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Ghrelin suppresses cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) in the intestine, and attenuates the anorectic effects of CCK, PYY and GLP-1 in goldfish (*Carassius auratus*)

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ABSTRACT

Ghrelin is an important gut-derived hormone with an appetite stimulatory role, while most of the intestinal hormones, including cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), are appetite-inhibitors. Whether these important peptides with opposing roles on food intake interact to regulate energy balance in fish is currently unknown. The aim of this study was to characterize the putative crosstalk between ghrelin and CCK, PYY and GLP-1 in goldfish (*Carassius auratus*). We first determined the localization of CCK, PYY and GLP-1 in relation to ghrelin and its main receptor GHS-R1a (growth hormone secretagogue 1a) in the goldfish intestine by immunohistochemistry. Colocalization of ghrelin/GHS-R1a and CCK/PYY/GLP-1 was found primarily in the luminal border of the intestinal mucosa. In an intestinal explant culture, a significant decrease in *prepro-cck*, *prepro-pyy* and *proglucagon* transcript levels was observed after 60 min of incubation with ghrelin, which was abolished by preincubation with the GHS-R1a ghrelin receptor antagonist [D-Lys3]-GHRP-6 (except for *proglucagon*). The protein expression of PYY and GLP-1 was also downregulated by ghrelin. Finally, intraperitoneal co-administration of CCK, PYY or GLP-1 with ghrelin results in no modification of food intake in goldfish. Overall, results of the present study show for the first time in fish that ghrelin exerts repressive effects on enteric anorexigens. It is likely that these interactions mediate the stimulatory effects of ghrelin on feeding and metabolism in fish.

1. Introduction

Regulation of food intake and energy homeostasis relies on complex interactions between various peripheral components transmitting the metabolic status of the organism, and the central nervous system, responsible for receiving, processing and responding to this information. Among the peripheral signals involved in this process, diverse gastrointestinal peptides are responsible for the short-term control of hunger and satiety (Konturek et al., 2004). One of these gastrointestinal peptides is ghrelin, particularly important for being so far the most potent peripherally-produced peptide exerting a stimulatory effect on food intake (Kaiya et al., 2013a; Kang et al., 2011; Müller et al., 2015). Ghrelin was purified in 1999 from the rat stomach and was described as a 28 amino acid peptide (Kojima et al., 1999), although it is shorter in many species, *e.g.* goldfish (*Carassius auratus*; Miura et al., 2009). Ghrelin is posttranslationally modified by an acylation catalyzed by ghrelin O-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008), modification that is essential for the peptide to bind to its receptor, the G protein-coupled growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999).

The orexigenic effects of ghrelin are mainly mediated *via* central mechanisms located primarily within the hypothalamus in both mammals (Goto et al., 2006; Wang et al., 2014) and fish (Miura et al., 2006). For this, ghrelin, mainly synthesized by enteric endocrine cells, reaches the central nervous system *via* the circulatory system by crossing the blood-brain barrier (Banks et al., 2002). Apart from secreting into circulation, enteric endocrine cells have been shown to have the potential for a secretory response *via* an intercellular (paracrine) route. Paracrine mechanisms of action of ghrelin in the intestine has

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been previously reported, for instance, in regulating cell proliferation in small intestinal IEC-6 cells (Yu et al., 2013).

The existence of paracrine or even autocrine actions for enteric endocrine cells suggests the existence of possible modulatory roles among the different enteric peptides. In mammals, several interactions were reported between ghrelin and peripheral anorexigens (Wisser et al., 2010), which includes cholecystokinin (CCK), peptide YY (PYY) and glucagon like peptide-1 (GLP-1). These hormones are postprandially released by I-cells in the duodenum (Gibbs et al., 1973) and by L-cells in the distal gastrointestinal tract (PYY and GLP-1; (Batterham and Bloom, 2003; Holst, 2007). Two forms of PYY are found in circulation in mammals: the full-length form PYY(1–36) and the truncated peptide PYY(3–36) (Ballantyne, 2006). Elevated food intake after peripheral ghrelin administration is antagonized by pre- or simultaneous injection of CCK in rats (Date et al., 2005; Kobelt et al., 2005). Similarly, peripheral ghrelin injections in rats attenuate the inhibition of food intake and gastric emptying induced by PYY(3–36) (Chelikani et al., 2006). Intracerebroventricular injection of GLP-1 significantly inhibits ghrelin-induced stimulation of food intake (Schusdziarra et al., 2008), as well as intravenous co-infusion of ghrelin attenuates the GLP-1-induced reduction of food intake and its inhibitory effect on gastric emptying (Chelikani et al., 2006).

While numerous observations point to the existence of important interactions between ghrelin and its anorexigenic counterparts in the regulation of food intake in mammals, whether ghrelin is interacting with other gastrointestinal peptides in fish is yet to be investigated. This study aimed to determine the possible interactions between ghrelin and CCK, PYY and GLP-1 in goldfish. We first determined whether ghrelin and its main receptor GHS-R1a are present in the same endocrine cells that produce these anorexigenic hormones in the goldfish gastrointestinal tract. Then, we studied whether ghrelin modulates CCK, PYY and/or GLP-1 gene and protein expression in cultured intestinal fragments *in vitro*. Finally, we tested the combined effects of ghrelin and each of these anorexigenic hormones in the regulation of food intake by intraperitoneal (ip) co-administration.

2. Materials and methods

2.1. Animals

Female and male goldfish (*Carassius auratus*) of the common variety, with a body weight (bw) of 27 ± 8 g, except for immunohistochemistry studies in which smaller fish were used (5 ± 1 g) to avoid perfusion, were obtained from a commercial supplier (Aquatic Imports, Calgary, Canada). Fish were housed in 300 L aquaria with filtered fresh water at 20 ± 2 °C and continuous aeration, and maintained under a 12 h light:12 h darkness (12L:12 D) photoperiod (lights on at 07:00 h). For the food intake experiment, fish were acclimated to 10 L aquaria (n = 3/aquarium), which received a constant flow of temperature-controlled water (21 ± 2 °C). Food from a commercial pellet diet (Goldfish granules, Aqueon, Franklin, USA) was offered daily at 10:00 h until visual apparent satiety. All fish studies adhered to the Canadian Council of Animal Care guidelines, and research protocols were approved by the Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 2012–0082).

2.2. Immunohistochemical localization of ghrelin/GHS-R1a and CCK/PYY/GLP-1 in the goldfish intestine

The anterior intestine (1 cm after the intestinal bulb) from goldfish was collected at the scheduled feeding time, and fixed as described in (Sánchez-Bretaño et al., 2015). Tissues were processed (dehydrated and embedded in paraffin) at the Prairie Diagnostic Services, University of Saskatchewan. The protocol for IHC was performed as previously described (Diotel et al., 2011) with slight modifications. Briefly, after deparaffination, rehydration and blocking, sections (7 μm thickness) were incubated overnight with a mixture (except in the cases listed below) of primary antibody again ghrelin/GHS-R1a and each of the other gastrointestinal hormones (mouse monoclonal to ghrelin, Catalog # ab57222; Abcam, Toronto, Canada; rabbit polyclonal to ghrelin, Catalog # H-031-31; rabbit polyclonal to GHS-R1a, Catalog # H-001-62; both from Phoenix Pharmaceuticals, Burlingame, USA; rabbit polyclonal to cholecystokinin, Catalog # ab83180; rabbit polyclonal to peptide YY, Catalog # ab22663; mouse monoclonal to GLP-1 (amidated), Catalog # ab26278; all from Abcam), each diluted 1:200, at room temperature. Antibodies used for GHS-R1a, CCK and PYY were not incubated as a mixture because they are all raised in the same host species; instead, colocalization was approached by staining consecutive sections with the different antibodies separately. Even though antibodies used here are specifically designed mammals, a BLAST analysis of the immunogens showed >70% identity with fish species (Supplemental Table 1). Indeed, some of them were previously used and characterized in fish (Kerbel and Unniappan, 2012). Nevertheless, it is likely that the use of heterologous antibodies leads to a certain degree of non-specificity in our findings. Therefore, ghrelin-like, GHS-R1a-like, CCK-like, PYY-like and GLP-1-like were used to refer to immunostaining obtained in this study. The following day, sections were washed and incubated for 1 h at room temperature with a mixture of secondary antibodies (1:2000 dilution each). Secondary antibodies used were: goat anti-mouse IgG Alexa Fluor 594, goat anti-rabbit IgG Alexa Fluor 488 (both from Invitrogen, Burlington, Canada), Texas Red anti-rabbit IgG (Vector Laboratories, Burlington, Canada) and FITC anti-mouse IgG (Abcam). A separate set of negative control slides were only treated with the secondary antibodies. Additionally, primary antibodies pre-absorbed with synthetic goldfish ghrelin, mouse CCK, goldfish PYY or mouse GLP-1 (1:10 M ratio) overnight were used as pre-absorption controls for confirming the antibody specificity. The percentage identity of antibody epitopes with fish peptide sequences is provided in Supplemental Table 1. All primary and secondary antibodies were diluted in antibody diluent reagent (Dako, Mississauga, Canada). Finally, slides were again washed, and mounted using VECTASHIELD Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Slides were then assessed using a Nikon Eclipse Ti-Inverted fluorescence microscope (Nikon Instruments, Melville, USA) connected to a Nikon DS-Qi1 MC camera. Micrographs were adjusted linearly for light and contrast using Photoshop CS6 (Adobe Systems Inc., San Jose, USA). For the quantification of immunopositive cells, the number of cells in the mucosa and submucosa, immunoreactive for ghrelin or GHS-R1a (red), CCK, PYY or GLP-1 (green), or colocalizing both ghrelin/GHS-R1a and CCK/PYY/GLP-1 (yellow) were counted in all sections assessed (n = 3 sections). The total number of cells counted for ghrelin or GHS-R1a alone and for CCK, PYY or GLP-1 alone was plotted as pie charts, showing the relative abundance of ghrelin/GHS-R1a and CCK/PYY/GLP-1 in each of the assessed combinations. Then, to calculate the percentage of ghrelin/GHS-R1a cells that colocalizes CCK/PYY/GLP-1 and *vice versa*, the number of cells positive for the two peptides was calculated as a percentage of the total number of ghrelin/GHS-R1a cells (for the first case) or of the total number of CCK/PYY/GLP-1 (for the second case). These percentages were indicated and shadowed in the corresponding pie charts.

2.3. In vitro study of the effects of ghrelin on CCK, PYY and GLP-1 gene and protein expression in goldfish intestine

Tissue culture was performed as previously described for goldfish (Sánchez-Bretaño et al., 2016) with slight modifications. Briefly, the anterior intestine (4–5 cm after the intestinal bulb) of 24 h-fasted goldfish (n = 6) was removed and cut into fragments of approximately 1–2 mm width. Intestinal portions were immersed in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, New York, USA) supplemented with 44 mM sodium bicarbonate, 10% penicillin-streptomycin and 0.5% gentamicin for 1 min, and then distributed (20 mg tissue/