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Distribution of neuromedin S and its receptor NMU2R in pigs

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ABSTRACT

Neuromedin S (NMS) has been found to be involved in the regulation of the reproductive, endocrine, and immune systems in mammals. However, its function in pigs is currently not well understood. Thus, it is essential and important to characterize the central distribution of NMS mRNA and its receptor, neuromedin U receptor-2 (NMU2R), in pigs for clarifying its physiological functions. In this study, we found that NMS mRNA were densely distributed in the hypothalamus, hypophysis, hippocampus, and brain stem of pigs by in situ hybridization. Moreover, NMS and NMU2R mRNAs was also expressed in the alimentary organs, endocrine and lymphatic organs, and ovaries by semi-Q RT-PCR. All these results suggest that the NMS/NMU2R system plays an important role in modulating various physiological functions in pigs. This research provides useful information for predicting the physiological functions of the NMS/NMU2R system in pigs.

1. Introduction

Neuromedin S (NMS), a brain–gut peptide containing 36 amino acids, has many physiological functions in mammals. It was identified as the second endogenous ligand for two orphan G protein-coupled receptors in rats (Mori et al., 2005). Neuromedin U receptor-2 (NMU2R), the second co-binding receptor of NMS and neuromedin U (MNU), was mainly found to be expressed in the paraventricular nucleus (PVN) and arcuate nucleus (Arc) of the rat hypothalamus. Structural analysis has revealed that NMS contains the same C-terminal 7-amino-acid sequences and it also binds to the same receptor as NMU; however, the gens encoding NMS and NMU are present in different locations (Minamino et al., 1985; Mori et al., 2005). Correspondingly, the expression of NMS and NMU mRNAs also differs. Other studies have shown that the tissue expression of NMS mRNA was more restricted than that of NMU mRNA (Ida et al., 2005). NMS mRNA was mainly found to be expressed in the suprachiasmatic nucleus (SCN), Arc, and PVN of the rat hypothalamus. As indicated by the pronounced rhythmic expression of NMS mRNA in the SCN of the rat hypothalamus (Miyazato et al., 2008; van Esseveldt et al., 2000; la Fleur et al., 2001; Kreier et al., 2003), NMS is involved in the regulation of the dark-light rhythm (Mori et al., 2005), anorexigenic behavior (Ida et al., 2005), and reproductive hormone secretion (Yang et al., 2009; Lei et al., 2009; Vigo et al., 2007). All these studies were mostly performed in rodents, and very few reports are available on farm animals, especially in pigs.

Owing to this lack of information, the distribution and biological role of NMS in pigs cannot be determined. In this study, we determined the tissue distribution of NMS in pigs by in situ hybridization (ISH) and the expression of NMS and NMU2R mRNA by semiquantitative reverse transcriptase-polymerase chain reaction (semi-Q RT-PCR).

2. Materials and methods

2.1. Animals and samples

Female long white pigs ($n = 10$) aged 1 month and weighing 10 ± 2 kg were used in this study. The pigs were killed by decapitation on specific days. The brain tissues ($n = 5$) were quickly removed and frozen in isopentane at −40 °C. The frozen brains were cut into 15-μm-thick transverse sections on a cryostat microtome and thaw-mounted as described previously (Guan et al., 1998; Su et al., 2008), and the sections were immediately stored at −80 °C. Brain and peripheral tissue ($n = 5$) samples for semi-Q RT-PCR were immediately frozen in liquid nitrogen and then stored at −80 °C until RNA extraction. The experiments were approved by the Institutional Animal Ethics Committee of Nanjing Agricultural University. In the present study, we attempted to minimize the number of animals used and their suffering.

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2.2. ISH

ISH analysis was performed on the frozen transverse brain sections using a specific oligonucleotide probe for pig NMS (5′-TGGAATCCCGCCGCC AAATGTCTTCATCCTTCGATCAGCA-3′, with 3′-end labeled with digoxin). One in every five sections of the brain was processed for NMS mRNA hybridization. The sections were pretreated with 0.3% Triton X-100/0.1 M PBS (15 min) and pepsin in 0.1 M citric acid (2 min) at RT. Briefly, sections were covered with 30 μl of hybridization buffer (50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 2× SSC, 100 mg/ml of yeast transfer RNA, 125 mg/ml sonicated salmon sperm DNA and 100 mM dithiothreitol) and allowed to prehybridize for 30 min at 37 °C. After prehybridization, the sections were quickly rinsed in 2× SSC buffer. The DIG labeled oligonucleotide probe was diluted with the hybridization buffer (the buffer should be heated to 37 °C before addition of probe) and used at a final saturating concentration of 0.5 μg/μl. Subsequently, the sections were evenly covered with sterile Parafilm about the same size as the tissues (avoiding air bubbles) to prevent evaporation and hybridized for 17 h at 37 °C (using a forceps to avoid contamination). Removed the Parafilm from sections by using forceps/tweezers before tipping off the hybridization buffer at the end of the hybridization, and orderly put the slides into different concentration of wash solution: 2× SSC buffer for 5 min at RT, 0.5× SSC buffer and 0.2× SSC buffer for 15 min at 37 °C. Slides remained in the last wash solution until next step. The sections were covered with blocking solution (0.5 M PBS + 0.3% Triton X-100 + 1% proprietary blocking agent from Roche) for 30 min at RT. Then, poured off blocking solution and incubated sections with antibody (anti-DIG-biotin, Fab fragments, made in rat by Wuhan Boster Biological Technology Co. Ltd., China) diluted 1:500 in 0.5 M PBS for 80 min at 37 °C. The expression of the bound probe mRNA was visualized by incubation in the streptavidin–biotin complex (SABC) reagent (containing horseradish peroxidase) and was developed in a freshly prepared 0.02% DAB (Wuhan Boster Biological Technology Co. Ltd., China) in distilled water as a chromogen for 3–4 min, resulting in the production of a profound brown color. The reaction was stopped by rinsing the slides several times in tap water. Finally, slides were then dehydrated in ethanol, cleared in xylene and cover slipped with resin. Specificity of hybridization was defined by signals that are completely displaceable by the addition of 100-fold molar excess of non-labeled probe.

2.3. Semi-Q RT-PCR

All samples were ground in liquid nitrogen and an approximately 1 g fraction was used to extract total RNA with the animal RNA purification reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed by incubation at 42 °C for 1 h in a 25-μl mixture consisting of 1× RT buffer (Promega, USA), 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, USA), 8 U RNase inhibitor (Promega, USA), 0.8 pmol/l oligo d (T) 15 primers (Invitrogen, Shanghai, China) and 0.8 mmol/l dNTPs (Invitrogen, Shanghai, China). The reaction was terminated by heating at 95 °C for 5 min and quickly cooling at 0 °C. The semi-Q RT-PCR protocols for analyzing pig NMS, NMU2R, and glyceraldehyde-3-phosphate dehydrogenase have been described previously (Yang et al., 2009).

2.4. Observation and statistical analysis

Images of representative tissue sections for determining the localization of NMS-positive cells or fibers were acquired using bright-field microscopy (Olympus BH2). All statistical analyses were performed by one-way ANOVA with the Statistical Package for the Social Sciences (SPSS 16.0, Chicago, IL, USA).

3. Results

3.1. Localization of NMS in the pig brain

3.1.1. Localization of NMS-positive cells in the pig brain

According to the instructions in Fig. 1, the localizations of some illegible nuclei containing NMS mRNA in the pig brain could be identified. In the diencephalon, NMS-positive cells typically appeared multipolar were mainly found in the anterior nuclei of the thalamus (Fig. 2A and A1); the positive cells were sparsely distributed in the ventromedial thalamic nucleus (Fig. 2B and B1) and central medial nucleus (Fig. 2C and C1), and they typically appeared ovoid, piriform, and multipolar. In the pig hypothalamus, NMS-positive cells were detected in the dorsal hypothalamic area (Fig. 2D and D1), anterior hypothalamic area (HAA), and tuberal region of the hypothalamus. In the HAA of the hypothalamus, NMS-positive cells were small-to-medium sized, mostly monopolar or bipolar; they were mainly concentrated in the hypothalamic periventricular nucleus (PEN) (Fig. 2E and E1), PVN (Fig. 2F and F1), SCN (Fig. 2G and G1), anterior hypothalamic nucleus (Fig. 2G and G2), and supraoptic nucleus (SON) (Fig. 2H and H1). The density of the NMS-positive cells in the tuberal region of the hypothalamus was lower than that in the HAA. In the tuberal region of the hypothalamus, NMS-positive cells were widely distributed in the

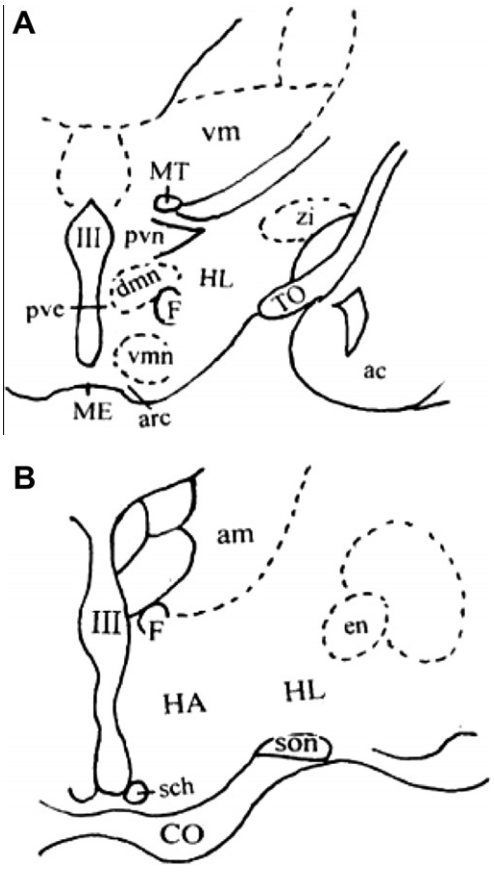

Fig. 1. The sketch map of some hypothalamic nuclei in pigs. (A) Transverse section through hypothalamic tuberal area; (B) transverse section through anterior hypothalamic area. (A) and (B) are maps adopted from the atlas which was made by one colleague of our laboratory (Li et al., 2005) according to the previous report (Fé lix et al., 1999). III, the third ventricle; F, Fornix; AH, anterior hypothalamic area; LH, lateral hypothalamic area; MT, Mammillothalamic tract; CO, Op tic chiasma; TO: Op tic tract; ME, median eminence; sch, Suprachiasmatic nucleus; son: supraoptic nucleus; pve, periventricular nucleus; pvn, paraventricular nucleus; dmn, dorsomedial nucleus; vmn, ventromedial nucleus; arc, arcuate nucleus; zi, zona incerta; ac, amygdala.