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Neuropeptide Y stimulates food intake and regulates metabolism in grass carp, *Ctenopharyngodon idellus*

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ABSTRACT

Neuropeptide Y (NPY) has been established as an important regulator of food intake in mammals. However, the involvement of NPY in the feeding behavior of fish has not yet been fully understood. The present study investigated the role of NPY in food intake in grass carp. The full-length cDNA sequence of gc-NPY was 797 bp, and an ORF of 96 amino acids, including the 28-residue signal peptide and 36-residue mature peptide, which had high identities to the NPY of cyprinid fishes. Two receptors Y1 and Y5 which mediate the appetite-stimulating effects of NPY in mammals lost from some of the euteleost genomes. However, sequences of other NPY receptors Y8a and Y8b were obtained from the ESTs library of grass carp in our previous studies. NPY and both Y8 receptors were predominantly expressed in the brain. Cumulative food intake was significantly increased by intracerebroventricular administration of NPY during a 72-h observation period. For better understanding of NPY's action in grass carp, the mRNA levels of appetite regulators, energy metabolism genes and key digestive enzymes were assessed. The results showed that the mRNA levels of Y8b were significantly increased, while the expressions of cholecystokinin (CCK), cocaine and amphetamine regulated transcript (CART), pituitary adenylate cyclase-activating peptide (PACAP), leptin, leptin receptor (leptinR) and leptin receptor overlapping transcript protein (LEPROT) were significantly decreased after NPY injection. In addition, the marked stimulation of insulin-like growth factor1 (IGF1), insulin-like growth factor 1 receptor (IGF1R), lipoprotein lipase (LPL), Stearoyl-CoA desaturase 1 (SCD1), amylase and trypsin mRNA levels in liver were caused by NPY administration, and the mRNA level of uncoupling protein 1(UCP 1) tended to decrease but not significantly. These results indicate that NPY acts as an orexigenic factor in the grass carp, and Y8b may involve in the feeding regulation. NPY not only affects food intake, but also has marked effects on energy metabolism in grass carp. Moreover, NPY seems to closely interact with other appetite regulators, and the present results provide novel insight into these complex interactions. Further understandings of appetite regulation in fish ultimately have the potential to yield methods for improving feed efficiency in aquaculture.

1. Introduction

Neuropeptide Y (NPY), a 36 amino acid peptide, is abundant in the central nervous system (CNS) (Volkoff, 2006). NPY has been implicated in several centrally mediated physiological functions such as regulation of circadian rhythms, body temperature, sexual behavior, blood pressure and neuroendocrine secretions (Dumont et al., 1992; Silva et al., 2005). Moreover, it has been established as an important regulator of food intake (Clark et al., 1984; Volkoff, 2006; Volkoff et al., 2005; Yokobori et al., 2012). NPY is one of the most potent orexigenic agents known in mammals (Halford et al., 2004). The effects are mediated via several G-protein-coupled receptors. In mammals, available evidence suggests that the Y1, Y5 or both receptors might be appetite receptors (Kanatani et al., 2000; Patel and Patel, 2010).

In non-mammalian vertebrates, there is limited information about NPY, its receptors and neuroendocrinological functions. The NPY gene or cDNA has also been characterized in fish such as Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), sea bass (*Dicentrarchus labrax*), and tiger puffer (*Takifuguru bripes*) (De Pedro et al., 1993; Filby et al., 2010; Kamijo et al., 2011; Murashita et al., 2009; Cerdá-Reverter et al., 2000). Several recent reports have

Abbreviations: AgRP, agouti-related peptide; AMY, amylase; BW, body weight; CART, cocaine and amphetamine regulated transcript; CCK, cholecystokinin; CNS, central nervous system; CRF, corticotropin-releasing factor; EST, expressed sequence tag; GH, growth hormone; ICV, intracerebroventricular; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; LEPROT, leptin receptor overlapping transcript protein; LPL, lipoprotein lipase; MCH, melanin concentrating hormone; MSH, melanocyte stimulating hormone; NPY, neuropeptide Y; ORF, open reading frame; PACAP, pituitary adenylate cyclase-activating peptide; POMC, pro-opiomelanocortin; SCD1, Stearoyl-CoA desaturase 1; TRY, trypsin; UCP1, uncoupling protein 1; UTR, Untranslated region.

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described NPY regulates feeding in fish, as both central and peripheral injections of mammalian or fish NPY increase food intake in goldfish (De Pedro et al., 2000; Narnaware et al., 2000), channel catfish (*Ictalurus punctatus*) (Silverstein and Plisetskaya, 2000), rainbow trout (*Oncorhynchus mykiss*) (Aldegunde and Mancebo, 2006) and tilapia (*Oreochromis mossambicus*) (Kiris et al., 2007).

However, it is unclear which receptor mediates the orexigenic actions of NPY in fish. Two of the receptors Y1 and Y5 seem to have been lost from some of the euteleost genomes (Salaneck et al., 2008). Nevertheless, the euteleosts that have been investigated physiologically still respond to NPY with increased feeding, thereby other NPY receptor subtypes may mediate the action of NPY on food intake. Obvious candidates for this role are Y8a and Y8b and also Y1 since it is likely to be present in other euteleost in addition to *D. rerio*. To date, seven receptors (Y1, Y2, Y2-2, Y4, Y7, Y8a and Y8b) for NPY and its related peptides have been identified in zebrafish (Fallmar et al., 2011; Larsson et al., 2008; Mathieu et al., 2005; Salaneck et al., 2008). Moreover, Yokobori et al. (2012) showed that the NPY-induced orexigenic action was blocked by treatment with a Y1 receptor antagonist in the zebrafish. In goldfish, Y1-receptor antagonist also decreased food intake whereas central injections with either a Y1- or a Y5-receptor agonist induced an increase in food intake (Narnaware and Peter, 2001). However, there is no direct or positive result to prove Y1 or Y5 as the orexigenic receptors in fish. Only Y8a and Y8b sequences were obtained from the ESTs library of grass carp, and those expressions were consistent with the expression of NPY (our unpublished data). So we hypothesize that Y8 receptors perhaps play important roles in the regulation of feeding behavior.

In mammals, NPY interacts with a number of appetite regulators, including cholecystokinin (CCK), cocaine and amphetamine regulated transcript (CART), and leptin (Mercer et al., 2011). In fish, there are only a few researches about coinjections with NPY and other regulators, and the interaction between these molecules is unknown. Considering the difference of NPY and receptors between mammals and fishes, preparation of species specific NPY is therefore a key step for characterizing the function of NPY. In this article, we report the cloning and characterization of grass carp NPY genes. In order to explore the biological functions of grass carp NPY, gc-NPY was synthesized, and its effects on food intake were examined in grass carp. Then the effects of gc-NPY treatments on the expression of some metabolism genes were assessed. The results can deepen understanding of appetite regulation in fish, and further studies ultimately have the potential to yield methods for improving feed efficiency in aquaculture.

2. Materials and methods

2.1. Animals

Juvenile grass carp were obtained from and reared in Guangdong Freshwater Fish Farm (Panyu, China). Fish were kept under controlled light–dark conditions (12 L/12 D) with a constant flow of filtered water and the water temperature regulated to 23–25 °C. The fish were fed uniformly a commercial floating diet (2% of body weight) (Haiwei, Guangzhou, China) at 11:00 every day. Waste diet was removed at 12:30, and dried for feed intake corrections. Feces were cleaned every day. Animals were acclimated to these conditions for at least 2 weeks before the experiment, showing a normal feeding pattern during this acclimation period.

2.2. Cloning and characterization of gc-NPY

To get the 5′-end and 3′-end of the NPY cDNA, the gene-specific primers (Table 1) were designed based on the core fragment cloned in our previous study (GenBank accession no. FJ641971). Total brain RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. 3′-RACE was performed using a 3′-Full RACE Core Set (Takara, Japan). Total RNA was reverse-transcribed to cDNA in the presence of oligo (dT)-3 site adaptor primer (provided in the kit). The 3′ ends were amplified by two rounds of PCR using primer pairs NPY 31F/3′-RACE outer primer (3OP) then NPY 32F/3′-RACE inner primer (3IP). The PCR parameters were 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with an additional initial 3-min denaturation at 94 °C and a 10-min final extension at 72 °C. The 3′-RACE-PCR products were purified from agarose gel, and cloned into the pEASY-T3 vector (TransGen Biotech, China). After transforming into the competent cells of *E. coli* DH5α, the recombinants were identified through blue-white color selection in ampicillin-containing LB plates and confirmed by PCR. Three positive clones in each PCR fragment were sequenced in both directions and these resulting sequences were verified and subjected to cluster analysis in NCBI.

For 5′-RACE, RNA from the brain was reverse transcribed with the oligo (dT) primer. Following cDNA synthesis, the first-strand cDNA was used in the TdT-tailing reaction. Then, tailed cDNA was amplified by prime pairs NPY 51R/ Abridged Anchor Primer (AAP). The annealing temperature of PCR was 55 °C. A dilution of the original PCR was

Table 1
Primer sequences for PCR.

Primer	Sequence 5′–3′
NPY31F	AGTCAACACCCACCGAGCAA
3OP	TACCGTCGTTCCACTAGTGATTT
NPY 32F	GCCTGCTTGGGAACTCTTA
3IP	CGCGGATCCTCCACTAGTGATTTCACTATAGG
NPY 51R	CCTTTTGCCATACCTCTGCC
AAP	GGCCACGCGTCGACTAGTACGGGGGGGGGG
NPY 52R	CCAAGCAGGCGAACAAGA
AUAP	GGCCACGCGTCGACTAGTAC

Table 2
Primer sequences for the quantitative real-time PCR.

Accession no.	Gene	Sequence 5′–3′	
M25013	β-actin	β-actin-F	GGCTGTGCTGTCCCTGTATG
		β-actin-R	GGTAGTCAGTCAGGTCACGGC
JQ951928	NPY	NPY-F	CTTCCTCTTGTTCGCCTGCT
		NPY-R	CCTTTTGCCATACCTCTGCC
ESTs	Y8a	Y8a-F	AATGTGTGCCCTCCCTCTGT
		Y8a-R	CGATGAGGATGTTGGTGACG
ESTs	Y8b	Y8b-F	GATTTTTGACTGGAACCACGAG
		Y8b-R	CGGCATCTGGAAAGCAGTG
JF912411	CCK	CCK-F	GGAACACACACGCCACACC
		CCK-R	GGAGAGGAACTTCTGCGGTATG
ESTs	CART	CART-F	AGTTTTACCCAAAGGACCCG
		CART-R	TGACCCTTTTCTGATGGCG
EF592488	PACAP	PACAP-F	CCAGAGAAAAGAACGGAAAGG
		PACAP-R	TCGTCTTCCTCGCTGCTTC
EU051323	IGF-1	IGF-1-F	GTGTGGAGACAGGGGCTTTTA
		IGF-1-R	CGTAGGGATCGTGGAGATTTG
EF062860	IGF-2	IGF-2-F	CGTGGGATTGTGGAAGAGTG
		IGF-2-R	TGGGACCTCCTGTTTTAATGC
EU816193	IGFR	IGFR-F	TGTGGTGCGTCTACTGGGC
		IGFR-R	TGGAGGTGTTCTCAGCGGA
EU719623	Leptin	Leptin-F	CAGGCAGACACCATCATCCA
		Leptin-R	CCCTTGGGCAGTGTTTGAA
JF825476	LeptinR	LeptinR-F	CCAGTGGAGGAAGGAAGCA
		LeptinR-R	GCAACTCCGCTGAATAAACG
ESTs	LEPROT	LEPROT-F	ACACTGAGTCAAGCAACGCAT
		LEPROT-R	GAAGTCATCTCCACCTCCGAA
FJ716100	LPL	LPL-F	ATTGTGGTGGACTGGTTG
		LPL-R	CTACATGAGCACCAAGACTG
AJ243835	SCD1	SCD1-F	ACTGGAGCTCTGTATGGAC
		SCD1-R	CGTAGATGTCATTCTGGAAG
FJ436059	UCP1	UCP1-F	GTGGACGTGGTGAAGACTC
		UCP1-R	GACACGAACATCACCACG
FJ641975	AMY	AMY-F	GACTGAGTTCAAGTATGGTGC
		AMY-R	TCCAGCACCATGTCCTC
FJ641976	TRY	TRY-F	CTGGACCATTGACAGTGAC
		TRY-R	CTCCAGACACTGAAGCTTG