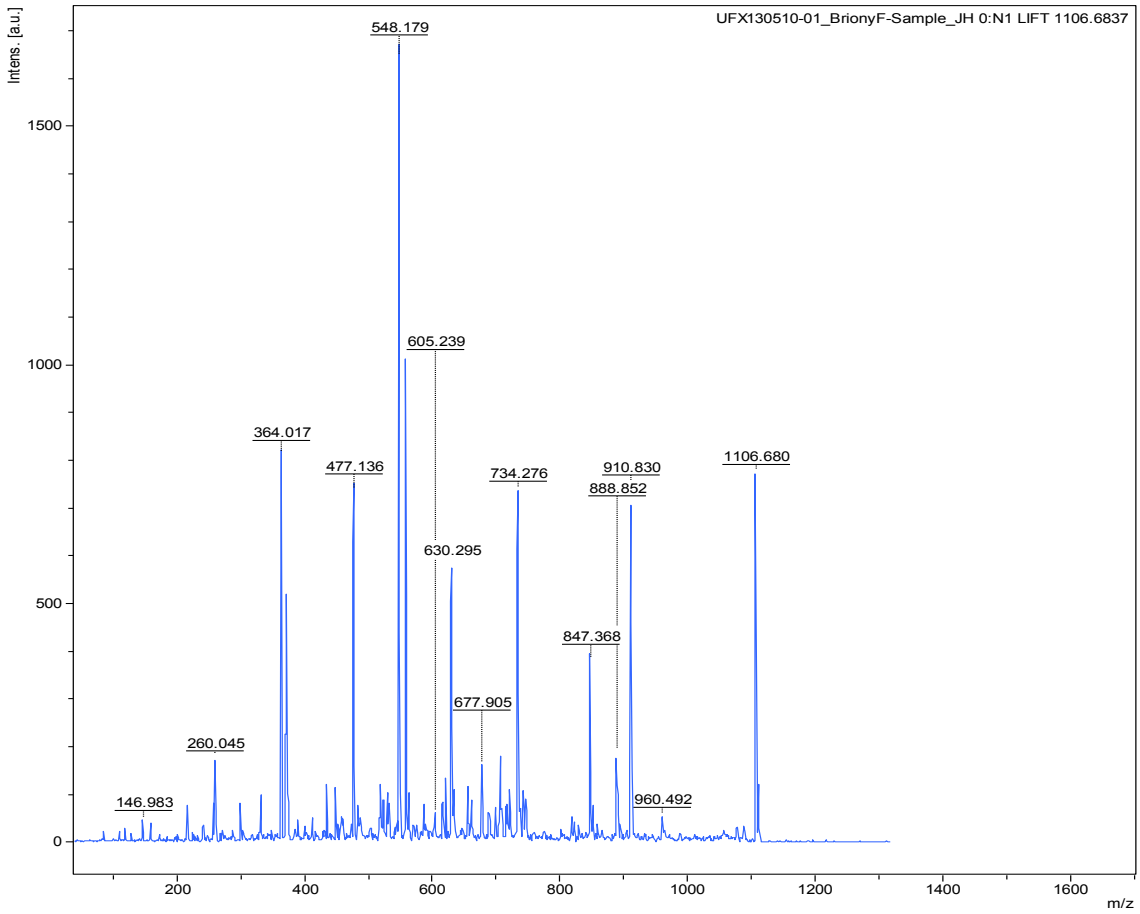
Fragmentation spectrum of pGLP-$1_{1\text{-}14}$
Mass 1589.8
Sequences: HSEGTFTNDVTRLL



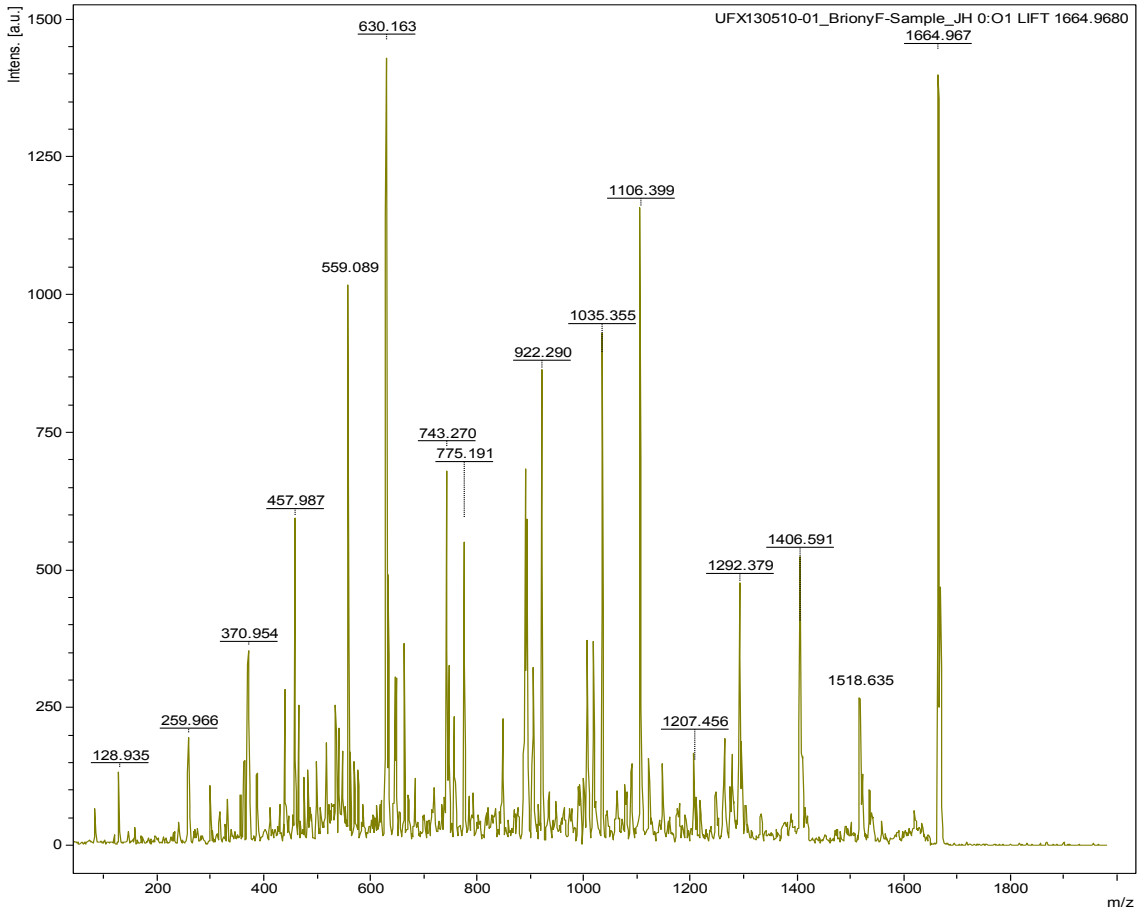
Fragmentation spectrum of pGLP-$1_{1\text{-}16}$
Mass 1847.9
Sequences: HSEGTFTNDVTRLLEE



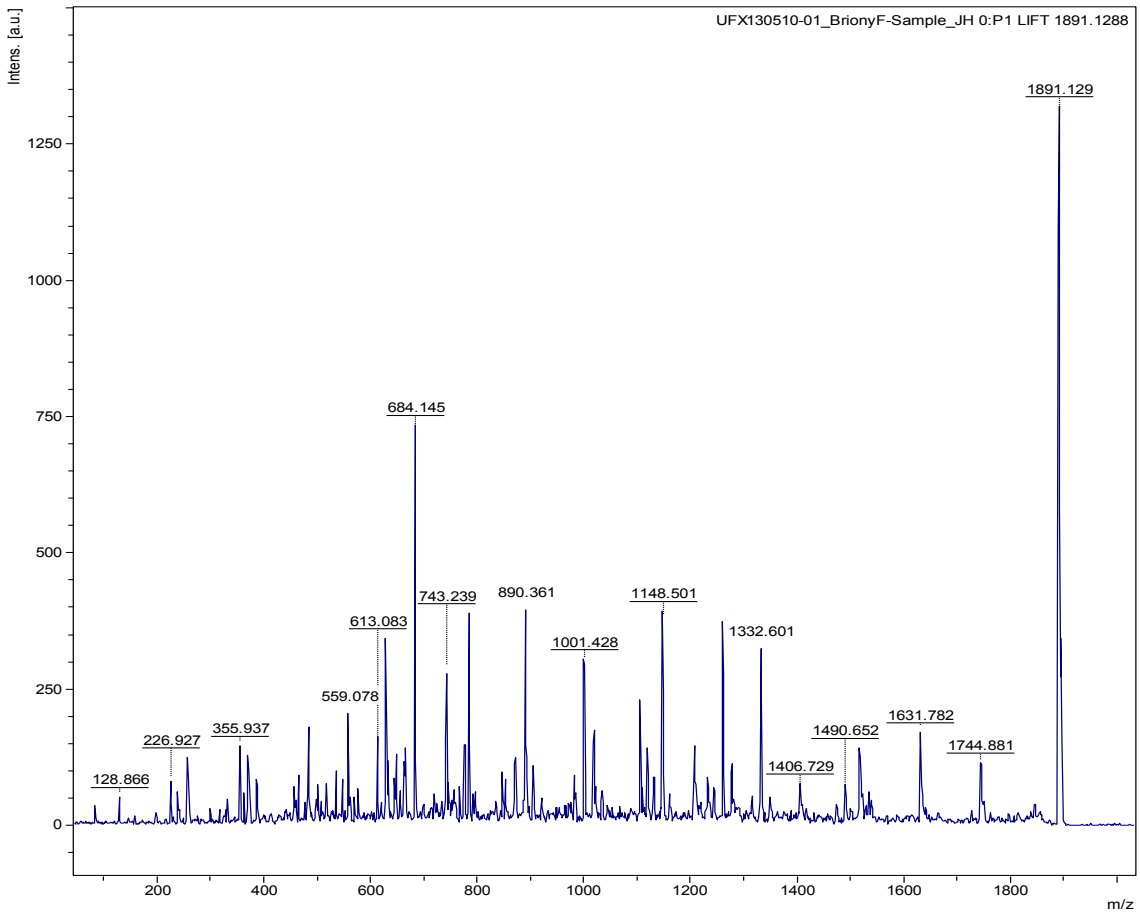
Fragmentation spectrum of pGLP-$1_{15\text{-}26}$
Mass 1423.7
Sequences: EEKATSEFIAWL



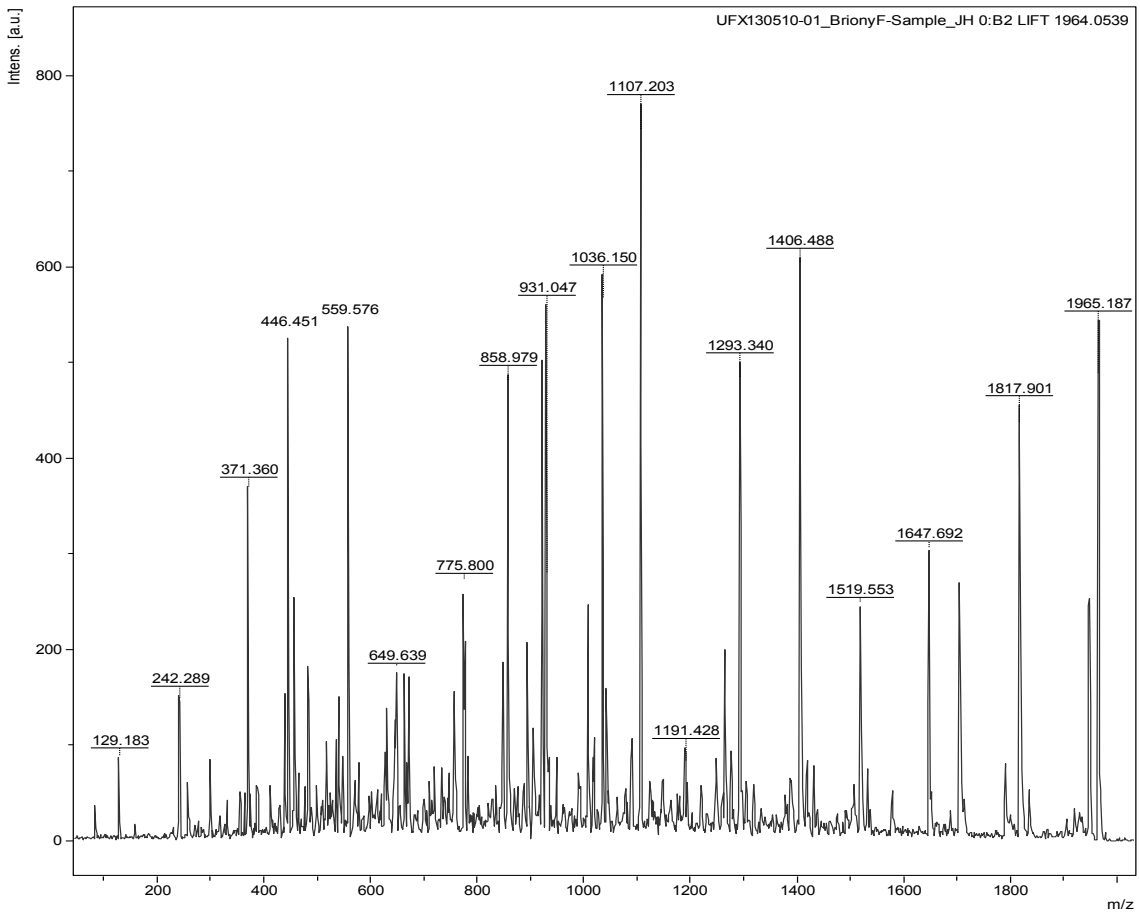
Fragmentation spectrum of pGLP-$1_{20\text{-}28}$
Mass 1106.68
Sequences: SEFIAWLLK



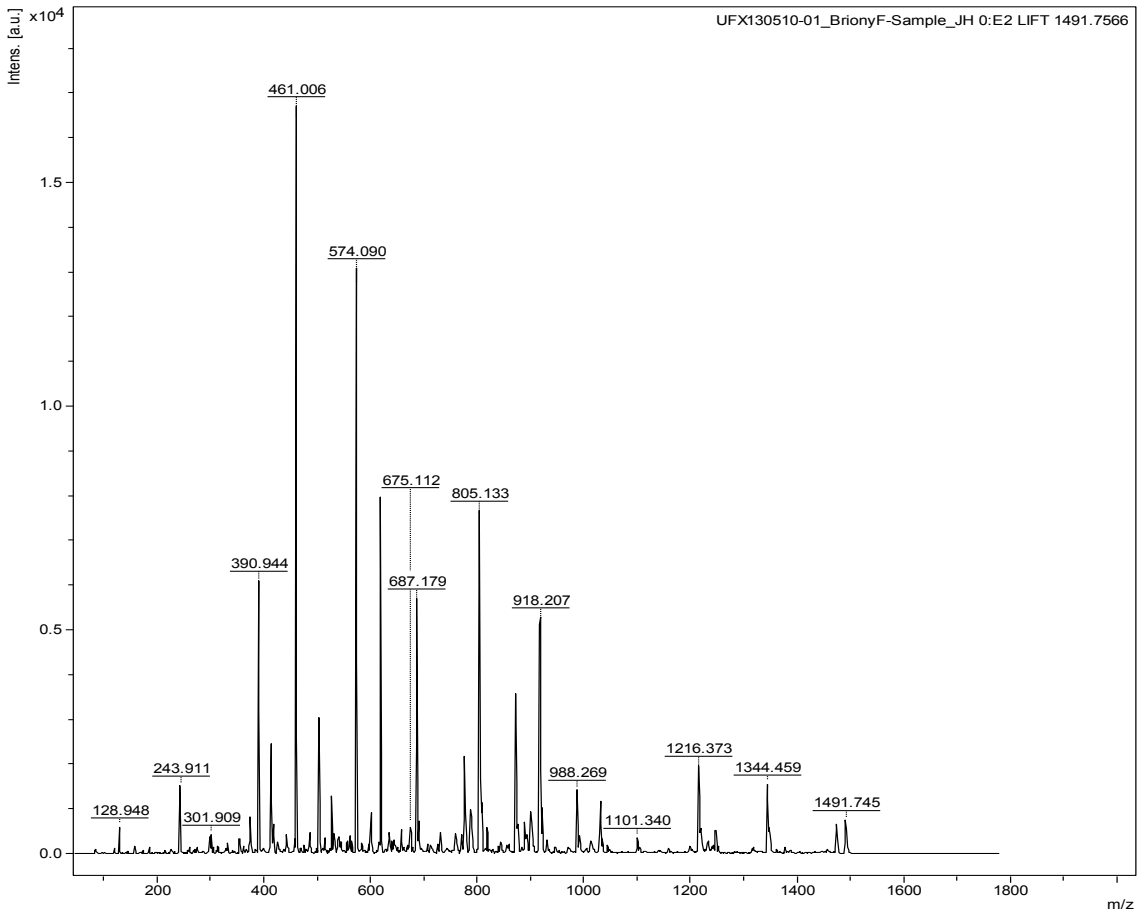
Fragmentation spectrum of pGLP-$1_{15\text{-}28}$
Mass 1664.96
Sequences: EEKATSEFIAWLLK



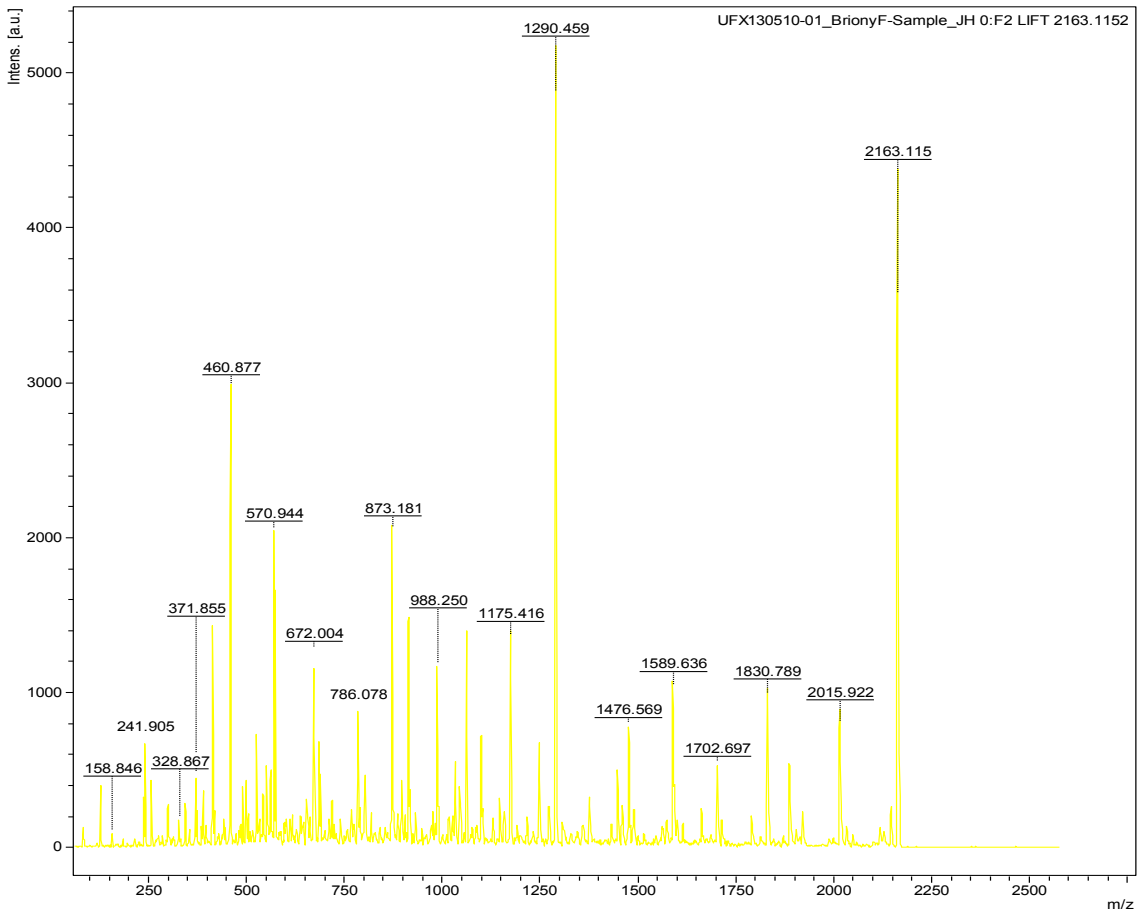
Fragmentation spectrum of pGLP-$1_{13\text{-}28}$
Mass 1891.05
Sequences: LLEEKATSEFIAWLLK



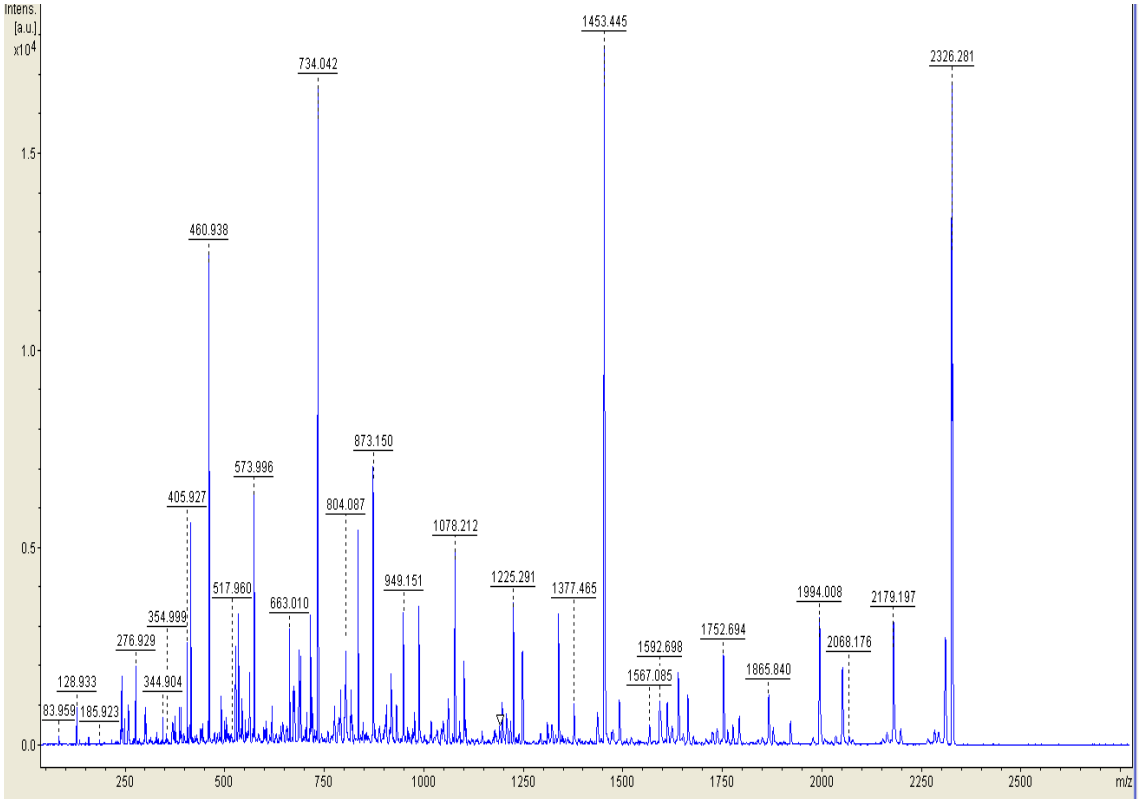
Fragmentation spectrum of pGLP-$1_{15\text{-}31}$
Mass 1964.05
Sequences: EEKATSEFIAWLLKGLE



Fragmentation spectrum of eGLP-$1_{20\text{-}31}$
Mass 1491.75
Sequences: NEFIDWLLKGQE



Fragmentation spectrum of eGLP-$1_{14\text{-}31}$
Mass 2163.11
Sequences: LEEKATNEFIDWLLKGQE



Fragmentation spectrum of eGLP-$1_{13\text{-}31}$
Mass 2326.28
Sequences: YLEEKATNEFIDWLLKGQE