Distinct functions of *neuromedin u* and *neuromedin s* in orange-spotted grouper

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Abstract

Neuromedin U (NMU) and neuromedin S (NMS) play inhibitory roles in the regulation of food intake and energy homeostasis in mammals. However, their functions are not clearly established in teleost fish. In the present study, *nmu* and *nms* homologs were identified in several fish species. Subsequently, their cDNA sequences were cloned from the orange-spotted grouper (*Epinephelus coioides*). Sequence analysis showed that the orange-spotted grouper Nmu proprotein contains a 21-amino acid mature Nmu peptide (Nmu-21). The Nms proprotein lost the typical mature Nms peptide, but it retains a putative 34-amino acid peptide (Nmsrp). *In situ* hybridization revealed that *nmu*- and *nms*-expressing cells are mainly localized in the hypothalamic regions associated with appetite regulation. Food deprivation decreased the hypothalamic *nmu* mRNA levels but induced an increase of *nms* mRNA levels. Periprandial expression analysis showed that hypothalamic expression of *nmu* increased significantly at 3 h post-feeding, while *nms* expression was elevated at the normal feeding time. I.p. injection of synthetic Nmu-21 peptide suppressed the hypothalamic neuropeptide y (*npv*) expression, while Nmsrp administration significantly increased the expression of *npv* and *orexin* in orange-spotted grouper. Furthermore, the mRNA levels of LH beta subunit (*lhβ*) and *gh* in the pituitary were significantly down-regulated after Nmu-21 peptide administration, while Nmsrp was able to significantly stimulate the expression of FSH beta subunit (*fshβ*), prolactin (*prl*), and somatolactin (*sl*). Our results indicate that *nmu* and *nms* possess distinct neuroendocrine functions and pituitary functions in the orange spotted grouper.

Key Words

- ▶ *nmu*
- ▶ *nms*
- ▶ structural diversity
- ▶ feeding regulation
- ▶ pituitary function

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Introduction

Neuromedin U (NMU), first isolated from porcine spinal cord, was named for its ability to cause uterine smooth muscle contraction (Minamino *et al.* 1985). Neuromedin S (NMS), first identified in rat brain, was named for its specific expression in the suprachiasmatic nucleus of the

hypothalamus (Mori *et al.* 2005). NMU and NMS are structurally related neuropeptides, bearing seven identical amino acid residues at their C-terminus, and they activate the same G-protein-coupled receptors, NMUR1 and NMUR2 (Mitchell *et al.* 2009). Both NM peptides have

now been implicated in a range of physiological activities, including smooth muscle contraction, circadian rhythm, and gonadotropic axis regulation (Mori *et al.* 2008, Budhiraja & Chugh 2009).

Evidence has also demonstrated that NMU and NMS play important roles in the regulation of feeding behavior and energy homeostasis in mammals (Howard *et al.* 2000, Hanada *et al.* 2004, Ida *et al.* 2005, Shousha *et al.* 2006, Peier *et al.* 2009). Central administration of NMU decreased food intake and resulted in loss of body weight in rodents, but also increased locomotor activities and body temperature (Howard *et al.* 2000, Ivanov *et al.* 2002, Peier *et al.* 2009). Mice deficient in *Nmu* are hyperphagic and obese with reduced energy expenditure (Hanada *et al.* 2004), while transgenic overexpression of *Nmu* can lead to leanness and hypophagia in mice (Kowalski *et al.* 2005). Despite low expression of NMS in the brain regions associated with feeding control, i.c.v. injections of NMS cause a decrease of food intake in rats (Ida *et al.* 2005). Recently, the actions of NMU and NMS in the control of feeding behavior have been clarified. The anorexigenic effects of NMU and NMS have been observed in WT and *Nmur1*^{-/-} mice, but were absent in *Nmur2*^{-/-} mice, indicating that the roles of these two peptides in feeding regulation are mediated by NMUR2 (Peier *et al.* 2009).

High expression levels of NMU and NMS are also observed in the pituitary in mammals (Fujii *et al.* 2000, Mori *et al.* 2005). The roles of NMU and NMS on gonadotropic hormone secretion have received much attention. Yet there is much controversy about their actions on gonadotropin release. Some studies showed that NMU and NMS suppressed luteinizing hormone (LH) release (Quan *et al.* 2004, Yang *et al.* 2010), but others found that they had stimulatory actions on LH secretion (Vigo *et al.* 2007a,b). On the other hand, their involvements in the regulation of other pituitary hormones are largely unclear.

In teleost fish, however, the significance of *nmu* and *nms* in feeding regulation and pituitary gland remains to be established. Only one report has found that i.c.v.-injected *Nmu* peptide could suppress food intake in goldfish (Maruyama *et al.* 2008). The function of *nms* gene has not been characterized in fish species thus far. Given that fish are composed of evolutionarily divergent species, further studies in different species are highly warranted to reveal their roles in the regulation of feeding and pituitary hormone secretion. In this study, we have therefore identified the *nmu* and *nms* genes and analyzed their protein structures in teleost fish. Then, we investigated their roles in feeding and pituitary hormones

regulation in the orange-spotted grouper (*Epinephelus coioides*), which is a commercially important marine fish and is widely cultured in South East Asia.

Materials and methods

Data mining and genomic synteny analysis

To identify the *nmu* and *nms* genes in teleost fish, tblastn was performed to search the Ensemble and NCBI genome databases, as well as the EST database at NCBI using the mammalian NMU and NMS peptides as the query sequences. Genomic synteny analysis was performed on the Ensemble genome database. The obtained *nmu* and *nms* gene sequences were searched against the genomes of human, chicken, zebrafish, and *Takifugu*, and the chromosomal locations of the *nmu* and *nms* genes were identified in these species. Genes around the fish *nmu* and *nms* were used to search for orthologs in the genome databases of human and chicken.

Animals and chemicals

Orange-spotted groupers were obtained from Daya Bay Aquaculture Center (Guangdong, China). Since orange-spotted grouper is a protogynous hermaphroditic fish in which individuals mature as female first and start to change sex around the age of 7 years, all fish used in this study were female. The fish were anesthetized with 0.05% tricaine methanesulfonate and sacrificed by decapitation. Tissue samples were collected immediately and snap-frozen in liquid nitrogen. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Sun Yat-Sen University.

Peptides corresponding to orange-spotted grouper *Nmu*-21 and NMS-related peptide (*Nmsrp*) were synthesized by GL Biochemistry (Shanghai, China). The purity was ≥95% as determined by analytical HPLC.

Molecular cloning of *nmu* and *nms* cDNAs in orange-spotted grouper and sequence analysis

Total RNA from the brain of orange-spotted groupers was prepared using TRIzol reagent (Invitrogen). One microgram of isolated RNA was used to synthesize the first-strand cDNA using the ReverTra Ace-First-strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Then GeneRacer Kit (Invitrogen) was used to amplify the full-length cDNA sequence by combining the gene-degenerated primers or specific primers

and 5'- or 3'-adaptor primers. Both 5'- and 3'-end sequences were amplified by two rounds of PCR respectively. All primers used in the study are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

PCR amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 15 s, 53–58 °C for 15 s, and 72 °C for 35–70 s. The reaction was terminated by a final extension cycle of 5 min at 72 °C.

The signal peptide and the neuropeptide prohormone cleavage sites of Nmu and Nms precursors were predicted using Signal P3.0 and Neuropred Software respectively (Bendtsen *et al.* 2004, Southey *et al.* 2006). Nmu and Nms amino acid sequences were aligned using Clustal X1.81 (Thompson *et al.* 1994).

A phylogenetic tree was constructed with MEGA3.1 using neighbor-joining with default settings (Kumar *et al.* 2004). One thousand bootstrap replications were conducted.

RT-PCR analysis of the tissue distribution of *nmu* and *nms* mRNA in the orange-spotted grouper

The tissue expression patterns of *nmu* and *nms* mRNA in various tissues were analyzed by RT-PCR. Total RNA was isolated from 11 tissues, i.e., brain, pituitary, liver, stomach, intestine, muscle, kidney, spleen, gill, heart, and ovary. One microgram of total RNA from each tissue was digested with DNase I (Invitrogen) and reverse-transcribed into cDNA as described above. Mock RT reactions without reverse transcriptase were used as negative controls. The primers are listed in [Supplementary Table 1](#). The PCRs were performed as described above.

Localization analysis of *nmu* and *nms* mRNA in the brain of the orange-spotted grouper

To investigate the distribution of *nmu* and *nms* cells in the brain of the orange-spotted grouper, frozen sections were used for *in situ* hybridization assays. Briefly, the whole brain was fixed with 4% paraformaldehyde (PFA) in PBS, kept at 4 °C overnight and dehydrated in 30% sucrose–PBS buffer for 12 h at 4 °C. The brain was then embedded in optimal cutting temperature compound (Sakura Finetek, Torrance CA, USA), and cross-sections were cut serially at 10 µm thick with frozen cryotomy (Leica 1900, Wetzlar, Germany). The sections were thaw-mounted onto aminopropylsilane-treated glass slides and dried in an oven.

The RNA probe sequences covering the open reading frame (ORF) sequence of the grouper's *nmu* and *nms* were amplified with primers listed in [Supplementary Table 1](#) and

constructed into the pGEM-T easy vector (Promega). Sense and antisense probes were synthesized with a DIG RNA Labeling Mix (Roche). The slides were permeabilized three times with PBS for 10 min and treated with proteinase K in PBS (10 µg/ml) for 15 min at 37 °C, refixed in 4% PFA, prehybridized at 55 °C for 1 h and hybridized with sense and antisense probes for *nmu* and *nms* at 55 °C overnight. After hybridization, sections were washed twice in 2× saline-sodium citrate (SSC) at room temperature for 15 min, and in 1× SSC and 0.1× SSC at 55 °C for 1 h sequentially. The hybridization signals were detected with an alkaline phosphatase-conjugated anti-DIG antibody and were visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Roche). The images were viewed with a Nikon eclipse Ni (Nikon, Japan) optical microscope.

Analysis of the changes in *nmu* and *nms* mRNA levels under different feeding regimes

The experiments were performed in the Daya Bay Aquaculture Center. Fish (body weight 52–60 g and body length 12–15 cm) used for fast experiment were cultured in indoor tanks with circulating seawater at temperatures between 26 and 28 °C.

Fish were randomly divided into five groups (eight animals per group) for fasting experiments, and fed with commercial fish feed (YueQun Ocean Biological Research Development Company, Jieyang, China) once a day at 0900 h. Fish were allowed to acclimate to the environment for 2 weeks. Two groups of fish were fed daily for 2 or 7 days while the remaining three groups were not fed. During the experiment, one fed group and one fasted group were sampled at 2 days. At the end of the 7th day, one of the unfed groups was re-fed to satiety. Then the fed, fasted, and re-fed groups were sampled at 3 h after the scheduled feeding time at end of the experiment. The hypothalamus of each fish was removed and snap-frozen in liquid nitrogen.

To investigate the periprandial expression of *nmu* and *nms* in the hypothalamus, fish were fed daily at a scheduled time (0900 h) for 4 weeks under the same conditions as described above. The daily scheduled feeding time was taken as time zero (0 h). The hypothalamuses were sampled at the following feeding times (–3, 0, 1, and 3 h).

The effects of *Nmu* and *Nms* on the expression of appetite-related genes and the pituitary hormones

Fish (body weight 750–800 g and body length 35–38 cm) whose oocytes were in the stage of cortical alveolus

were used for these experiments, being cultured in indoor tanks with circulating seawater at temperatures between 26 and 28 °C.

The test peptide was dissolved in 0.8% saline. Fish were anesthetized with 0.05% tricaine methanesulfonate and intraperitoneally injected with the test peptides: negative control fish were administered saline only, while experimental fish were injected with Nmu-21 and Nmsrp (250 ng/g body weight). The hypothalamus and pituitary were sampled at 6 h after the injection.

Quantitative real-time PCR

Quantitative real-time PCR was performed on a Roche LightCycler 480 Real-Time PCR System using SYBR Green Real-time PCR Master Mix (Toyobo) according to the manufacturer's protocol. The 10 µl real-time PCRs contained 5 µl SYBR Green Realtime PCR Master Mix, 0.5 µl cDNA, 4.1 µl H₂O, 0.2 µl forward primer, and 0.2 µl reverse primer. PCR conditions were as follows: denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. After amplification, a melting curve was obtained by reading the fluorescence value from 72 to 94 °C. The standard curve was generated between cycle threshold (C_t) value and the logarithm of vector dilutions using serial dilution of quantified pTZ57R vector (MBI Fermentas, Vilnius, Lithuania) containing the fragment of interest. The amplification efficiency of *nmu*, *nms*, neuropeptide y (*npv*), *orexin*, proopiomelanocortin (*pomc*), corticotropin-releasing hormone (*crh*), follicle-stimulating hormone beta subunit (*fshβ*), LH beta subunit (*lhβ*), thyroid-stimulating hormone beta subunit (*tshβ*), growth hormone (*gh*), somatolactation (*sl*), prolactin (*prl*), and β -actin genes was 83, 100, 97, 83, 83, 91, 92, 85, 91, 91, 100, 89, and 100% respectively. The concentration of the template in the sample was determined by relating the C_t value to the standard curve. Quantitative results were expressed as the ratio of target gene/ β -actin. The primers used in the study are listed in Supplementary Table 1.

Statistical analyses

All data are expressed as mean values $\pm$ S.E.M. Statistical differences were assessed by using unpaired Student's *t*-test or a one-way ANOVA followed by a Duncan's multiple range test. *P* value <0.05 was used as the definition of statistical significance. All analyses were performed using the SPSS Software 13.0 (SPSS).

Results

Identification and synteny analysis of *nmu* and *nms* in teleost fish

A number of *nmu* and *nms* candidates were found in the genomes of zebrafish, medaka, and *Takifugu* and in the EST database (Supplementary Information, see section on supplementary data given at the end of this article). To clarify the orthologous relationship of the predicted *nmu* and *nms* sequences, we performed genomic synteny analyses: *nmu* genes were found on the zebrafish chromosome 20 and on the *Takifugu* scaffold 13; *nms* genes were located on the zebrafish scaffold Zv9-NA923 and on the *Takifugu* scaffold 38. Gene clusters around the fish *nmu* and *nms* homologs were determined and compared to those found around human and chicken *NMU* and *NMS* genes. As shown in Fig. 1, by comparing the *NMU* and *NMS* gene loci among these organisms, highly conserved synteny was observed. *NMU* is located in the genomic region that contains several common loci, including steroid 5 alpha-reductase 3 (*SRD5A3*), transmembrane protein 165 (*TMEM165*), and clock circadian regulator

Figure 1

Genomic synteny analysis of (A) *NMU* and (B) *NMS* gene loci in the genome of several vertebrates. The chromosome or scaffold name of synteny blocks are shown on the left (Chr, chromosome; Sd, scaffold). Genes showing syntenic relationships are color-coded. *SRD5A3*, steroid 5 alpha-reductase 3; *TMEM165*, transmembrane protein 165; *CLOCK*, clock circadian regulator; *PDCL2*, phosducin-like 2; *EXOC1*, exocyst complex component 1; *CEP135*, centrosomal protein 135 kDa; *LONRF2*, LON peptidase N-terminal domain and ring finger 2; *CHST10*, carbohydrate sulfotransferase 10; *PDCL3*, phosducin-like 3.

(CLOCK). The neighboring genes around *NMS*, including phosducin-like 3 (*PDCL3*) and LON peptidase N-terminal domain and ring finger 2 (*LONRF2*), are conserved in zebrafish, *Takifugu*, chicken, and human.

Cloning and sequence analysis of *nmu* and *nms* cDNAs in the orange-spotted grouper

Based on the known and predicted sequences, *nmu* and *nms* cDNA sequences were cloned from the brain of the orange-spotted groupers. The cloned *nmu* cDNA fragment is 728 bp in length with a 42 bp 5'-UTR, a 257 bp 3'-UTR, and a 429 bp ORF. The Nmu precursor protein comprises 142 amino acids with a predicted signal peptide of 30 amino acids and a putative mature peptide of 21 amino acids (Fig. 2A). The cloned *nms* cDNA fragment is 1024 bp in length with a 204 bp 5'-UTR, a 457 bp 3'-UTR,

and a 363 bp ORF. The Nms precursor protein is composed of 120 amino acids with a predicted signal peptide of 26 amino acids and a putative mature peptide of 34 amino acids (Fig. 2B).

Sequence comparisons among the deduced amino acid sequences of the vertebrate NMU and NMS precursor proteins showed that the putative functional regions and some proteolytic cleavage site in these two precursor proteins are well conserved across vertebrates (Fig. 3). Interestingly, sequence comparison revealed that two putative peptides, the typical NMU peptides and a 33- or 32-amino acid peptide that we have named NMU-related peptide (NMURP), were found in mammals, chicken, goldfish, and zebrafish NMU precursor proteins. However, only the typical Nmu peptide containing 21 amino acids was observed in the orange-spotted grouper Nmu precursor protein (Fig. 3A). For NMS, two putative

A

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1          TTCCTCAAGACGAGACAGAGCAGCGAGAGCAAGACAGGGAC 42
43  ATGAGGACCGTCCAGAGCCAAAGTCAGCATGCGCAGCGGAGCCTCCAGCAGCCTGCACAGCCTCAGCAGCGGGCGTGAGTCCCCTC 132
    M R T V Q S Q S Q H A Q R G A S S S L H S L S S G G V S P L
133  AGCACCACCAGCGTCACCCCTCACAGCCCTGCTCATCATCTCCACATCCAGTCTGCCAAAGTGCTCCAGCAGAAGTTCAGCAAGCCACA 222
    S T T S V T L T A L L I L T I P V C Q S A P A E L Q Q A T
223  ACAGATCAGAGGAGCTCCTCAGCCAGATAGACGCTGTGTGCTCATCTACCTCTCTCAGAGCCTGAAGTTTGGGCATCTGATGTCTTA 312
    T D Q R Q L L S Q I D A V C S S Y L S A D L K F W A S D V L
313  GGAGAAGCTGTGTCTTGTGATGCTGGTTCAGAAAGTCAAAGGAGCTGAAAGTGCGAGAAAATAGTAAAGGGGTGAAGTCCAGGGAGCTGGG 402
    G E L C V L M L V Q K S K E L K V R E N S K R A E L Q G P G
403  GGAATCCAGAGCAGAGGTTACTTCTCTATCGGCCACGAAATGGAAGAAGATCCTTAGAATATGAGTAAATTAAGAGTCGCCACCTCTG 492
    G I Q S R G Y F L Y R P F N G R R S L E Y E *
493  GAGCTGAGTGACAGAGACAACACAGACCCCTGAGACCCCGAGCCGCTGCACCACGACTGAGTGATCCAGACTTTAACCACAGTCCTTTGC 582
583  CACTGCTGTACAGTGATGTTTGAATGTACACAGAGAATCATGATTAATACTCTCCATTGTCCAGATTTTATTTTGTGTATATGTGTG 672
673  TCATTTCGATTAAACTGCAACAATGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 728

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B

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1          ATGTCTGGGCCATTACGGCCGGGG 24
25  GACACCTCACGGCTGTTAGACCAACCAGGAGAGCTGAGCTGAGCCAAGACACACCGAGACAACTCAGTGGAAACAGAGCTGAGCCACACC 114
115  AAGTGGGCTGGTGTGGATCCACCAAGGAAGAACAACTCTCCAAACAAAGCGCACAGAGTTGAGTTGAGTGATGAGGAGAGAGAGAGAC 204
205  ATGTCTGTACCAACTGTGAGACAACCTTTTCTCTGTGTTTCTTCTGTTGCTGGGATCATGGAGCTCAGCAGATGCCAGTCCGTTTGAT 294
    M S V P T V R Q L F L L C F F W C L G S W S S A D A S P F D
295  CAGTGGGAGGAGCGGATCCAGTTAAGGAAGGTGCGAGGCTCCAGAGTATGACCTGAGTGATGCATTGTGGGAGGATCAGAAGAGGAG 384
    Q W E D G I Q L R K V R G V Q S D D L S D A L W E D Q K E E
385  CAGGTCCAGAAGCTCTTCAAAGAGTTCCTGTTTCTTACTCTAAGCAGCAAACTCAGTAGGAGCGGTGCAGCAGGAGTCTCACTCAGTC 474
    Q V Q N V F K R F L F H Y S K A R N S V G A V Q Q E S H S V
475  CATCTCTGATGAGACTTCTCCCAACTATCCAGAGGAGAAAGAAGGTTTACTGTTGATTTCGCGTCTGCCTGAGGGGATGTTG 564
    H P L M R L S P K L S Q R R K K K V L L L I R G L P E G M L
565  TAGAAGAATCGACCACAAGAGACAGAGCTCAAGCTGAAGAGAAGAGGAGCAGCAGGCAGCAGCTGAACGTCAACATCACTGAGCCGAGGA 654
    *
655  GCCATGTTTACAGACACACACACACACACACACACACACACACACAGGACCTTTTTTTATGAGAAAATAAATCATTTGTACAG 744
745  ATGAGAGACAGCAAGCACTCAACATGGCTGCCATGTGTCTTCTGCTTCCAGCTCAATCAGTGATCGTGAAGTGTGTTGTTT 834
835  TTACACAAGACACAAGCGTCACCAAAATACACACAAACACATTCTTTTAAACAAGTCTTTATGATTCCAGAACACAGATTAACATG 924
925  GCATCGAGGTGAAGTCACTTTCATTTGATTCAACAGATTTACAGTTTGTGACATCATGAAATAAAAAGTTAAACTCAAAAAAAAAAAAAA 1014
1015  AAAAAAAAAA 1024

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Figure 2

Nucleotide sequences and the deduced amino acid sequences of the orange-spotted grouper Nmu (A) and Nms (B). The putative signal peptides

are underlined. The mature peptides are boxed and shown in bold. The proteolytic cleavage sites are italic and shown in bold.

Figure 4

Phylogenetic analysis of NMU and NMS in vertebrates. The phylogenetic tree was constructed using MEGA 3.1 and the neighbor-joining method. Data were re-sampled by 1000 bootstrap replicates. The sequences and their accession numbers are: human NMU (NP_006672.1), mouse NMU (NP_062388.1), pig NMU (XP_003129080.1), chicken NMU (XP_420701.1), goldfish NMU (BAH57726), zebrafish NMU (NP_001292548), tilapia NMU (XP_003439981.1), human NMS (NP_001011717.1), mouse NMS (NP_001011684.3), zebrafish NMS (XP_003201803.1), rainbow trout NMS (BX297606.3), and medaka NMS (AM146637.1).

hypothalamus of the orange-spotted grouper (Fig. 6). *nmu* signals were strongly found in the lateral tuberal nucleus, ventral part (NLTV), while *nms*-positive cells were found in the ventral zone of the periventricular hypothalamus (Hv). No staining was detected using the sense riboprobes of *nmu* and *nms* (data not shown). The neuroanatomy in this study was identified by using the orange-spotted grouper atlas (Nagarajan *et al.* 2013).

Changes of hypothalamic *nmu* and *nms* mRNA levels under different feeding regimes

Real-time PCR was used to study *nmu* and *nms* mRNA levels under different feeding regimes. As shown in Fig. 7A, there was no significant change in *nmu* mRNA levels in hypothalamus after 2 days of food deprivation, but a significant increase of hypothalamic *nms* mRNA levels was observed. Seven days of food deprivation induced a significant reduction in *nmu* mRNA levels in the hypothalamus, but its expression was markedly increased in the 7-day fasted fish after re-feeding. However, there was no significant change in the *nms* mRNA levels in the hypothalamus after 7 days of food deprivation and re-feeding (Fig. 7A).

Periprandial changes in *nmu* and *nms* mRNA levels in the hypothalamus of the orange-spotted grouper are shown in Fig. 7B. The hypothalamic expression of *nmu* did not change at 3 h before feeding and at 0 and 1 h after feeding. However, there was a significant increase in *nmu*

expression at 3 h post-feeding compared to that at other time point. The hypothalamic expression of *nms* was elevated at the normal feeding time (0 h), and then dropped to pre-feeding level at 1 and 3 h post-feeding.

Expression of appetite-related genes in the hypothalamus after the Nmu-21 and Nmsrp administration

The effects of Nmu-21 and Nmsrp administration on the expression of appetite-related genes, *npv*, *pomc*, *crh*, and *orexin*, were investigated in the orange-spotted grouper. As shown in Fig. 8, peripheral administration of grouper Nmu-21 peptide slightly suppressed the expression of hypothalamic *npv*, but had no effect on the hypothalamic expression of *orexin*, *pomc*, and *crh*. Grouper Nmsrp significantly stimulated the expression of *npv* and *orexin* in the hypothalamus, but could not change the hypothalamic expression of *pomc* and *crh* (Fig. 8).

The effects of Nmu-21 and Nmsrp on the mRNA levels of pituitary hormones

The effects of Nmu-21 and Nmsrp administration on the expression of pituitary hormones, including *fshβ*, *lhβ*, *tshβ*, *gh*, *prl*, and *sl*, were investigated in the orange-spotted grouper. As shown in Fig. 9, the mRNA levels of *lhβ* and *gh* in pituitary of the orange-spotted grouper were significantly down-regulated after peripheral injection of Nmu-21 peptide. There was no obvious effect of Nmu-21 peptide on the expression of other pituitary hormones. On the other hand, Nmsrp could significantly up-regulate the mRNA levels of *fshβ*, *prl*, and *sl* but showed no effect on the expression of *lhβ*, *tshβ*, and *gh* in the pituitary.

Discussion

In the present study, we have identified *nmu* and *nms* in several fish species and have cloned the cDNAs of these

Figure 5

RT-PCR analysis of tissue expression patterns of *nmu* and *nms* in the orange-spotted grouper. Amplification of *β-actin* was used as the housekeeping gene control. B, brain; P, pituitary; L, liver; ST, stomach; I, intestine; M, muscle; K, kidney; SP, spleen; G, gill; H, heart; O, ovary; NC, negative control.

Figure 6

Localization of *nmu* (A) and *nms* (B) mRNA-expressing cells in the brain of the orange-spotted grouper. Dp, dorsal posterior thalamic nucleus; Hv, ventral zone of the periventricular hypothalamus; NAPv, anterior periventricular nucleus; NLT, lateral tuberal nucleus; NLTl, lateral tuberal

nucleus, lateral part; NLTv, lateral tuberal nucleus, ventral part; NRL, nucleus of the lateral recess; NSC, suprachiasmatic nucleus; TeO, tectum opticum; TL, torus longitudinalis; Tpp, periventricular nucleus of the posterior tuberculum.

two genes from the orange-spotted grouper. Some differences were found in the precursors of Nmu and Nms between fish species, as well as between fish and other vertebrates. For example, two putative peptides are present in the precursor of NMU in other vertebrates and in some fish species such as common carp and goldfish. But Nmurp

could not be found in the present cloned sequence of orange-spotted grouper. Studies in mammals, common carp and goldfish have shown that *nmu* genes could produce several alternative splicing variants during transcription (Chen *et al.* 2006, Maruyama *et al.* 2008, Kono *et al.* 2012). It was found that the nucleotide sequences

Figure 7

mRNA expression of *nmu* and *nms* under different feeding regimes. (A) Relative mRNA levels of *nmu* and *nms* in the hypothalamus of orange-spotted grouper after 2- and 7-day fasting. Re-feeding was introduced at 7 days after food deprivation. The data obtained at different groups were normalized as % 2-day ctrl or 7-day ctrl. $P < 0.05$ vs the corresponding control (unpaired Student's *t*-test or one-way ANOVA with the Duncan's multiple

range tests). (B) Periprandial changes of *nmu* and *nms* in the hypothalamus of orange-spotted grouper. Fish food was introduced at 0900 h (taken as time zero, 0 h). The data obtained at different groups were normalized as % ctrl at time 0 h. Values are expressed as mean $\pm$ s.e.m. ($n = 8$ for each group). $P < 0.05$ vs the group at time 0 h (one-way ANOVA with the Duncan's multiple range tests). Different letters denote statistically significant differences.

Figure 8

In vivo effects of Nmu-21 and Nmsrp on the expression of appetite-related genes in the hypothalamus of orange-spotted grouper. Fish were injected intraperitoneally with Nmu-21 and Nmsrp at a dose of 250 ng/g body-weight. Hypothalamus samples were collected at 6 h post-injection.

The data obtained at different groups were normalized as % ctrl without peptide treatment. Values are expressed as mean values $\pm$ s.e.m. ($n=6-8$).

* $P<0.05$ vs the corresponding control.

encoding Nmurf were absent in some cDNA variants in goldfish and common carp as well (Maruyama *et al.* 2008, Kono *et al.* 2012). Whether the loss of Nmurf in orange-spotted grouper is caused by alternative splicing regulation is uncertain, as we did not successfully isolate other cDNA variants in this study. It was observed that *nmu* variants exhibited organ-specific expression in the goldfish. Although four cDNAs encoding *nmu* were identified in goldfish, only one splice variant, which did not contain Nmurf and also encodes a putative 21 amino acids Nmu peptide, was highly expressed in the brain and its expression was reduced by fasting (Maruyama *et al.* 2008). The aim of this study was to investigate the role of *nmu* in the regulation of feeding and energy balance. Therefore, the cloned cDNA from the brain of orange-spotted grouper is suitable for our subsequent analysis.

As for the Nms precursor protein, it is likely that the typical Nms peptide was lost in the teleost fish during evolution. We searched the sequence of *nms* gene in the genome databases and EST database. Some transcript variants were predicted in fish, but no typical Nms peptide was found in the precursors of these variants either (Supplementary Information). Moreover, the nucleotide sequence corresponding to Nms peptide could not be found in the genomes of any of fish species analyzed here. Although the existence of NMSRP protein in vertebrates remains to be confirmed, according to the potential protease cleavage sites, it has been proposed that NMURP and NMSRP may be generated from their proproteins (Austin *et al.* 1995). One report mentioned that an endogenous 33-residue peptide NMURP had been purified from rat brain and small intestine extracts, and found that both synthetic NMURP and NMSRP showed potent PRL-releasing activity in rats (Mori *et al.* 2005). In another study, synthetic NMURP was found to

significantly induce feeding behavior, body weight, and metabolic rate in mice (Bechtold *et al.* 2009). These data imply that the NMURP and NMSRP in vertebrates possess biological functions.

Figure 9

In vivo effects of Nmu-21 and Nmsrp on the expression of the pituitary hormones in the orange-spotted grouper. Pituitaries were collected at 6 h post-injection. The data obtained at different groups were normalized as % ctrl without peptide treatment. Values are expressed as mean values $\pm$ s.e.m. ($n=6-8$). * $P<0.05$ vs the corresponding control.

RT-PCR showed that *nmu* and *nms* are broadly expressed in brain and peripheral tissues in the orange-spotted grouper, in a manner similar to that observed in goldfish (Maruyama *et al.* 2008). Brain is the key organ in which NMU and NMS exert their functions. *In situ* hybridization analysis in the orange-spotted grouper revealed that *nmu*-expressing cells are abundant in the NLT of the hypothalamus, while the cells expressing *nms* are located in the Hv. The NLT and Hv may be associated with feeding control in fish as well. Studies have demonstrated that several important neuropeptides associated with food intake are expressed in the NLT and Hv, including *npv*, *pomc*, *mch*, and *crh* (Castro *et al.* 1999, Cerdá-Reverter *et al.* 2000, 2003, Pepels *et al.* 2002, Alderman & Bernier 2007, Amano *et al.* 2009, Berman *et al.* 2009). Although we do not know whether these genes are also localized in the NLT and Hv in the orange-spotted grouper, studies in other fish species have demonstrated that the functions and localizations of these peptides in the brain are well conserved (reviewed by Lin *et al.* (2000), Volkoff *et al.* (2005) and Matsuda *et al.* (2012)). Thus, *nmu* and *nms* are highly likely to play roles in feeding regulation and energy homeostasis in the orange-spotted grouper, and are implicated in interaction with the appetite-related genes.

In agreement with our assumptions above, *nmu* and *nms* gene expression was regulated by changes in energy balance. *nmu* expression was reduced after a 7-day period of food deprivation and increased dramatically after feeding in orange-spotted grouper. This result is in accordance with those found in rodents and goldfish (Howard *et al.* 2000, Ivanov *et al.* 2002, Maruyama *et al.* 2008), indicating that the anorexic roles of *nmu* are evolutionarily conserved from fish to mammals. The present study of the periprandial expression of *nmu* also provided evidence for anorexic roles in the orange-spotted grouper, as *nmu* mRNA levels were significantly elevated at 3 h after the normal feeding. There is a similar report of *nmu* expression increasing under positive energy balance in rodents. *nmu* mRNA levels were elevated in the dorsomedial hypothalamic nucleus of obese *ob/ob* mice compared to lean litter-mates (Graham *et al.* 2003). A number of studies have demonstrated that *nms* acts as an anorexigenic factor (reviewed by Novak (2009)). Unexpectedly, in the orange-spotted grouper, *nms* expression was up-regulated by 2-day fasting and *nms* mRNA levels had gone up at the start of normal feeding. These expression patterns are similar to that of the orexigenic gene *npv* and *orexin* in orange-spotted grouper. Previous studies showed that the expression of

npv and *orexin* were dramatically increased at the time of feeding and after the food deprivation (Yan *et al.* 2011, Tang *et al.* 2013). These data suggest that the *nms* gene may play an orexigenic role in the orange-spotted grouper.

In mammals, the anorexigenic actions of NMU and NMS are mediated by the activation of CRH neurons (reviewed by Malendowicz *et al.* (2012)). Administration of NMU could not suppress food intake or increase oxygen consumption and body temperature in CRH-knockout mice (Hanada *et al.* 2003). Administration of NMS could upregulate the *CRH* mRNA levels in the brain, and the NMS-mediated inhibition of food intake could be attenuated by pretreatment with the antagonist of CRH (Ida *et al.* 2005, Nakahara *et al.* 2010). In goldfish, a significant increase in the expression of *crh* mRNA was observed after injection with Nmu-21 peptide, and the anorexigenic effect of this peptide was erased by treatment with the antagonist of Crh receptor (Maruyama *et al.* 2008). However, this pathway appears to be not conserved in the orange-spotted grouper. Peripheral administration of Nmu-21 peptide had little effect on the *crh* mRNA levels, but slightly suppressed the expression of the orexigenic gene *npv* in hypothalamus of the orange-spotted grouper. Additionally, Nmu-21 peptide could not change the mRNA levels of other feeding-related factors such as *orexin* and *pomc*. These data suggest that the anorexigenic action of Nmu might be mediated by the suppression of Npy or other unknown factors in the orange-spotted grouper. On the other hand, Nmsrp could significantly increase the mRNA levels of *npv* and *orexin*, corresponding well to its putative orexigenic role in the orange-spotted grouper. Although there is limited information about the exact biological functions of NMSRP, a study have found that a significant but transient increase in feeding was observed in mice after central administration of NMURP (Bechtold *et al.* 2009). In vertebrates, NMSRP shares relative high sequence similarity to the NMURP, implying its involvement in the regulation of energy balance. Taken together, it appears that the fish *nms* shows an opposite function to its mammalian counterparts. The structural discrepancy may be responsible for the functional difference of NMS genes between fish and mammals. Nevertheless, its roles in regulating the feeding behavior of the orange-spotted grouper need further confirmation.

Additionally, Nmu-21 peptide and Nmsrp were found to have effects on the expression of some pituitary hormones in the orange-spotted grouper. Nmu-21 inhibited the *lhβ* transcript expression but Nmsrp elevated the *fshβ* mRNA levels, suggesting that these peptides are involved in the regulation of the gonadotropic axis in the

orange-spotted grouper. Our data also further confirm the negative role of NMU in vertebrate reproduction, and first disclose the potential involvement of *nms* in the regulation of *fsh* expression. Furthermore, Nmu-21 peptide was found to suppress *gh* expression, and Nmsrp stimulated *prl* and *sl* expression. These data suggest that *nmu* and *nms* may possess pituitary functions in the orange-spotted grouper, and also provide some indications for further study on the function of NMSRP in vertebrates.

In summary, we have identified the *nmu* and *nms* genes in fish species. And for the first time we have simultaneously characterized the putative roles of *nmu* and *nms* in a single species, the orange-spotted grouper, in terms of their sequence analysis, localization analysis, expression profiles under different energy statuses, interaction with feeding related factors and the regulation on pituitary hormones. Our study is highly suggestive of distinct roles for *nmu* and *nms* genes in regulation feeding and of possessing different pituitary functions in the orange-spotted grouper, paving the way for further evaluation of their physiological significance in fish species.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0018>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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