SPANDIDOS PUBLICATIONS

Expression of neuromedins S and U and their receptors in the hypothalamus and endocrine glands of the rat

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Abstract. Neuromedin S (NMS) and neuromedin U (NMU) are regulatory peptides that share the C-terminal amino-acid sequence and act via common G protein-coupled receptors called NMUR1 and NMUR2. Semiquantitative real time-PCR showed that in the rat hypothalamus and testis NMS gene expression was markedly higher than that of the NMU gene, while the reverse occurred in the anterior pituitary and thyroid gland. Low expression of both genes was detected in the thymus, adrenal gland and ovary, whereas in the pancreatic islets only the expression of NMU mRNA was detected. In the rat hypothalamus the expression of the NMUR2 gene was strikingly higher than that of the NMUR1 gene; in contrast, in the testis and ovary the very low expression of NMUR2 contrasted with the relatively high expression of the NMUR1 gene. In the other glands examined only expression of the NMUR1 gene was found. The marked differences in the level of expression of NMU, NMS and their receptors in the hypothalamus and endocrine glands of the rat suggest that in this species such neuromedins may play different roles in the functional regulation of neuroendocrine axes.

Introduction

Neuromedin S (NMS) is a 30-amino acid peptide, which has been recently isolated from the human, rat and mouse brain as an endogenous ligand of the orphan G protein-coupled receptors FM-3/GPR66 and FM-4/TGR-1 that in turn have been identified as neuromedin U (NMU) receptors (NMUR) 1 and 2, respectively (1-6). This neuropeptide shares with NMU the C-terminal amino-acid sequence, and was named NMS because it is highly expressed in the hypothalamic suprachiasmatic nucleus (7-9).

Available findings stress numerous differences between NMS and NMU. NMS is a neuropeptide mainly involved in the modulation of the immune response, regulation of circadian rhythms and spermatogenesis. Moreover, NMS has been also suggested to be a potent anorexigenic hormone that acts, among others, via CRH neurons (7-9). NMU is a brain-gut peptide involved in the regulation of energy homeostasis and neuroendocrine functions (4,5,10-18).

The aim of the present study was to compare by semi-quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR) the level of expression of NMS, NMU, NMUR1 and NMUR2 genes in the hypothalamus and endocrine glands of the rat.

Materials and methods

Animals. Adult male and female Wistar rats (200-250 g body weight) were kept under a 14:10-h light-dark cycle (illumination onset at 8:00 a.m.) at 23˚C, and maintained on a standard diet and tap water *ad libitum*. Rats were decapitated, and the hypothalamus, anterior pituitary, thyroid gland, thymus, pancreas, adrenal glands, ovaries and testes were promptly removed and placed in RNA later (Qiagen, Hilden, Germany) (19). Pancreatic islets were isolated by collagenase digestion (20). The study protocol was approved by the local Ethics Committees for Biomedical Studies.

RT-PCR. Total RNA was extracted from 30 mg of tissues, and reverse transcribed to cDNA (21-23). Conventional PCR was performed as previously described (24-26), using the following program: predenaturation step at 94˚C for 10 min to activate Taq DNA polymerase, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 60 sec and extension at 68˚C for 120 sec. Primer sequences and the predicted sizes of amplicons were as follows: i) NMS, sense (276-293) 5'-AGTTTGCTCCCGTCCACT-3' and antisense (444-465) 5'-TTGTCAGTGTATCTTCCATTCC-3' (190 bp; NM012233); ii) NMU, sense (50-69) 5'-GCTTTAACACCC GCACAACA-3' and antisense (346-365) 5'-GGCAAAGCTT CCTCAATGCA-3' (316 bp; NM022239); iii) NMUR1, sense (481-500) 5'-GCCATCTGGGTCTTCGCTAT-3' and antisense

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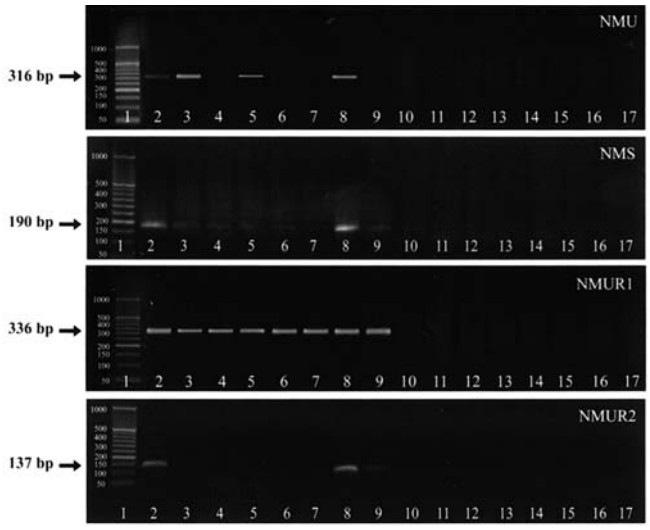

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat NMU, NMS, NMUR1 and NMUR2 specific primers from exemplary rat hypothalamus (2), anterior pituitary (3), adrenal gland (4), thyroid gland (5), thymus (6), pancreatic islets (7), testis (8) and ovary (9). Respective negative controls (no RT of the RNA) are shown in lanes 10-16. Lane 1 was loaded with 200 ng of a DNA size marker (50 bp DNA Ladder; MBI Fermentas, Vilnius, Lithuania).

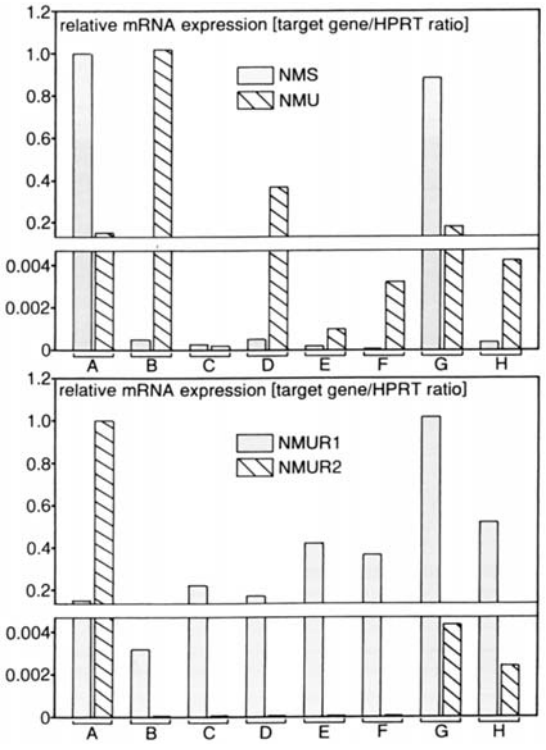

Figure 2. Real-time PCR semiquantitative analysis of NMU, NMS (upper panel), NMUR1 and NMUR2 gene expression (lower panel) in the hypothalamus (A), anterior pituitary (B), adrenal gland (C), thyroid gland (D), thymus (E), pancreatic islets (F), testis (G) and ovary (H) of the adult rat. Bars are means of two independent estimations.

(797-816) 5'-CACCTGTCTGCGTTCCCTAT-3' (336 bp; AF242873); and iv) NMUR2, sense (841-859) 5'-GAATCCC TTGAGGCGAACA-3' and antisense (958-977) 5'-CTGAAG AAGAGCCGGTCCAC-3' (137 bp; NM022275). To rule out the possibility of amplifying genomic DNA, one PCR was performed without prior RT of the RNA. Detection of the PCR amplification products was performed by size fractionation on 2% agarose gel electrophoresis. The specificity of the PCR was further verified by sequencing analysis (27).

Real-time PCR. Semiquantitative real-time PCR was carried out in a Roche Light Cycler 2.0 (Roche Applied Science, Basel, Switzerland) with software version 4.0 (28-31), using the following program: predenaturation step at 95˚C for 10 min, and 45 cycles of three-step amplification (denaturation, 95˚C for 10 sec; annealing, 58˚C for 5 sec; and extension, 72˚C for 10 sec). Subsequently, melting curve (60-90˚C with a heating rate of 0.1˚C/sec) was performed to check the specificity of amplification and the presence of byproducts. All samples were amplified in duplicate, and the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) gene was used as a reference to normalize data. The primer sequences were as follows: i) NMS, see conventional PCR; ii) NMU, sense (50-69) 5'-GCTTTAACACCCGCACAACA-3' and antisense (230-249) 5'-TGAGGCGATATTGGCGTACC-3' (200 bp; NM022239); iii) NMUR1, sense (1-20) 5'-ATGCTCTCCC CAAATGCTTC-3' and antisense (172-190) 5'-CCAGAGT GCCCACTACGAA-3' (190 bp; NM023100); iv) NMUR2, see conventional PCR; and v) HPRT, sense (391-412) 5'-CAGTCAACGGGGGACATAAAAG-3' and antisense (515-536) 5'-ATTTTGGGGCTGTACTGCTTGA-3' (146 bp; NM012583).

Western blotting. Prepro-NMS protein was identified in the rat hypothalamus and testis. Samples were homogenized in Tris-sucrose-EDTA buffer (10 mM Tris, 250 mM sucrose and 0.1 mM EDTA; pH 7.4), and centrifuged at 600 x g for 30 min at 4˚C to remove cell debris. Protein concentration was determined by the Bradford method. Samples of 20 μg of protein were loaded into each lane, separated on a 15% SDS-polyacrylamide electrophoretic gel, and then transferred onto a PVDF membrane (Millipore, Bedford, MA). Transferred proteins were stained with Ponceau S, and non-specific binding was blocked by immersing the membrane in 5% bovine serum albumine at 4˚C overnight. Membranes were incubated with rabbit anti-NMS primary antibody (Phoenix Pharmaceuticals, Belmont, CA) (1:2,000 dilution) for 60 min at 37˚C. Then, after washing in TBST [10 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween-20] three times for 10 min, membranes were incubated with anti-rabbit IgG peroxidase-conjugated secondary antibody (Sigma-Aldrich Corporation, St. Louis, MO) (1:10,000 dilution) for 60 min at room temperature. Membranes were washed four times in TBST, and the target protein was detected by an ECL advanced Western blotting detection kit (Amersham, Aylesbury, UK). Chemiluminescence signal was then transferred on a CL-X Posure Film (Pierce Biotechnology, Rockford, IL).

Results

Conventional PCR detected the presence of NMU mRNA in the anterior pituitary, thyroid gland and testis of the adult rat, and of NMS mRNA in the hypothalamus and testis. NMUR1 mRNA was present in all tissues examined, while NMUR2 mRNA was found only in the hypothalamus and testis (Fig. 1).

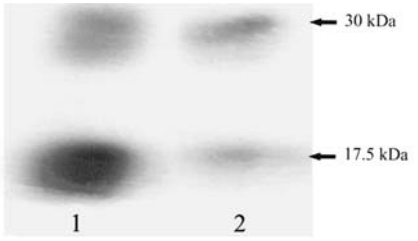

Figure 3. Western blotting of prepro-NMS in the hypothalamus (1) and testis (2) of the adult rat.

Real-time PCR revealed that in the rat hypothalamus and testis NMS gene expression was from 2- to 3-fold higher than that of the NMU gene, while in the anterior pituitary and thyroid gland expression of the NMU gene was nearly 1000 times higher than that of the NMS gene. Low expression of both NMU and NMS genes was observed in the thymus, adrenal gland and ovary, while in pancreatic islets only NMU expression was detected (Fig. 2, upper panel). In the hypothalamus expression of the NMUR2 gene was approximately 10 times higher than that of the NMUR1 gene, whereas in the testis and ovary very low expression of the NMUR2 gene contrasted with a relatively high expression of the NMUR1 gene. In the anterior pituitary, thyroid gland, thymus, pancreatic islets and adrenal gland only expression of the NMUR1 gene was detected (Fig. 2, lower panel).

Western blotting showed the expected NMS-protein size-band of 17.5 kDa in the hypothalamus and testis. An additional band of approximately 30 kDa was also observed (Fig. 3).

Discussion

Earlier studies revealed the presence of NMU-like immunoreactivity in the central nervous system and alimentary tract, as well as in the excretory, respiratory, endocrine and immune systems. The NMU protein expression was very intense in the hypothalamus, thalamus, anterior pituitary and thyroid gland (32-42). In the hypothalamus, NMU-positive nerve fibers were observed in the supraoptic and paraventricular nuclei, while NMU-positive perikarya were restricted to the rostrocaudal part of the arcuate nucleus (34-36). However, *in situ* hybridization demonstrated the presence of NMU mRNA in the tuberal part of the hypothalamus, but not in the arcuate nucleus (40). On the other hand, the NMS gene was found to be specifically expressed only in the hypothalamic suprachiasmatic nucleus, as well as in the rat testis (8). Our study confirms the expression of NMS as mRNA and protein in the rat hypothalamus and testis. However, Western blotting revealed two bands, one with a molecular weight typical for prepro-NMS protein (17.5 kDa) and one of approximately 30 kDa. Similar bands for hypothalamic NMS protein are also shown in the manual for applied methods of Phoenix Pharmaceuticals, and it remains to be established whether the higher molecular weight band corresponds to a prepro-NMS protein dimer. In contrast with previous studies, NMU mRNA expression was not detected in the rat hypothalamus, and this negative results requires further investigation.

Both NMU and NMS interact with specific NMUR1 and NMUR2 (8), the former being the peripheral receptor and the latter the central one (43-44). Accordingly, the widespread distribution of the NMUR1 gene, among others in several endocrine glands, has been consistently reported (2,4,6,43,45,46), while the presence of the NMUR2 gene was restricted to different brain regions, including numerous hypothalamic nuclei (4,43,45,47), and rat testis (43,46,47). Our present findings confirm this distribution of NMUR1 and NMUR2. They also show the expression of NMUR2 in the rat ovary, at a level comparable to that in the testis but markedly lower than that of the NMUR1 gene.

As far as the physiological role of NMU and NMS is concerned, experimental data suggest their involvement in the regulation of neuroendocrine axes, including the gonadotropic one (7-18,48,49). Our semiquantitative real-time PCR data indicate great differences in the level of expression of these neuromedins and their receptors in the endocrine glands, thereby suggesting that NMU and NMS may play different roles in the functional regulation of the endocrine system. It is believed that such neuromedins, and especially NMS, exert their biological effects mainly via autocrine-paracrine mechanisms (8). However, Phoenix Pharmaceuticals has recently made available kits for NMU and NMS assay in the human, rat and mouse serum that showed that NMS concentration may reach values of approximately 30 pg/ml in humans. This finding makes it reasonable to suggest that NMU and NMS may modulate neuroendocrine axes by also acting as true circulating hormones.

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