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Two chicken neuromedin U receptors: Characterization of primary structure, biological activity and tissue distribution ☆

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ABSTRACT

Neuromedin U (NMU) is a bioactive peptide that is involved in a variety of physiological functions. Two of its receptors, NMUR1 and NMUR2, have been identified and characterized in mammals. In this study, we performed cDNA cloning of chicken NMUR1 and NMUR2, and characterized their primary structure, biological activity, and expression patterns in chicken tissues. The chicken NMUR1 and NMUR2 cDNAs encoded 438 and 395 amino acid sequences, respectively. Chicken NMUR1 showed 54.8%–56.5% sequence identity with human, rat, and mouse NMUR1, and NMUR2 shared 67.3%–70.1% sequence identity with mammalian orthologs. Both chicken receptors have typical characteristics of G-protein-coupled receptors with seven transmembrane domains and the D/ERY motif. An increase in intracellular Ca^{2+} mobilization was observed in HEK293 cells transfected with chicken NMUR1 or NMUR2 cDNA and treated with chicken or rat NMU. Real-time PCR analysis revealed that NMUR1 mRNA was preferentially expressed in the intestinal tissues such as the duodenum, jejunum, ileum, cecum, and colon/rectum, and brain regions such as the midbrain and optic lobe, and the ovary in adult hens. NMUR2 mRNA was exclusively expressed in the brain regions such as the cerebrum and midbrain. These results indicate that NMUR1 and NMUR2 mRNAs, which encode functional receptor proteins, are expressed in chicken tissues with different distribution patterns.

1. Introduction

Neuromedin U (NMU) is a bioactive peptide comprising 8–25 amino acid residues with the conserved C-terminal five amino acid sequence that is responsible for the biological activity. NMU involved in a wide range of central and peripheral functions in various vertebrate species [1,2,17]. The functions of NMU are mediated through its specific receptors. In mammals, two distinct NMU receptors, NMUR1 and NMUR2, have been identified. NMUR1 was initially cloned as an orphan G-protein-coupled receptor (GPCR), FM3/GPR66, which is a homolog of the growth hormone secretagogue receptor (GHS-R)/ghrelin receptor and neurotensin receptor (NTR) [6,11,28]. This receptor was later identified as a NMU receptor and cloning of NMUR2 revealed another form of NMU receptor [10,11,22,24]. NMUR1 mRNA is distributed in a broad range of tissues [6,11,27], whereas NMUR2 mRNA is expressed in restricted regions of the brain [10,11,22]. Recently, non-mammalian orthologues to NMUR1 and NMUR2 have been identified in goldfish [16].

In birds, two forms of NMU, NMU-25 and NMU-9, have been isolated from the intestine and brain of a chicken [5,19], respectively, and NMU cDNA has been identified in Japanese quails [25]. Central effects of NMU have been observed in these avian species. In chickens, central administration of rat NMU-23 suppresses food intake and induces wing-flapping behavior [13]. In addition, hypothalamic mRNA levels of corticotropin-releasing factor and arginine vasotocin are increased by the NMU administration [13]. In Japanese quails, both peripheral and central injections of Japanese quail NMU reduce food intake and increase body temperature and locomotor activity [25]. To understand the molecular mechanisms of NMU function in birds, characterization of the structure and tissue distribution of NMUR is indispensable. NMUR is classified as a member of the ghrelin receptor family including GHS-R, motilin receptor (MTL-R), NTR1, NTR2, and the orphan receptor GPR39 [9]. We have previously identified and characterized the ghrelin receptor family in chickens (GHS-R [29], MTR [31], NTR1 [18], and GPR39 [32]) and Japanese quails (GPR39 [33]). NMURs have not been identified in any avian species although the nucleotide sequences of the coding regions for chicken NMUR1 and

☆ These sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession Nos. HQ833216 (NMUR1) and HQ833217 (NMUR2).

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NMUR2 mRNAs can be predicted from the chicken genome sequence.

In this paper, we report the identification and molecular characterization of chicken NMUR1 and NMUR2 mRNAs together with their tissue distribution.

2. Materials and methods

2.1. Animals

Adult hens (White Leghorn, 36-weeks-old) were obtained from Gen Corporation (Gifu, Japan) and housed for a week under standard conditions with food and water available ad libitum. The animals were then sacrificed by decapitation, tissues were immediately frozen in liquid nitrogen, and stored at −80 °C until further use. All procedures were performed in accordance with NIH guidelines for animal care.

2.2. Cloning of cDNA for chicken NMUR1 and NMUR2

Total RNA was extracted from the jejunum and brain of a 36-week-old hen using TRIzol (Invitrogen, Carlsbad, CA). The amount of RNA was determined by spectrophotometry. cDNA libraries were prepared from the total RNA using a FirstChoice RLM-RACE kit (Ambion, Austin, TX). Primers were designed based on the sequences of chicken NMUR1 (GenBank accession number XM_426705) and NMUR2 (GenBank accession number XM_425209) predicted from the genomic sequences recorded in the National Center for Biotechnology Information (NCBI) DNA database. The 5′- and 3′-ends of the cDNA were amplified by RACE-PCR according to the manufacturer's instructions using primers 1–1 (antisense) and 1–2 (sense) for NMUR1 and primers 2–1 (antisense) and 2–2 (sense) for NMUR2 with an adapter primer supplied by the manufacturer. The positions of the PCR primers are shown in Figs. 1 and 2. cDNA clones for chicken NMUR1 and NMUR2 were obtained from the jejunum and brain cDNA libraries, respectively. PCR was performed for 30 cycles of 98 °C for 10 s and 68 °C for 90 s in 50 μl of GC buffer containing 1.25 units of PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) and 0.2 μM each of sense or antisense primer. The amplified fragments were cloned into a pCR-Blunt II-TOPO plasmid vector (Invitrogen). Sequencing was performed with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

2.3. Functional assay of chicken NMUR1 and NMUR2

The coding region of NMUR1 and NMUR2 was amplified by PCR with primers 1–3 (sense) and 1–4 (antisense) for NMUR1 and primers 2–3 (sense) and 2–4 (antisense) for NMUR2. The positions of the primers are shown in Figs. 1 and 2. Primers 1–3 and 2–3 contained a HindIII restriction enzyme site at their 5′-ends, and primers 1–4 and 2–4 contained an XhoI restriction enzyme site at their 5′-ends. The plasmid DNA containing the coding region of chicken NMUR1 or NMUR2 was digested with restriction enzymes HindIII or XhoI, and the DNA fragment was subcloned into a pcDNA3.1/Hygro (+) expression vector (Invitrogen).

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 10% fetal bovine serum at a density of 1×10^5 cells in a collagen-coated 6 cm diameter dish for 24 h. The expression vectors (1 μg each) for chicken NMUR1 and NMUR2 were transfected with FuGENE-6 (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's protocol. Twenty-four hours after transfection, the cells were plated onto a collagen-coated 96-well black plate at a density of 3×10^4 cells per well. Twenty hours after plating, the cultured medium was aspirated, and 100 μl of a fluorescent dye solution containing 4.4 μM Fluo-4AM (Invitrogen), 1% fetal bovine serum, and 0.045% pluronic acid (Sigma–Aldrich, St. Louis, MO) in a working buffer consisting of 1× Hank's BSS and 20 mM HEPES buffer containing 250 μM probenecid (Sigma–Aldrich) was loaded into each well. The plate was incubated for 1 h at 37 °C in a CO^2 incubator. After three washes with the working buffer, 100 μl of the designated concentration of synthetic chicken NMU-25 (YKVDEDLQGAGGIQSRGYFFFRPRN–NH_2, Hayashi-Kasei Co., Ltd., Osaka, Japan) or rat NMU-23 (YKVNEYQGPVAPSGGFFLFRPRN–NH_2, Peptide Institute, Osaka, Japan) in working buffer containing 0.001% Triton X-100 was automatically added to each well using the FLIPRtetra system (Molecular Devices, Menlo Park, CA). Intracellular Ca^{2+} changes were measured by excitation at 488 nm and emission at 500–560 nm.

2.4. Reverse transcription (RT) and real-time PCR analysis

Total RNA (1 μg) extracted from various chicken tissues was reverse-transcribed at 50 °C for 60 min in 12.5 μl of reaction mixture containing 25 units of Superscript III Transcriptase (Invitrogen), 0.4 mM dNTPs, 10 mM dithiothreitol, 64.2 μM random primers, and 1× First Strand buffer supplied by the manufacturer. After inactivation of the reverse transcriptase by heating at 70 °C for 15 min, the cDNA product was subjected to real-time PCR performed according to the user instructions for Real-time PCR system 7500 (Applied Biosystems). PCR was performed at 95 °C for 5 s and 60 °C for 34 s in 20 μl buffer containing 1× SYBR premix ExTaq II (Takara) and 0.2 μM each of primers 1–5 (sense) and 1–6 (antisense) for NMUR1 or primers 2–5 (sense) and 2–6 (antisense) for NMUR2. Quantitative measurement was performed by establishing a linear amplification line from serial dilutions of the coding-region cDNAs of chicken NMUR1 and NMUR2.

The specificity of the PCR reaction was confirmed by melting curve analysis with the real-time PCR system.

3. Results

3.1. Nucleotide and deduced amino acid sequences of chicken NMUR1 and NMUR2 cDNAs

The chicken NMUR1 cDNA consisted of 31 bp of the 5′-untranslated region (UTR), 1314 bp of the coding region, and 531 bp of 3′-UTR (Fig. 1). Chicken NMUR2 cDNA comprised 179 bp of 5′-UTR, 1185 bp of the coding region, and 1280 bp of 3′-UTR (Fig. 2). A potential polyadenylation signal was present close to the 3′-ends of both cDNAs (Figs. 1 and 2). The deduced 438 amino acid sequence of chicken NMUR1 showed moderate overall sequence identity with NMUR1 of humans (56.3%), mouse (54.8%), and rat (56.5%), while the deduced 395 amino acid sequence of chicken NMUR2 displayed high sequence identity with humans (70.1%), mouse (68.7%), and rat (67.3%). Fig. 3 shows an alignment of the amino acid sequences of chicken and mammalian NMUR1 (Fig. 3A) and NMUR2 (Fig. 3B). The hydrophobic seven transmembrane (TM) regions were conserved in both chicken receptors. An ERY motif is present at the junction of TM3 and the second cytoplasmic loop in both types of chicken receptors as well as in mammalian receptors. The two cysteine residues, which are known to form a disulfide bridge, are also conserved at the first and second extracellular loops of both chicken receptors. Chicken NMUR1 and NMUR2 contain two N-linked glycosylation sites, NX(S/T), in their extracellular N-terminal regions, as is observed in the mammalian receptors. NMUR2 has an additional conserved N-linked glycosylation site in the second extracellular loop.