



Ghrelin, neuropeptide Y (NPY) and cholecystokinin (CCK) in blunt snout bream (*Megalobrama amblycephala*): cDNA cloning, tissue distribution and mRNA expression changes responding to fasting and refeeding



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ABSTRACT

Blunt snout bream (*Megalobrama amblycephala* Yih, 1955) is an endemic freshwater fish in China for which the endocrine mechanism of regulation of feeding has never been examined. Ghrelin, neuropeptide Y (NPY) and cholecystokinin (CCK) play important roles in the regulation of fish feeding. In this study, full-length cDNAs of ghrelin, NPY and CCK were cloned and analyzed from blunt snout bream. Both the ghrelin and NPY genes of blunt snout bream had the same amino acid sequences as grass carp, and CCK also shared considerable similarity with that of grass carp. The three genes were expressed in a wide range of adult tissues, with the highest expression levels of ghrelin in the hindgut, NPY in the hypothalamus and CCK in the pituitary, respectively. Starvation challenge experiments showed that the expression levels of ghrelin and NPY mRNA increased in brain and intestine after starvation, and the expression levels of CCK decreased after starvation. Refeeding could bring the expression levels of the three genes back to the control levels. These results indicated that the feeding behavior of blunt snout bream was regulated by the potential correlative actions of ghrelin, NPY and CCK, which contributed to the defense against starvation. This study will further our understanding of the function of ghrelin, NPY and CCK and the molecular mechanism of feeding regulation in teleosts.

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1. Introduction

Food intake plays an important role in fish growth and production performance, which is regulated by both central and peripheral signals, involving the central nervous system (CNS), gastrointestinal (GI) tract, adrenals, pancreas and adipose tissue (Naslund and Hellstrom, 2007). Like other vertebrates, fish regulate their feeding by key appetite-stimulating (orexigenic) and appetite-inhibiting (anorexigenic) endocrine factors. Orexigenic factors include ghrelin, neuropeptide Y (NPY), galanin, orexins and agouti-related protein (AgRP). Anorexigenic factors include cholecystokinin (CCK), leptin, cocaine- and amphetamine-regulated transcript (CART), and corticotropin-releasing factor

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(CRF) (Volkoff et al., 2010). Ghrelin, NPY and CCK are important endocrine factors in the regulation of feeding, as well as growth and reproduction of fish.

Ghrelin is a brain-gut peptide first purified from rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R). It has two main physiological actions: regulation of appetite and hormone (including GH (growth hormone)) release (Kaiya et al., 2008; Kojima et al., 1999). So far, ghrelin has been isolated from several teleost and elasmobranch fish species (Kaiya et al., 2011). Previous studies showed that ghrelin mRNA was mainly expressed in the fish stomach and in the intestine of stomachless fish, but it was also detected in the hypothalamus, spleen, gill, skin, kidney, heart and liver of fish (Feng et al., 2012; Xu and Volkoff, 2009). The tissue distribution patterns vary from species to species in fish and other vertebrates (Kaiya et al., 2011). Ghrelin can stimulate GH release. For example, Unniappan and Peter (2004) reported that intracerebroventricular (ICV) or intraperitoneal (IP) injections of ghrelin stimulated the release of GH in

15 min in the goldfish (*Carassius auratus*). In addition, both IP and ICV injections of ghrelin stimulated food intake in goldfish (Unniappan et al., 2004a) and this effect was mediated by NPY and orexin pathways (Miura et al., 2006, 2007). Ghrelin mRNA expression increased during starvation in goldfish (Unniappan et al., 2004b), sea bass (*Dicentrarchus labrax*) (Terova et al., 2008) and zebrafish (*Danio rerio*) (Amole and Unniappan, 2009). In addition, many studies have shown that ghrelin is a multifunctional hormone involved in the regulation of various physiological processes in fish (Kaiya et al., 2008).

NPY is a peptide with 36 amino acid residues belonging to the NPY family, which is present in the central and peripheral nervous systems and controls the appetite and blood pressure (Kalra et al., 1999). NPY is highly conserved among vertebrates, including fish, and it is one of the most potent orexigenic agents in mammals (Halford et al., 2004; Hoyle, 1999). Numerous studies show that NPY is involved in the regulation of feeding in fish. Both central and peripheral injections of NPY increase food intake in goldfish (López-Patiño et al., 1999), channel catfish (*Ictalurus punctatus*) (Silverstein and Plisetskaya, 2000) and tilapia (*Oreochromis mossambicus*) (Kiris et al., 2007). Fasting induces an increase of NPY expression in the hypothalamus of goldfish (Narnaware and Peter, 2001) and salmon (*Oncorhynchus spp.*) (Silverstein et al., 1999), and refeeding can reverse these effects (Narnaware and Peter, 2001). As reported for some mammals, NPY in fish species also interacts with a number of appetite regulators, including CART, leptin, orexins, and ghrelin (Mercer et al., 2011; Volkoff, 2006).

CCK, a peptide hormone which can stimulate pancreatic secretion (Murashita et al., 2006), is dominantly distributed in the brain and digestive tract of vertebrates, including fish (Johnsen, 1998). In mammals, CCK is released from intestinal endocrine cells during a meal and decreases gastric emptying, stimulates pancreatic and gastric secretions and reduces food intake via vagal afferent pathways (Chandra and Liddle, 2007; Rehfeld et al., 2007). CCK also influences appetite in fish which has been identified in several fish, including red drum (*Sciaenops ocellatus*) (Webb et al., 2010), winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a), yellowtail (*Seriola quinqueradiata*) (Murashita et al., 2006), winter skate (*Raja ocellata*) (MacDonald and Volkoff, 2009b) and grass carp (*Ctenopharyngodon idellus*) (Feng et al., 2012). Central or peripheral injection of sulfated CCK-8 (CCK-8 has a well conserved C-terminal octapeptide among vertebrates, and it is sulphated at the tyrosine residue and is the most abundant form of CCK) suppressed food intake in goldfish (Himick and Peter, 1994), while oral administration of CCK receptor antagonists increased food intake in rainbow trout (Gelineau and Boujard, 2001), which revealed the suppression role of CCK as an appetite-regulating hormone. In addition, CCK mRNA levels increased in the brain of goldfish (Peyon et al., 1999) and in the pyloric caeca of yellowtail (Murashita et al., 2007) following a meal.

The blunt snout bream (*Megalobrama amblycephala* Yih, 1955) is an endemic freshwater fish in China. It was originally distributed in the middle reaches of the Yangtze River and a few accessory lakes, of which the Liangzi Lake, Poyang Lake and Yuni Lake are three main sources (Li et al., 1991). In addition, it was also introduced to North America, Africa and Eurasia (Li et al., 2012). In recent years, it has been intensively cultured in China because of its wide range of food sources, fast growth, tender flesh and high larval survival rate. In 2012, the total production of the bream reached 705,821 tonnes (FBAMC, 2013).

Our previous study showed that ghrelin, NPY and CCK were all expressed throughout the embryonic and larval development stages, and higher expression levels were found in larval stages than in embryonic stages. The mRNA expression levels of these

three genes in larvae varied significantly until 30 days after hatching (Ping et al., 2014). To further investigate the regulation of the appetite endocrine factors ghrelin, NPY and CCK in blunt snout bream, we cloned the full-length cDNAs of these appetite-regulating hormones, examined the mRNA expression in various adult tissues. Because juveniles are harder to culture than adults (and therefore the juvenile stage represents an important step in the developmental process for producing an aquaculture product), we assessed the effects of fasting and refeeding on gene expression of these hormones in juvenile brain and intestine. The aim of our research was to contribute to the further development of the blunt snout bream aquaculture industry by improving our understanding of the effect of fasting and refeeding on the gene expression of ghrelin, NPY and CCK in this important fish.

2. Materials and methods

2.1. Fish and samples

For cDNA cloning and mRNA expression analyses of ghrelin, NPY and CCK in various tissues, individuals of adult blunt snout bream (~400 g each), were obtained from the Nanhu fish breeding base of Huazhong Agricultural University. The blunt snout breams were acclimated in indoor tanks with freshwater at 20 °C and fed daily at 09:00 for at least 1 week before the experiment. Five fish individuals were sampled at 4 h post-feeding and were anesthetized with tricaine methanesulfonate (MS-222, 100 mg/L) before dissection. Tissues were isolated and immediately immersed in liquid nitrogen and then stored at -80 °C until RNA isolation.

To evaluate the effects of starvation and refeeding on the gene expression of ghrelin, NPY and CCK, approximately 100 individuals of juvenile blunt snout bream (~10 g each) were randomly divided into two groups (experimental and control groups, respectively). The two groups of breams were respectively maintained in two indoor tanks with sufficient dissolved oxygen at 25 ± 1 °C and were fed with a commercial diet (Hubei Haid Feeds Company, Wuhan, China) twice a day (08:00 and 16:00) for 2 weeks to acclimate them to the artificial culture environment. After acclimation, the individuals of the experimental group were starved for 15 days and then were refed to apparent satiety twice a day for 15 days, while the individuals of the control group were kept under the same feeding condition as the acclimation period. Five individuals were randomly sampled from each group at the following time points: 0, 1, 4, 7, 15 days of starvation, and 1, 4, 7, 15 days of refeeding. To avoid the short-term effects of feeding the fish were sampled 4 h post-feeding (12:00 each day). The sampled individuals were anesthetized with MS-222 (50 mg/L), and then the whole brain and the intestine (foregut) were rapidly isolated for RNA extraction.

This study was approved by the Institutional Animal Care and Use Committees (IACUC) of Huazhong Agricultural University.

2.2. Molecular cloning of ghrelin, NPY and CCK cDNAs

Total RNA was extracted from the brain tissue of adult blunt snout bream using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The integrity and purity of RNA were assayed by agarose gel electrophoresis and a Nanodrop ND-2000 spectrophotometer (Thermo Electron Corporation, USA), respectively. cDNA was reverse transcribed from total RNA using a moloney murine leukemia virus (M-MLV) Reverse Transcriptase kit (Toyobo, Osaka, Japan) following the manufacturer's protocol. Based on the partial cDNA sequences of ghrelin, NPY and CCK genes that were previously obtained (Ping et al., 2014), the full-length

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