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NPY and NPY receptors in the central control of feeding and interactions with CART and MC4R in Siberian sturgeon

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ABSTRACT

Neuropeptide Y (NPY) is the most powerful central neuropeptide implicated in feeding regulation via its receptors. Understanding the role of NPY system is critical to elucidate animal feeding regulation. Unlike mammal, the possible mechanisms of NPY system in the food intake of teleost fish are mostly unknown. Therefore, we investigated the regulatory mechanism of NPY and NPY receptors in Siberian sturgeon. In this study, we cloned the cDNA encoding NPY, and assessed the effects of different energy status on *npy* mRNAs abundance. The expression of *npy* was decreased in the brain after feeding 1 and 3 h. Besides, the expression of *npy* was increased after fasting within 15 days, while exhibiting significant decrease after refeeding. In order to further characterize the role of NPY receptor in fish, we performed acute intraperitoneal (i.p.) injection of NPY Y1 and Y2 receptor agonists, which is [Leu 31, Pro 34] NPY and NPY13-36 respectively. The results showed that the food intake of Siberian sturgeon was increased within 30 mins after injection of both Y1 and Y2 receptor agonist. To explore the relationship between NPY, NPY receptors and another appetite peptides, we examined the level of *npy*, *cocaine- and amphetamine-regulated transcript* (*cart*) and *melanocortin-4 receptor* (*mc4r*) by injected Y1 and Y2 receptor agonist. The results suggested that *cart* expression was regulated by NPY which acts on Y1 receptor or Y2 receptor. While *mc4r* expression just was mediated by NPY and Y1 receptor.

1. Introduction

The regulation of appetite and satiety had appeared to be a complex process, which involves numerous neuroendocrine peptides and signaling pathways (Lenard and Berthoud, 2008). There are two major of central signaling pathways, one consists of the powerfully orexigenic peptides Neuropeptide Y (NPY) and Agouti-related protein (AgRP) and the second one consists of the anorexigenic peptides proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). These two pathways are located in the arcuate nucleus (ARC) of the hypothalamus, and influenced appetite regulation by acting on melanocortin-4 receptor (MC4R) and activating downstream pathways (Atkinson, 2008).

NPY is one of the most effective and abundant neuropeptides (Matsuda et al., 2012), which is constituted by 36 amino acid peptides. NPY was first isolated from the porcine brain (Tatemoto et al., 1982). After that, NPY has identified in many vertebrate (Higuchi et al., 1988) and invertebrate species (de Jong-Brink et al., 2001). NPY is a highly conserved neuropeptides in vertebrates (Ramos et al., 2005). In the central nervous system (CNS), NPY is predominant expressed in the hypothalamus, telencephalon, forebrain, optic tectum and pituitary (Amiya et al., 2011; Hosomi et al., 2014; Zahid et al., 2014). In the periphery, NPY widely expressed in intestinal tract, stomach, eye and heart (Hosomi et al., 2014; Volkoff, 2016). NPY has powerful and complex effects on energy homeostasis (Morton and Schwartz, 2001), regulation of stress and anxiety (Thorsell, 2010), and sexual behavior

Abbreviations: NPY, neuropeptide Y; AGRP, agouti gene-related protein; POMC, proopiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; ARC, arcuate nucleus; CNS, central nervous system; BW, body weight; MS-222, tricaine methane sulfonate; i.p., intraperitoneal; i.c.v., intracerebroventricular; qPCR, real-time quantitative PCR; HPT, hypothalamus-pituitary–thyroid; TRH, thyrotropin-releasing hormone; PVN, paraventricular nucleus; FSGD, fish-specific genome duplication

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(Argiolas and Melis, 2013). NPY exerts these physiological functions via its multiple receptor subtypes (Loh et al., 2015). Seven types of NPY receptors (Y1, Y2, Y4, Y5, Y7, Y6, and Y8) have been identified in vertebrates to date, of which up to 5 (Y1, Y2, Y4, Y5, Y6) are present in mammals (Sundström et al., 2015). All of NPY receptors are belong to G-protein coupled receptor, and these receptor can be divided into two groups: the Y1-Y4-Y6-Y8 and the Y2-Y7 groups. Y1-Y4-Y6-Y8 receptors mainly expressed in neural tissue, while Y2-Y7 receptors in visceral organs, such as kidney and intestine (Yi et al., 2018). NPY receptors are differ in their ligand affinity profiles, of which Y1, Y2 and Y5 have high affinity for NPY (Mercer et al., 2011; Thorsell, 2010).

NPY is one of the most potent orexigenic peptide that plays a critical role in regulating appetite. Food deprivation up-regulated the level of NPY in the ARC (Sainsbury and Zhang, 2010). Furthermore, ARC NPY neurons as a feeding center can receive and integrate central and peripheral signals (e.g. AgRP, leptin, ghrelin, PYY) related to appetite regulation (Kohno and Yada, 2012; Mercer et al., 2011). Among the seven NPY receptors, only Y1 and Y2 have been strongly implicated in the appetite regulation with NPY (Mercer et al., 2011; Nguyen et al., 2012). In mice, central administration of Y1 receptor agonist remarkably increased body weight, adiposity and respiratory quotient (Henry et al., 2005). Conversely, intrahypothalamic administration of Y1 receptor antagonist GI264879A to rats significantly decreased food intake and body weight (Danielsa et al., 2001). Y2 receptor is also involved in appetite regulation, but the role is complicated. Hypothalamic-specific Y2 knockout decreased body weight, and germ-line Y2 knockout in mice produced a sustained reduction in bodyweight and adiposity (Sainsbury et al., 2002). Central administration of Y2 receptor agonist (NPY3–36) delayed gastric emptying in rats (Ishiguchi et al., 2001). Peripheral administration of PYY 3–36, the other Y2 receptor agonist, inhibited gastric emptying (Witte et al., 2009), and reduced food intake (Shi et al., 2013). The above studies indicated that the appetite regulation by NPY and receptors is much more complex than previously expectation.

Unlike the mammal, there is limited information of NPY system in the control of feeding in teleost fish. NPY has identified in many teleost species, such as goldfish (Narnaware and Peter, 2001), zebrafish (SundstrÖm et al., 2005), Atlantic salmon (Murashita et al., 2009), *Schizothorax prenanti* (Wei et al., 2014), and the preliminary studies of appetite regulation function of NPY in these species have been conducted. Intracerebroventricularly (i.c.v.) administration of NPY stimulated food consumption in goldfish (Narnaware and Peter, 2001), and promoted food intake in zebrafish (Yokobori et al., 2012), these two roles suggesting that NPY exerts as an orexigenic action in fish. After long-time fasting, the NPY expression was notably increased in *Schizothorax prenanti* (Wei et al., 2014), whereas there was no significant change in Atlantic salmon (Murashita et al., 2009). And another study indicated that two or four weeks of fasting induced an increase in hypothalamic *npy* expression of winter flounder in the summer, but did not affect hypothalamic *npy* expression in the winter (MacDonald and Volkoff, 2009a). The above results suggest that the roles of NPY in fish might be more complex and involved species-specific mechanisms that attributed to high biodiversity of fish, since fish have different feeding habits, digestive tract and physiology, and also are affected by various extrinsic and intrinsic factors (Volkoff, 2016). So more extensive and in-depth studies about the role of NPY in appetite regulation in fish are still needed to be conducted.

Siberian sturgeon (*Acipenser baerii*) is one of the most ancient of the actinopterygian fish. It is a widely farmed species in China, exhibits fast growth (Sadati et al., 2011), and high rate of food consumption and conversion (Ruchin, 2007). The economic benefits of fish farming highly dependent on fish growth, which is strongly related to food intake. Appetite is a major factor for food intake, so knowing the effect of appetite regulatory peptides on food intake can be useful to improve fish growth and optimize production in aquaculture. In this study, we identified and characterized the *npy* from Siberian sturgeon, investigated the expression levels in different tissues, and then conducted postprandial and feeding status related changes in brain *npy* mRNA expressions. Considering that most published studies about NPY in fish are limited to gene identification and expression, little is known about the interaction effect between NPY, NPY receptor and other appetite peptides, we also study the effect of NPY on other appetite regulatory peptides via receptors. To be specific, the intraperitoneal (i.p.) injections of Y1 receptor agonist [Leu 31, Pro 34] NPY and Y2 receptor agonist NPY13-36 were performed, and the food intake and level of *npy*, *cart* and *mc4r*of the injected fish were examined in this study.

2. Material and methods

2.1. Peptide

[Leu 31, Pro 34] NPY and NPY13-36 was synthesized by Shanghai Top-peptide Biotechnology Co., Ltd. (Shanghai, China). The purity of the peptide was confirmed by using high performance liquid chromatography (HPLC) and mass spectrometry. The peptide was synthesized with > 98% purity, with a single peak confirming the predicted quality. Peptide was dissolved in fish physiological saline and diluted to appropriate concentrations that based on the results from pre-experimental study, and then stored at −20 °C until i.p. injection experiments.

2.2. Experimental animals

The experimental Siberian sturgeon were obtained from the farms of Runzhao Fisheries Co., Ltd. (Sichuan, China). Fish were acclimated in Aquaculture Laboratory of Sichuan Agricultural University (Chengdu, China) indoor tanks (60 × 50 × 40 cm), which were supplied with a continuous flow of the fresh water system at 18 ± 1 °C and kept under natural photoperiod. All fish were fed with commercial sinking pellets (nutrient content: crude protein ≥ 40%, crude fat ≥ 12%, coarse fiber ≤ 6%, crude ash ≤ 18%, water ≤ 18% and total phosphorus ≥ 1.2%; Tongyi, Suzhou, China). Fish were fed to satiety once a day at 14:00 and the proportion was 3% of the body weight (BW). The residual diets were removed with a dip net. Before the experiments, fish were acclimated under these conditions for three weeks. The formal experiment was started at 30th, September 2015.

All experimental procedures performed on fish in this study were done in accordance with the guidelines for animal experiments of Sichuan Agricultural University with the approval of the Institutional Animal Care and Use Committee.

2.3. Molecular cloning, RNA extraction and cDNA synthesis

Six Siberian sturgeon with an average BW of 438.77 ± 59.72 g were used for cloning. The fish were killed with an overdose of MS-222 (SciYoung, Suzhou, China), and the whole brain was sampled, frozen and ground into powder by liquid nitrogen and then stored at −80 °C until RNA was isolated. Total RNA was isolated by using Trizol® reagent (TaKaRa, Japan) and treated with RNase free DNase I (TaKaRa, Japan) according to the manufacturer's protocol. Final RNA concentrations were determined by a photometer (Bio-Rad) fixed at 260 nm and 280 nm wavelength. The samples with an absorbance ratio between 1.8 and 2.1 were used for cDNA synthesis. RNA from sample was reverse-transcribed (RT) into cDNA using the PrimeScript™ RT Reagent Kit (Takara, Japan). First-strand cDNA of whole brain with 5′ or 3′ adaptors added was synthesized using SMART RACE cDNA Construction Kit (Clonetech, USA) for rapid amplification of cDNA ends (RACE) PCR. The RACE products were purified from agarose gel using the Universal DNA Purification Kit (TIANGEN, China), and cloned into the pMD-19T vector (TaKaRa, Japan). The inserts were sequenced at BGI (Beijing, China). The primers for cloning were designed using Primer premier 5.0 program and are listed in Table 1 (primer set for partial *npy* fragment,

Table 1
Primers sequences and function used in this study.

Gene	Primer	Primer sequence (5′to3′)	Applications
npy	*npy*-F	CCCCCCTCCTTTTACTTA	Partial cDNA PCR
	npy-R	GTTTCTCTTTGGAAGTCTGG	
	*npy*5′R1	CTTTCCCTCCACAGAAGGTCGG	5′RACE PCR
	*npy*5′R2	TAGCCAGCCACAACCCCAGG	
	*npy*3′F1	TTGTGGCTGGCTACCGTGGCTT	3′RACE PCR
	*npy*3′F2	TACGGAAAGAATCCCAAGACCAAG	
	npy-qF	GCTGGCTACCGTGGCTTTC	qPCR
	npy-qR	GACTGGACCTCTTCCCATACCT	
cart	*cart*-qF	ACGAAAAACAACTTCTGGGAGC	qPCR
	cart-qR	GACAGTCACACAACTTGCCGAT	
mc4r	*mc4r*-qF	ATGAAGAGAATCGCAGTCCT	qPCR
	mc4r-qR	GGTGGAGAAAGAATGGTGC	
β-actin	*β-actin*-qF	AGAGGCTCCCCTGAACCC	qPCR
	β-actin-qR	CACCAGAGTCCATCACAATACC	
gadph	*gadph*-qF	CATTTGATGTTGGCTGGGT	qPCR
	gadph-qR	CTTTCTGGGAAGGTGGAGGT	

β-actin and *gadph* were used as housekeeping genes.

npy-F and *npy*-R; 5′RACE outer for *npy*, *npy*-R1; 5′RACE inner for *npy*, *npy*-R2; 3′RACE outer for *npy*, *npy*-F1; 3′RACE inner for *npy*, *npy*-F2).

2.4. Structural analysis

The nucleotide and deduced protein sequences were analyzed by using BLASTn and BLASTp (http://www.ncbi.nlm.nih.gov), and the ORF was predicted with Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple sequence alignments were generated with clustalx1.83. The cleavage site of the signal peptide was estimated by using the SignalP Ver. 4.0 program (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree based on the amino acid sequences was constructed by the neighbor-joining method of the ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) and MEGA 5.1 program (http://www.megasoftware.net/index.html). The analysis reliability was assessed by 1000 bootstrap replicates.

2.5. Tissue distribution of npy mRNA in Siberian sturgeon

As molecular cloning experiment, six Siberian sturgeon with an average BW of 438.77 ± 59.72 g (n = 6) were obtained. The following tissues were collected from the whole brain, pancreas, liver, spleen, kidney, rectum, duodenum, intestinum valula, oesophagus, pyloric caeca, and stomach to examine the distribution of *npy* mRNA expression. Tissues were flash-frozen and ground into powder by liquid nitrogen, and stored at −80 °C until RNA isolations were performed. Total RNA was extracted and cDNA was synthesized as described above. The real-time quantitative PCR (qPCR) were conducted to determine tissue distribution. Primer sets for the qPCR of *npy* was designed in the obtained nucleotide sequence (primer sets for *npy*, *npy*-qF and *npy*-qR; Table 1). Siberian sturgeon *β-actin* and *gadph* were both used as reference genes for the purpose of controlling for error between samples to analyze the target genes mRNA expressions (Vandesompele et al., 2002), and the primers also were presented in Table 1. The qPCR was performed in triplicate for each sample on a CFX96 Real Time PCR Detection System (Bio-Rad, USA), and the procedures and methods were described as the previous way (Yuan et al., 2014). The comparative C_T method was used to analyze the expression of the target genes (Schmittgen and Livak, 2008). The geometric mean of *β-actin* and *gadph* gene was validated as an accurate normalization factor of the relative expression values (Vandesompele et al., 2002). All the dates of *npy* were normalized by the geometric mean of reference genes. In the figure, all data were normalized to the value of the highest expression.

2.6. Preiprandial and postprandial expression of npy mRNA in the brain of Siberian sturgeon

For these studies, the weight-matched fish (average BW 29.46 ± 3.56 g) were randomly distributed in 21 tanks (n = 3/tank), and divided into 7 groups, and then numbered Group1 to Group 7 (n = 9/group). The fish were sampled 3 h prior to feeding (11:00, −3 h) in Group 1, 1 h prior to feeding (13:00, −1 h) in Group 2, upon commencement of feeding (14:00, 0 h) in Group 3, 1 h after feeding (15:00, +1 h) in Group 4 and 3 h after feeding (17:00, +3 h) in Group 5. Group 6 and 7 were served as the control groups for the unfed fish. The fish of Group 6 were not feed at “0 h”, and fish of group 7 were not feed at “0 h” and “+1 h”. The fish were sampled at +1 h in group 6, and sampled at +3 h in group 7. The whole brain was sampled and respectively numbered then flash frozen and ground into powder by liquid nitrogen, and then stored at −80 °C. Total RNA extraction, cDNA synthesis, and qPCR were conducted as described above.

2.7. Fasting and refeeding induced changes in npy mRNA in the brain of Siberian sturgeon

For the fasting experiment, the weight-matched fish (average BW 29.45 ± 2.84 g) were randomly distributed in 33 tanks (n = 3/tank), of which 15 tanks as feeding group, 15 tanks as fasting group and 3 tank as re-feeding group (n = 9/group). As feeding group, fish were fed daily at 14:00 and samples were collected at 15:00 after the diet on the 1st, 3rd, 6th, 10th, and 15th day. As fasting group, fish fasted for 1, 3, 6, 10 and 15 days were marked, and the samples were collected on each of these days at 15:00. As re-feeding group, fish were fasted for 15 days and fed again at 14:00, and then the samples were collected in the 15th day at 15:00. The procedure of sampling, total RNA extraction, cDNA synthesis, and qPCR as described above.

2.8. Effect of i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36 on food intake

The weight-matched fish (average BW 84.68 ± 2.00 g) were randomly distributed in 21 tanks (n = 3/tank), and divided into 7 groups (n = 9/group). Six experiment groups were i.p. respectively injected with 0.1 μg/g, 0.5 μg/g and 1 μg/g BW of [Leu 31, Pro 34] NPY and NPY13-36, and one control group were IP injected with 100 μL saline at 13:30. Then, fish were returned to tanks and allowed to recover from anesthesia. Prior experiment showed that fish were return to the normal physiology state within 30 min after injection. So fish were fed the pre-weighed diet at 14:00, then the residual diets were collected at 14:30, and weighed after drying for determined the food intake.

2.9. Effect of i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36 on expression of npy, cart and mc4r

The weight-matched fish (average BW 84.68 ± 2.00 g) were randomly distributed in 9 tanks (n = 3/tank), and divided into 3 groups (n = 9/group). Two groups were IP respectively injected with 0.5 μg/g BW of [Leu 31, Pro 34] NPY and NPY13-36, and one control group were IP injected with 100 μL saline at 13:30 (30 min before feeding time for fish return to the normal physiology state). Then, fish were returned to their tanks and allowed to recover from anesthesia. Fish were fed the pre-weighed diet at 14:00, and then the whole brain of fish were sampled at 15:00. The procedure of sampling, sampling, total RNA extraction, cDNA synthesis and qPCR as described above. Primers set for qPCR of *cart* and *mc4r* were designed base on nucleotide sequences obtained by our laboratory (primer sets for *cart*, *cart*-qF3 and *cart*-qR3; primer sets for *mc4r*, *mc4r*-qF3 and *mc4r*-qR3; Table 1).

Fig. 1. Nucleotide and predicted amino acid sequences of Siberian sturgeon NPY. The putative signal peptide is shaded. The mature peptide is underlined. The asterisk indicates the stop codon.

2.10. Statistical analysis

Quantitative data are shown as mean ± SEM. Statistical analysis was performed with the SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Student's t-tests were used for the comparison between two groups. For multiple group designs, one-way ANOVA followed by the *LSD* post-hoc test were performed. Significant differences were identified when their values were < 0.05 ($P < 0.05$).

3. Results

3.1. Molecular cloning of Siberian sturgeon npy

From the RACE PCR, full-length cDNA sequence of Siberian sturgeon *npy* gene were obtained (GenBank accession No. MF805788). The Siberian sturgeon *npy* nucleotide sequence was 651 bp in length and contained a 72 bp sequence of the 5′-untranslated region (5′-UTR), a 285 bp sequence of the 3′-untranslated region (3′-UTR) and a 294 bp sequence of the ORF. The deduced NPY protein is composed of 97 amino acids including 18-residue signal peptide on the N-terminus (Fig. 1). A potential processing signal (KR) was identified at the C-terminus of the putative mature peptide. Both the processing signal and mature peptide are highly conserved in vertebrates (Fig. 2). The Siberian sturgeon NPY has high percent similarity to the NPY of other species including: Atlantic salmon (85%), Atlantic cod (83.84%), zebrafish (82.47%) and channel catfish (77.32%).In the phylogenetic analysis, the amino acid sequence of Siberian sturgeon NPY was clustered with the another teleost fish, and the teleost NPY paralogous were all divided from mammalian and amphibian homologues with high bootstrap value (Fig. 3).

3.2. Tissue distribution of npy in Siberian sturgeon

The high *npy* mRNA levels of Siberian sturgeon were observed in the brain, followed by the rectum, pancreas, intestinum valula, stomach, oesophagus, spleen and duodenum. Expression levels of the *npy* mRNA appeared to be lower in liver, pyloric caeca and kidney (Fig. 4).

3.3. Preiprandial- and postprandial expression of the npy mRNA in the brain of Siberian sturgeon

Quantitative analysis showed that the *npy* mRNA expressions had no significant changes at −3 h and −1 h prior to feeding. Similar result was found that the *npy* mRNA expressions at −1 h and 0 h. However, *npy* mRNA expression of fed fish were significantly decreased in the brain after a meal compared to the unfed control groups. At postprandial +1 h and +3 h, the *npy* mRNA expression level of fed fish in the brain had a 55% and 60% decrease compared to unfed control group, respectively ($P < 0.01$) (Fig. 5).

3.4. Fasting and refeeding induced changes of npy mRNA expression in the brain

The *npy* mRNA expression in the brain of unfed fish had a significant increase compared to fed animals ($P < 0.01$). When 15-day food-deprived fish were re-fed, *npy* mRNA expression had a remarkable decrease than that in the fasting group and fed group ($P < 0.01$). No significant changes in *npy* mRNA levels were observed between the fed groups (Fig. 6).

3.5. Effects of i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36 on food intake

After i.p. injection of 0.1 μg/g, 0.5 μg/g and 1 μg/g BW [Leu 31, Pro 34] NPY, the food intake of Siberian sturgeon was dramatically increased ($P < 0.01$). But no differences in food intake were found among the groups that injected with the dose of 0.1 μg/g, 0.5 μg/g and 1 μg/g BW [Leu 31, Pro 34] NPY. Likewise, the food intake of Siberian sturgeon was notably increased after i.p. injection of 0.5 μg/g and 1 μg/g BW NPY13-36 ($P < 0.01$). There is no difference in food intake between the groups that injected with the dose of 0.5 μg/g and 1 μg/g BW NPY13-36. However, the food intake of Siberian sturgeon was not effected after injection of 0.1 μg/g BW NPY13-36 (Fig. 7).

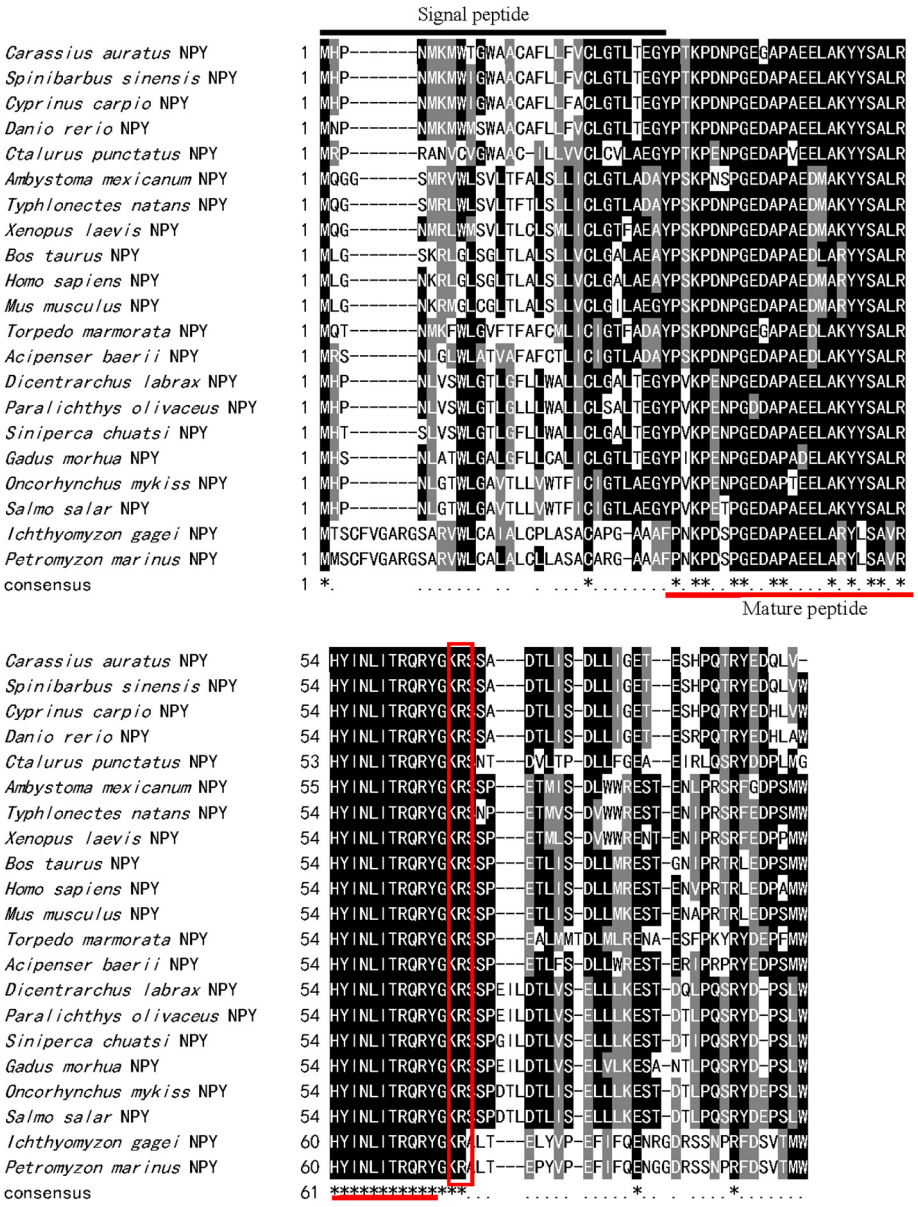

Fig. 2. Molecular characterization of Siberian sturgeon NPY. Residues that are conserved in more than half of the listed peptides are shadowed. Identical amino acid is indicated by an asterisk. The potential processing signal (KR) are boxed in red. The mature NPY is underlined in red.

3.6. Effects of i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36 on expression of npy, cart and mc4r

After injection of 0.5 μg/g BW [Leu 31, Pro 34] NPY, the mRNA expression of *npy*, *cart* and *mc4r* in the brain of Siberian sturgeon were significantly decreased ($P < 0.01$), and with different degree in each gene. Among these genes, the decline degree of *mc4r* level was larger than other two genes. In contrast, the mRNA expression of *npy* and *cart* were significantly increased ($P < 0.01$) after injection of 0.5 μg/g BW NPY13-36, while the *mc4r* mRNA expression was not affected (Fig. 8).

4. Discussion

Two forms of *npy* have been identified in several teleost, whereas Siberian sturgeon appears have only one form of *npy* just like goldfish (Bernier et al., 2004), zebrafish (SundstrÖm et al., 2005), grass carp (Zhou et al., 2013) and *Schizothorax prenanti* (Wei et al., 2014). We speculated that this difference of gene type is relate to biological diversification of fish. Fish are the most diversified group of vertebrates, and many fish might have even more duplicated genes than mammal, in which these additional genes generated through a complete fish-specific genome duplication (FSGD) (Meyer and Van de Peer, 2005). Amino acids alignment showed that NPY in teleost is highly conserved and retains the proposed processing signal site, which suggests that the physiological role of NPY might also be conserved.

Our study indicated that *npy* was primarily expressed in the brain of Siberian sturgeon, while immunohistochemical localization results also showed that Siberian sturgeon NPY were ubiquitously expressed in the CNS and peripheral nervous systems (Amiya et al., 2011). Brain region, especially the hypothalamus, is the feeding center that controls food intake and energy balance in mammal. Contrary in teleost, the feeding center seems to be more widespread in fish brains and it does not only include the hypothalamus but also extrahypothalamic regions such as the ventral telencephalon and olfactory tract (Cerdá-Reverter and Canosa, 2009; Volkoff, 2016). So in this study, the change of *npy* levels in the brain by the feeding status was conducted to evaluate the role of NPY in Siberian sturgeon.

NPY system responds rapidly during short- and long-term energy

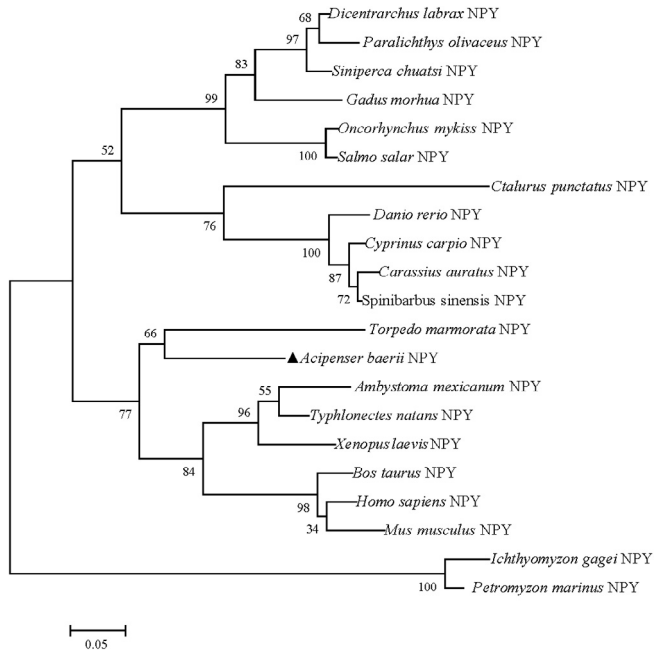

Fig. 3. Phylogenetic analysis of NPY amino acid sequences. Scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1000 replicates. GenBank accession numbers: *Homo sapiens* NPY (AH002914.2); *Mus musculus* NPY (AF273768.1); *Bos Taurus* NPY (NM_001014845.3); *Xenopus tropicalis* NPY (NM_001079062.1); *Ambystoma mexicanum* NPY (AAT66407.1); *Danio rerio* NPY (BC162071.1); *Spinibarbus sinensis* NPY (DQ462412.2); *Cyprinus carpio* NPY (XP_018919109.1); *Carassius auratus* NPY (ALO79070.1); *Salmo salar* NPY (NP_001140153.1); *Ctalurus punctatus* NPY (NP_001187016.1); *Dicentrarchus labrax* NPY (CAB64935.1); *Siniperca chuatsi* NPY (ABS83815.1); *Gadus morhua* NPY (AAX19943.1); *Oncorhynchus mykiss* NPY (NP_001117738.1); *Typhlonectes natans* NPY (AAD48033.1); *Torpedo marmorata* NPY (P28674.1); *Ichthyomyzon gagei* NPY (AAW47388.1); *Petromyzon marinus* NPY (AAW47393.1); *Paralichthys olivaceus* NPY (Q90WF4.1).

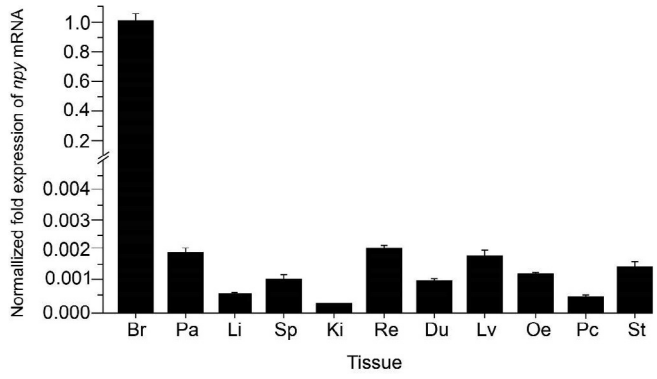

Fig. 4. Tissue distribution of *npy* mRNA in Siberian sturgeon. The results were expressed as relative expression levels after standardization by *β-actin* and *gadph* gene. Error bars represent standard error of the mean (n = 6). Br, brain; PA, pancreas; Li, liver; Sp, spleen; Ki, kidney; Re, rectum; Du, duodenum; Iv, intestinum valula; Oe, oesophagus; Pc, pyloric caeca; St, stomach.

intake patterns. Preiprandial- and postprandial study indicated that Siberian sturgeon *NPY* mRNA expression were significantly decreased in the brain after a meal, suggesting that NPY regulates the orexigenic response to energy status. Consistent with this notion, the *npy* levels of Siberian sturgeon in CNS was strikingly increased under the situation of food deprivation. The similar results also are observed in the other fish. In zebrafish, *npy* levels in the hypothalamus were higher in the fish when fasted for 7 days (Yokobori et al., 2012). In *Schizothorax prenanti*,

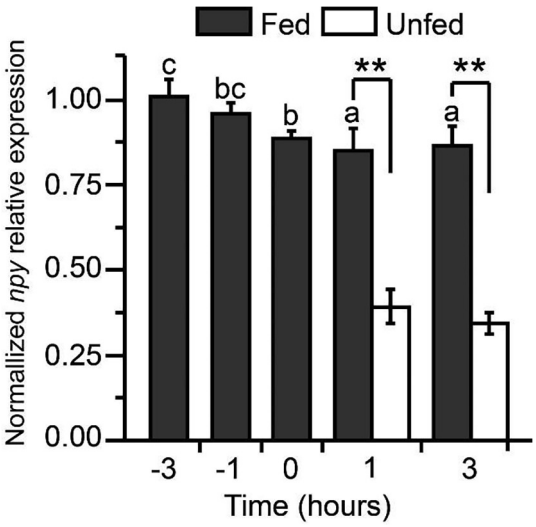

Fig. 5. Preiprandial and postprandial expression of *npy* mRNA expression in the brain. The mRNA expression of *npy* was normalized to *β-actin* and *gadph* gene. Error bars represent standard error of the mean (n = 9). Bars with different letters represent significant differences between preiprandial and postprandial groups. Asterisks represent significant differences between the groups at the same time point. $^{**}P < 0.01$.

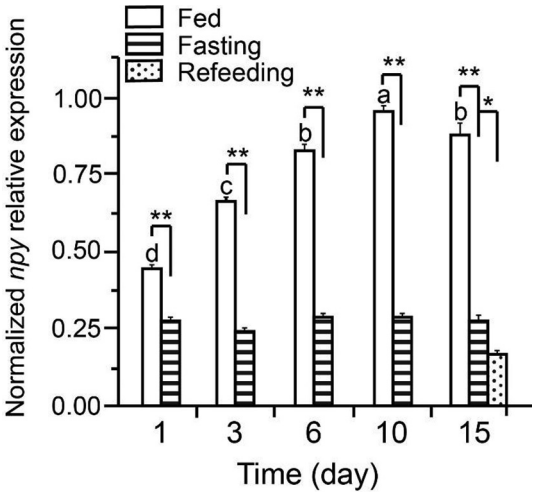

Fig. 6. Effects of fasting and refeeding on *npy* mRNA expression in the brain. The mRNA expression of *npy* was normalized to *β-actin* and *gadph* gene. Error bars represent standard error of the mean (n = 9). Bars with different letters represent significant differences between experimental groups. Asterisks represent significant differences between the groups at the same time point. $^{*}P < 0.5$, $^{**}P < 0.01$.

the expression of *npy* in the brain was decreased after a meal, and increased after fasting for 14 days (Wei et al., 2014). In the winter skate, after 2 weeks of fasting showed an increase in *npy* mRNA expression in their telencephalon (MacDonald and Volkoff, 2009b). The *npy* mRNA expression in the hypothalamus of yellowtail (Hosomi et al., 2014) and winter flounder (MacDonald and Volkoff, 2009a) also responded to fasting. These studies support a role for NPY as an appetite-regulating peptide in teleost, but the further studies about the specific regulation mechanism would be needed to be conducted.

NPY has many types of receptor, but the studies in mammals indicated that Y1 and Y2 receptor mainly take part in appetite regulation. The previous study (Salaneck et al., 2008) and transcriptome database from our laboratory showed that the type of Y1 and Y2 exists in Siberian sturgeon. i.p. injection is still useful and reproducible way to study various biological role of peptide in fish (Samaee et al., 2017;

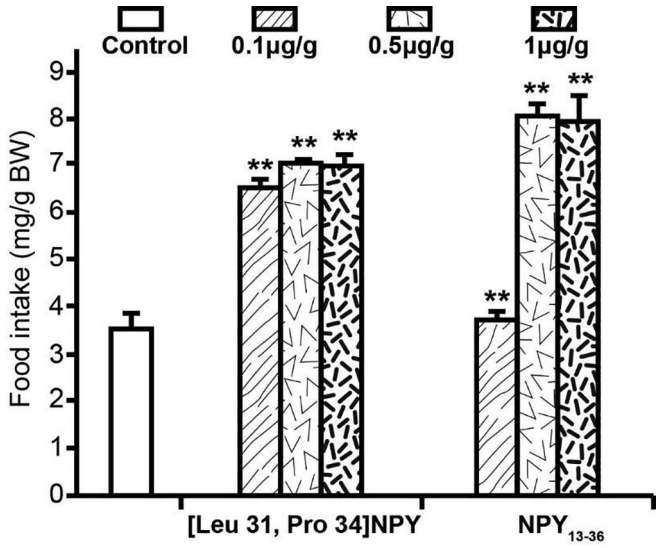

Fig. 7. Effects of i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36 on food intake. * represents significant difference between experiment group and control group. # represents significant difference within same experiment group. Error bars represent standard error of the mean (n = 9). $^{**}P < 0.01$, $^{\#\#}P < 0.05$.

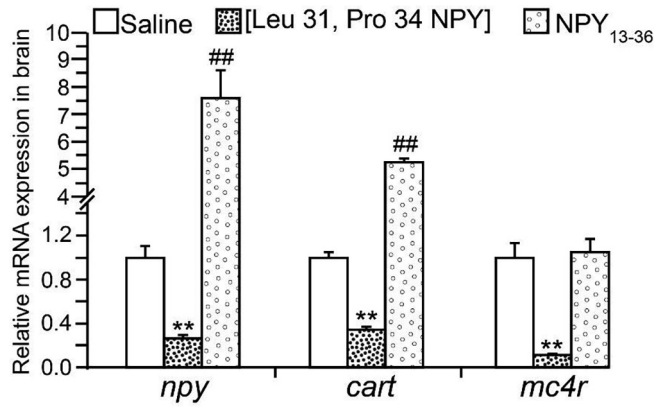

Fig. 8. Effects of i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36 on expression of *npy*, *cart* and *mc4r*. The mRNA expression of *npy*, *cart* and *mc4r* was normalized to *β-actin* and *gadph* gene. Error bars represent standard error of the mean (n = 9). * represents significant difference between experiment group which is injected [Leu 31, Pro 34] NPY and control group. # represents significant difference experiment group which is injected NPY13-36 and control group. $^{**}P < 0.01$, $^{\#\#}P < 0.05$.

Volkoff, 2016), and NPY can cross the blood-brain barrier intact via a non-saturable transporter (Kastin and Akerstrom, 1999). So for better understand the regulation function of NPY system in fish, i.p. administration of Y1 and Y2 receptor agonist, [Leu 31, Pro 34] NPY and NPY13-36, in Siberian sturgeon was performed. [Leu 31, Pro 34] NPY is a recognized Y1 receptor agonist, which has high affinity for Y1 receptor and less affinity for Y5 and Y4 receptor. NPY13-36 is a Y2 receptor agonist that gives it has a high affinity to NPY C-terminal fragments such as NPY3–36 and NPY13–36 (Zhang et al., 2014). In the present study, found that, food intake of Siberian sturgeon was significantly improved after injected injection of [Leu 31, Pro 34] NPY, this observation was similar to those observed in rat (Stanley et al., 1992) and goldfish (De Pedro et al., 2000). Interestingly, the injection of [Phe7, Pro34] pNPY (Y1 receptor agonist), in rats, inhibited the rat hypothalamus-pituitary–thyroid (HPT) axis and reduced thyrotropin-releasing hormone (TRH) expression in the paraventricular nucleus (PVN) (Fekete et al., 2002). These results suggested that NPY and Y1 receptor play an important role in regulate energy metabolism. Meanwhile, the food intake of Siberian sturgeon also was increased after injection 0.5 μg/g and 1 μg/g BW NPY13-36. The similar results were observed in the neonatal chick (Ando et al., 2001) and rainbow trout (Aldegunde and Mancebo, 2006). Besides NPY13-36, PYY 3–36 also can combine with Y2 receptor as an agonist. However, ARC injection of PYY 3–36 reduced food intake in hamster after fasting 56 h (Teubner and Bartness, 2013). Although there are some controversies between these findings, it can be confirmed that Y2 receptor is involved in food intake regulation, and this regulation is more complicated than expected.

Numerous neuropeptides implicated in the process of appetite regulation, but the interactions between neuropeptides have not been well studied. NPY/AgRP neurons and POMC/CART neurons, are two distinct populations of neurons, exist in the CNS and act at downstream MC4R neurons to regulate appetite and energy metabolism (Atkinson, 2008; Krashes et al., 2016). To investigate the relationship between these appetite peptides, we examined *npy*, *cart* and *mc4r* mRNA expression after i.p. administration of [Leu 31, Pro 34] NPY and NPY13-36. The *npy*, *cart* and *mc4r* level were significantly decreased when fish were injected 0.5 μg/g BW [Leu 31, Pro 34] NPY, suggesting that NPY mediated the effect of CART and MC4R via Y1 receptor. Furthermore, injection of 0.5 μg/g BW NPY13-36 caused significant increase in levels of *npy* and *cart* while *mc4r* level was unchanged. This explained that NPY exerts on Y2 receptor to regulate CART expression but not MC4R. Although NPY induced different response on CART expression by different receptors, our results showed that NPY pathway are connected to other pathways implicated in appetite regulation. Analogous results were observed in rat that i.c.v. infusion of NPY antisense or Y1R inhibited the expression levels of MC3R and CART (Chu et al., 2015). The lack of inhibitory Y2 signaling in NPY neurons increased *npy* mRNA expression, and decreased *pomc* mRNA expression in contiguous POMC/CART neurons (Qi et al., 2016). Previous studies in rat has demonstrated that knockdown of NPY decreased *npy* expression and increased *mc4r*, *mc3r*, and *cart (55–102)* expressions (Hsieh et al., 2014). The above studies suggested NPY has a powerful role regulatory in food intake, and interact with other regulators of energy balance via NPY receptors.

To conclude, it is clear that, NPY, Y1 and Y2 receptor are involved in the regulation of appetite in the Siberian sturgeon. Besides, NPY can mediates *cart* and *mc4r* expression in the brain via receptors. The *cart* expression was regulated by NPY, which acts on both Y1 receptor and Y2 receptor. However, *mc4r* expression was just mediated by NPY and Y1 receptor. These findings would shed new light on the role of vertebrate NPY system in feeding and metabolism homeostasis.

Disclosure summary

The authors have nothing to disclose.

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Appendix A. Supplementary data

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