General and Comparative Endocrinology 255 (2018) 56–63

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

The evolution and functional characterization of lined seahorse (*Hippocampus erectus*) CCKs involved in fasting and thermal stress response

Huixian Zhang [a], Geng Qin [a], Jinhui Sun [b], Bo Zhang [a,c], Qiang Lin [a,c,*]

[a] CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, Guangdong 510301, PR China
[b] Tianjin Key Lab of Aqua-Ecology and Aquaculture, College of Fisheries, Tianjin Agricultural University, Tianjin 300384, PR China
[c] University of Chinese Academy of Sciences, Beijing 100049, PR China

ARTICLE INFO

Article history:
Received 11 July 2017
Revised 27 September 2017
Accepted 14 October 2017
Available online 16 October 2017

Keywords:
Cholecystokinin
Tissue distribution
Fasting
Feeding
Hippocampus erectus

ABSTRACT

The peptide cholecystokinin (CCK) plays an important role in the regulation of vertebrate appetite and feeding behaviour. In the present study, the full-length cDNA and genomic DNA sequences of two CCK precursors were cloned and analysed in the Syngnathidae fish, the lined seahorse (*Hippocampus erectus*). Both CCK1 and CCK2 in the seahorse consist of four exons. The sequence of the octapeptide of seahorse CCK1 (DYMGWMDF) was the same as that of the chicken and human, while the octapeptide of seahorse CCK2 (DYEGWMDF) was unique among vertebrates. According to the phylogenetic analysis, two types of CCKs were produced by teleost-specific genome duplication (TGD). Both CCK1 and CCK2 were highly expressed in the brain, while detectable amounts of CCK1 mRNA in the brood pouch and CCK2 mRNA in the intestine were also found. Both CCK1 and CCK2 mRNA levels significantly increased during the transition from endogenous to exogenous nutrition. Additionally, fasting induced a significant increase in the CCK1 mRNA expression in the brain of juvenile seahorses but had no effect on CCK2 transcript levels. In addition, the CCK1 and CCK2 mRNA levels in the seahorse brain significantly increased after a high-temperature treatment. Thus, the mRNA expression of CCK had obvious tissue specificities and this preliminary study opens new avenues for further functional studies on the endocrine regulations of CCK in the transition from endogenous to exogenous nutrition, food intake regulation and metabolism in the seahorse.

1. Introduction

Cholecystokinin (CCK) is a neuroendocrine peptide with the common C-terminal tetrapeptide sequence Trp-Met-Asp-Phe-NH2 (Larsson and Rehfeld, 1977). CCK is found mainly in the brain and the gastrointestinal tract and has multiple biologically active forms. The C-terminal octapeptide (CCK-8) is the main form in the brain (Moran and Kinzig, 2004). Pro-CCK has three sulphated tyrosine residues that are considered important for its activity at CCK receptors (Beinfeld, 2003).

In mammals, CCK has been implicated in many physiological actions, including the regulation of feeding behaviour, anterior pituitary hormone release, pancreatic secretion, gallbladder contraction, stomach secretion and gut motility (Morley, 1987). However, CCK functions primarily as a satiety signal (Cupples, 2002). Thus, CCK is essential for the efficient digestion and absorption of nutrients.

In teleost, the CCK genes have been cloned in the Japanese flounder (*Paralichthys olivaceus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*) (Peterson et al., 2012), goldfish (*Carassius auratus*) and grass carp (*Ctenopharyngodon idellus*) (Feng et al., 2012; Volkoff et al., 2005). Cleavage site analyses of fish CCK cDNAs suggest that pro-CCK may be processed into octapeptides. Pro-CCK is also cleaved into fragments of different lengths, but CCK-8 is the major product of posttranslational processing (Jensen et al., 2001).

In all examined fishes, CCK mRNAs are mainly expressed in the brain and intestine (Feng et al., 2012; Johnsen, 1998; Kurokawa et al., 2003). In goldfish and grass carp, CCK mRNA was widely expressed in the brain, and the highest levels were found in the hypothalamus and pituitary, but CCK is also highly expressed in the gastrointestinal tract (Feng et al., 2012; Peyon et al., 1999). CCK has been demonstrated to be expressed at the protein level

* Corresponding author at: CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, Guangdong 510301, China.
E-mail address: linqiang@scsio.ac.cn (Q. Lin).

https://doi.org/10.1016/j.ygcen.2017.10.006
0016-6480/

in the nervous system and gut of several fish species (Barrenechea et al., 1994; Kamisaka et al., 2001).

Similar to that in mammals, CCK in fish can influence digestion and feeding processes. In the presence of food in the intestine, CCK-related peptides are released and induce the contraction of the gallbladder and gastric emptying (Aldman and Holmgren, 1995) (Olsson et al., 1999). In goldfish, central or peripheral injections of sulphated CCK-8 suppress food intake (Volkoff et al., 2003), whereas the oral administration of CCK antagonists increase food intake in the rainbow trout (Gélineau and Boujard, 2001). Recently, CCK was reported to play an important role in the regulation of glucose metabolism in the rainbow trout (Polakof et al., 2011). To date, information regarding the physiological roles of CCK in ovoviviparous fish remains limited.

The seahorse is an ovoviviparous fish whose embryos can obtain paternal nutrients during pregnancy through the male's brood pouch and maternal nutrients from the yolk (Foster and Vincent, 2004). The lined seahorse (*Hippocampus erectus*), which was imported from America, became the main species of seahorse aquaculture in China (Lin et al., 2008; Lin et al., 2009). To enhance the growth rate of the lined seahorse, it is important to increase its food intake and feed conversion efficiency. To provide more information regarding the mechanism of appetite regulation in the lined seahorse, we cloned the full-length cDNAs of the CCKs and detected the mRNA expression levels of CCK in different adult tissues. We also investigated the expression profiles of the CCK mRNA in larval seahorses under different feeding regimes and thermal stress.

2. Materials and methods

2.1. Animals and experimental conditions

The lined seahorses used in the experiments were cultured at the Shenzhen Seahorse Center of the South China Sea Institute of Oceanology, Chinese Academy of Sciences (SCSIO-CAS), and animal ethics approval for experimentation was granted by the Chinese Academy of Sciences. The seahorses were maintained in re-circulating holding tanks (90 × 70 × 60 cm), and seawater was pumped directly from the South China Sea and treated with double sand filtration. The seahorses were fed twice a day (09:00 and 16:00 h) with frozen *Mysis* spp. Faeces and uneaten fish bait were siphoned off daily. The temperature, salinity, pH, light intensity, dissolved oxygen (DO), and photoperiod were maintained at (mean ± S.D.) 22 ± 0.5 °C, 25 ± 1.0‰, 7.9 ± 0.4, 2000 lx, 6.5 ± 0.5 mg L^{-1}, and 16L: 8D, respectively.

2.2. Cloning of the seahorse CCKs

Total RNA was extracted from the seahorse brains using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The first-strand cDNA was synthesized using a SMART cDNA Library Construction Kit (Clontech, USA), and 3′ and 5′ adaptors were added. The fragments of the seahorse CCK genes were searched for in the seahorse RNA-sequencing (RNA-Seq) data from our laboratory (Lin et al., 2016) using the BLAST program with the puffer CCK protein sequences. To confirm the cDNA sequences of the seahorse CCK genes, 3′ and 5′ rapid amplification of cDNA ends (RACE) was performed. The primers used for the RACE analysis were designed using the sequences shown in Table 1. The PCR parameters were 35 cycles of 94 °C for 30 s, 58–60 °C for 1 min, and 72 °C for 2 min. The RACE-PCR products were purified from an agarose gel and cloned into a pGEM-T easy vector (Promega, USA). The inserts were sequenced using an automated DNA sequencer (Applied Biosystems, 3730).

Table 1
Nucleotide sequences used in 5′ RACE PCR, 3′RACE PCR and qPCR assays for CCK and the internal reference gene β-actin.

Primer sequence			
Gene	Purpose	Primer	5′-3′ sequence
CCK1	Partial cDNA	CCK1 F1	GTGGTTCTGGTGGTCCTGTGC
		CCK1 R1	GCCGTCCAAAGTCCATCCAG
	5′RACE	CCK1 R2 (first)	CGCCTCTGCTGTTGGGTGA
		CCK1 R3 (nest)	TCCGACGCACCGAACCTTT
	3′RACE	CCK1 F2 (first)	GAACTTGTCACCCAACAGCAG
		CCK1 F3 (nest)	GGGCTGGATGGACTTTGGA
	Real-time PCR	CCK1 qF	TGTGGTTCTGGTGGTCCTG
		CCK1 qR	GCCTCTGCTGTTGGGTGA
CCK2	Partial cDNA	CCK2 F1	GCTGCTGTCTCCAAGGTCTC
		CCK2 R1	CCATCCAGCCCTCGTAATCT
	5′RACE	CCK2 R2 (first)	AGGGAGCGTTCCAGCAGAGC
		CCK2 R3 (nest)	CAGGGAGACCTTGGAGACAGC
	3′RACE	CCK2 F2 (first)	GGCTTTGAACGCCTTGAGT
		CCK2 F3 (nest)	AGCGGCTGAGCACAGGATA
	Real-time PCR	CCK2 qF	GCTGCTGTCTCCAAGGTCTC
		CCK2 qR	TCCTGGAGATGAGTCTGACC
β-actin	Real-time PCR	β-actin qF	TTCACCACCACAGCCGAGA
		β-actin qR	TGGTCTCGTGGATTCCGCAG

2.3. Tissue expression analysis

To analyse the tissue expression pattern of the seahorse CCKs, total RNA was prepared from the brain, gill, liver, intestine, kidney, muscle, testis and brood pouch of the adult male seahorse using TRIzol reagent. The first-strand cDNA was synthesized using an oligo (dT) primer with the RNA Eraser Reverse Transcription Kit (TaKaRa, Japan). The primers used for the RNA expression analysis were designed using seahorse CCK genes at positions flanking an intron (Table 1). Real-time PCR was performed on a Roche LightCycler 480 using SYBR *Premix Ex Taq*™ (TaKaRa, Japan), and the PCR parameters were 40 cycles of 94 °C for 20 s, 52 °C for 20 s, and 72 °C for 15 s. The fluorescence was acquired at the end of each cycle, and the melting curve from 50 to 99 °C was then obtained. Seahorse β-actin was amplified to confirm the steady expression level of the housekeeping gene.

2.4. Expression profiles of the CCKs during the transition from endogenous to exogenous nutrition in larval seahorses

After birth, the larvae (0.035 ± 0.0041 g; 1.82 ± 0.26 cm total length, n = 150) were divided into three groups. The first group (n = 8) of larvae were fed once a day (09:00 h) with frozen *Mysis* spp. The second group of larvae were fasted continuously and sampled at 11:00 am on each day (1–5 days). The third group of larvae were fasted and refed on the next day at 9:00 am, then sampled at 11:00 am on each day (1–5 days). Thus, the first group of larval seahorses mainly relied on exogenous nutrition; the fasted larvae mainly relied on endogenous nutrition; and the third group of larvae was in a transition from endogenous to exogenous nutrition. After the treatment, eight seahorses from each group were anaesthetized using MS222 (100 mg/L) and individually weighed. Whole larvae were sampled for the real-time quantitative PCR measurements of the mRNA, frozen in liquid nitrogen and stored at −80 °C until the RNA extraction.

2.5. Fasting treatment in juvenile and adult seahorses

The juvenile seahorses (0.85 ± 0.46 g; 6.32 ± 0.75 cm total length) and adult seahorses (2.94 ± 0.72 g; 9.31 ± 0.51 cm total length) were fed frozen *Mysis* spp. twice a day (09:00 h and 16:00 h) and maintained in re-circulating holding tanks (90 × 70 × 60 cm). After a two-week acclimation period, the sea-