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Molecular cloning, tissue distribution, and effect of fasting and refeeding on the expression of neuropeptide Y in *Channa argus*

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ABSTRACT

Neuropeptide Y (NPY) is a 36 amino-acid amidated peptide of the pancreatic polypeptide (PP) family, which plays an important role in appetite regulation and energy expenditure in mammals. Although several teleost NPY have been identified, its roles remain unclear in fish. We herein reported on the molecular cloning, tissue distribution and the effect of fasting on the expression of NPY in *Channa argus*, and designated as CaNPY. It consisted of a 300 bp open reading frame predicted to encode a prepro-NPY of 99 amino acids. Sequence analysis revealed that CaNPY was highly conserved (>60%) with other vertebrate NPY. Phylogenetic analysis highly supported CaNPY was closely related to piscine NPY. In addition, except for muscle and spleen tissues, *CaNPY* was found to extensively expressed in all other detected tissues, with the highest level in brain. Futhermore, the CaNPY transcript was found to significantly increase after short-term and long-term food deprivation, and dramatically decrease following refeeding. These findings suggested that CaNPY might be involved in food intake regulation and it could be as a potential target locus to improve commercial production of this kind of fish.

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 (Ji et al., 2015; Naslund and Hellstrom, 2007). In fish, as in other vertebrates, feeding behavior and food intake are regulated by hormones produced by both the brain and peripheral organs, which are referred to as appetite or feeding-regulating hormones (Hoskins and Volkoff, 2012; Volkoff et al., 2005). These include appetite stimulators (or orexigenic factors, such as orexins, neuropeptide Y (NPY) and ghrelin) and inhibitors (or anorexigenic factors, such as cocaine- and amphetamine-regulated transcript (CART), cholecystokinin (CCK), leptin and amylin) (Gorrssen et al., 2006; Volkoff et al., 2009).

NPY is a peptide with 36 amino acid residues belonging to the NPY family, which was first purified from porcine brain in 1982 (Tatemoto, 1982). To date, lots of studies about this kind of neuropeptide were widely investigated in vertebrates, such as mammals (Naslund and Hellstrom, 2007), birds (Blomqvist et al., 1992), reptile (Kumar and Rai, 2011), amphibian (Sundstrom et al., 2012) and teleost (Ma et al., 2013; Mancebo et al., 2013). In both mammals and fish, NPY is one of the most abundant neuropeptides within the brain and has a major regulatory role in energy homeostasis and food intake (Hoskins and Volkoff, 2012; Volkoff, 2016). Thus far, the orexigenic actions of NPY have been well investigated thoroughly over the past decades. Injection of NPY into the third ventricle of the brain or directly into the hypothalamus increases food intake in rats (Clark et al., 1985, 1987), while central injections of NPY anti-sense oligodeoxyneucleotides inhibit feeding (Akabayashi et al., 1994; Schaffhauser et al., 1997). Similar to mammals, central administration of homologous and heterologous NPY in fish species increases food intake in a dose dependent manner (Aldegunde and Mancebo, 2006; Silverstein and Plisetskaya, 2000; Zhou et al., 2013). In addition, short-term and long-term fasting induce an increase in hypothalamic NPY expression compared to normal feeding fish (Babichuk and Volkoff, 2013; Kehoe and Volkoff, 2007), and refeeding reverses the effects of starvation on NPY peptide levels and mRNA expression (Ji et al., 2015; Narnaware and Peter, 2001; Narnaware et al., 2000). Thus far, although several teleost NPYs have been identified, its physiological roles still remain unclear and need more researches for further investigation (Volkoff, 2016).

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The northern snakehead, *Channa argus*, a member of the Channidae family of the Perciformes, is an economically important freshwater fish native to East Asia (Xu et al., 2017). It is mainly cultivated in Asia and Africa for food, especially in China with an annual production of about 510,000 tons. However, genetic degradation caused by inbreeding of *C. argus* cultivation has led to higher susceptibility to diseases recent years (Xu et al., 2017). As our known, NPY is functionally related to growth and reproduction of fish (Breton et al., 1991; Peng et al., 1993; Zohar et al., 2010). Studies on CaNPY will be undoubtedly helpful for understanding the growth traits of *C. argus* and might also be of benefit for future breeding programs. We herein report the molecular cloning, tissue distribution and a potential role involved in food take regulation of CaNPY.

2. Materials and methods

2.1. Fish sampling

The juvenile northern snakehead, *C. argus* (weight 71.3 ± 5.6 g) used in this study were obtained from Neijiang Fish Farm (Neijiang, China) and transported to the experimental aquarium in the College of Life sciences (Neijiang Normal University). Fish were kept in 100 L tanks under natural light-dark conditions (12 L/12 D) with a constant flow of filtered water and the water temperature regulated to 18–20 °C. Fish were fed with fish meat (5–8% of body weight) at 19:00 every day. Fish were acclimated to these conditions for 2 weeks before the experiment, showing a normal feeding pattern during this acclimation period.

Following acclimation, six femal-fish were randomly selected for cloning and tissue distribution studies. Fish were anesthetized on ice and were sacrificed by decapitation. Tissue samples from brain, eye, gill, heart, intestine, kidney, liver, muscle, gonad (ovary), spleen and adipose tissue, were collected and frozen in liquid nitrogen immediately. For short-term food deprivation experiments, fish were assigned to 5 tanks (with 15 tails per tank), these fish were fed at the same time, and then five fish from each tank were selected and brains were sampled at 19:00 (0 h), 22:00 (3 h), 01:00 (6 h), 07:00 (12 h) and 19:00 (24 h) after feeding. For long-term food deprivation experiments, fish were assigned to 3 experimental tanks (with 20 tails per tank, 1 tank fed and 2 tanks unfed) for 2 weeks, subsequently, five fish were randomly selected and brains were sampled at 19:15, the control group and one fasted tank (refed group) were fed at 19:00, the other fasted tank was not fed (fasted group). All samples were kept at −80 °C until further analysis.

All animal experiments were performed with the approval of the Neijiang Normal University Animal Care and Use Committee and in full compliance with its ethics guidelines.

2.2. Molecular cloning of NPY from C. argus

Total RNA was isolated from whole brain with the Trizol reagent (Invitrogen, USA) according to manufacturer's protocol, and 1 μg of the RNA was reverse transcribed to cDNA using Super Script™ II RT reverse transcriptase (Takara, Japan). The partial cDNA sequence was obtained from a transcriptome library constructed by our research team (data not shown), and then two pairs primers were designed to amplify the full-length cDNA sequence. The basic cycling conditions of the PCR were a denaturing stage at 94 °C for 30 s, gene-specific annealing temperature for 45 s and elongation stage at 72 °C for 60 s. The product was purified from agarose gel using the Universal DNA Purification Kit (TIANGEN, China), and cloned into the pMD-19T vector (TaKaRa, Dalian, China). The insert was sequenced at Huada (Beijing, China). Primers used to amplify the NPY gene were listed in Table 1.

2.3. Sequence analysis and data processing

The coding sequence (CDS) was determined using online software ORF finder (https://www.ncbi.nlm.nih.gov/gorf/gorf.html), and then the putative protein sequence was translated with Primer Premier 5.0 software. Moreover, the Bioedit software was used to determine the electronic point (PI), and the online tool SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. Furthermore, multiple sequence alignments were as described in our previous work (Wen et al., 2017).

2.4. Phylogenetic analysis

The predicted CaNPY amino acid sequences together with other teleost NPY genes as well as those identified in other vertebrates were aligned by CLUSTAL X2.1. The aligned amino acid data sets were used to reconstruct the phylogenetic tree with maximum likelihood (ML) approach using Mega 6.0 software (Zou et al., 2017). The best-fitting model was calculated by Mrmodeltest 2.0 (Nylander, 2004) and ProtTest 2.4 (Abascal et al., 2005), and finally the JTT + I model was selected as the best model. The robustness of the tree topology was assessed by nonparametric bootstrap analysis with 1000 resampling replicates. Moreover, the *lampetra fluviatilis* was selected as an outgroup. All protein sequences IDs used in present study are shown in Table 2.

2.5. Quantification real-time PCR

Extraction of total RNA from fish tissues and first strand cDNA synthesis were performed as described above. Then real-time PCR was used to detect the mRNA expression of NPY with Light Cycler Real-Time system. Reverse transcription product was used for real-time PCR in a final volume of 10 μL. The end products of PCR were verified with the melting curves that showing a single peak specific for the target gene. The relative expression levels were calculated according to the method described in our previous study (Wen et al., 2015), and Tubα1 was selected as the reference gene. Quantification primers used to amplify the NPY and Tubα1 genes were listed in Table 1. Values are expressed as means ± SE M (N = 5).

2.6. Statistical analysis

All data were expressed as the mean ± SEM. Statistical analysis was performed with SPSS19.0 software. Significant differences were found using one-way analysis of variance (ANOVA), followed by the post hoc test (least significant difference test and Duncan's multiple range test), after confirming for data normality and homogeneity of variances. Differences were considered to be significant if $P < 0.05$.

Table 1
PCR primers used for cloning and gene expression studies.

Primers	Primer sequence (5′-3′)
NPY01-F	CCTTGACGGAGGGATA
NPY01-R	ACTGTGGAAGCGTGTCT
NPY02-F	AAGAGACGCCACGACGC
NPY02-R	TGCAGCTGATGGGTAAGG
NPY01-qF	AGACACGCTTCCACAGT
NPY01-qR	CTTCAAACAACATATT
Tubα1-qF	AGCCTGATGGTCAAATGC
Tubα1-qR	TTCCAATGGTGTAGTGCC