Animal Reproduction Science 181 (2017) 115–124

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Postnatal developmental of Neuromedin S and its receptor in the male Xiaomeishan pig reproductive axis

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ARTICLE INFO

Keywords:
Neuromedin S (NMS)
NMUR
Xiaomeishan pigs
Reproductive axis
Postnatal

ABSTRACT

Neuromedin S (NMS) has been identified as an endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 which are NMU receptors NMUR1 and NMUR2, respectively. The NMS molecule is present in some peripheral tissues and the central nervous system (CNS), and it had been documented that NMS has fundamental and important roles in multiple physiological functions and processes such as circadian rhythm, energy balance, feeding behavior, stress responses and reproduction. The possible role of NMS in sexual development postnatally, however, is still obscure. This study aims to determine the change of NMS and its receptor gene expression in the reproductive axis of male Xiaomeishan pigs, postnatally. Firstly, the cDNA of the NMS and its receptors was cloned and sequenced. The results showed that there was a lack of 12 amino acids in the C-terminal of the male Xiaomeishan pig NMS amino-acid sequences compared with other animal species, but the main protein structure of prepro-NMS was high in homology. In addition, the nucleotide sequence and amino acids of the male Xiaomeishan pig's NMUR1 and NMUR2 had high homology. The NMS and NMUR2 mRNA in the male Xiaomeishan pig was detected in the reproductive axis at postnatal development stages, including postnatal day 3, 30, 60, 90 and 120, using real-time PCR and immunohistochemistry. The data showed that there were developmental changes in NMS and NMUR2 in the reproductive axis of the male Xiaomeishan pigs, postnatally, which suggested that NMS and NMUR2 might have a role in the development of the boar reproductive axis, but its regulatory mechanism remains to be elucidated.

1. Introduction

The neuromedin S (NMS) molecule, a 36-amino acid brain-gut peptide, was isolated and purified as the endogenous ligand of orphan G protein coupling receptors NMUR1 (FM-3/GPR66) and NMUR2 (FM-4/TGR-1) in 2005 (Mori et al., 2005). It was named "neuromedin S" due to its specific expression of this gene in the suprachiasmatic nuclei (SCN) of the hypothalamus (Ida et al., 2005; Mori et al., 2005). The NMS molecule shares the same C-terminal core structure with NMU, and binds to the same receptors; however, the genes encoding NMS and NMU are present in different locations (Minamino et al., 1985; Ida et al., 2005). Correspondingly, the relative abundance of NMS and NMU mRNA also is different. Expression of the NMS gene was markedly greater than that of the NMU gene in the rat hypothalamus and testis, while the reverse occurred in the anterior pituitary and thyroid gland (Rucinski et al., 2007). The NMS mRNA was more abundant in the suprachiasmatic nucleus (SCN), Arc, and PVN of the rat hypothalamus (Mori et al., 2012),

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http://dx.doi.org/10.1016/j.anireprosci.2017.03.023
Received 29 September 2016; Accepted 31 March 2017
Available online 02 April 2017
0378-4320/

and the gene was also highly expressed in the central nervous system and spleen (Mori et al., 2005). The NMS mRNA was densely distributed in the hippocampus and brain stem of pigs and was also present in the alimentary organs, endocrine and lymphatic organs, and ovaries (Yang et al., 2012). The wide distribution of NMS suggests that NMS is involved in various physiological functions. It has been documented that NMS has fundamental and important roles in physiological regulation via its receptors, including dark-light rhythms (Mori et al., 2005; Miyazato et al., 2008), energy metabolism (Kirsz and Zieba, 2012), stress responses (Jaszberenyi et al., 2007; Tachibana et al., 2010), feeding regulation behavior (Ida et al., 2005; Miyazato et al., 2008; Tachibana et al., 2010), antidiuretic action (Sakamoto et al., 2007) and reproduction (Vigo et al., 2007; Mori et al., 2008; Yang et al., 2009, 2010).

Although NMS has important roles in regulating the physiological functions of animals via its receptors, NMUR1 and NMUR2, interestingly, NMUR1 and NMUR2 have quite different distribution patterns, with NMUR1 being detected in a wide range of peripheral tissues and NMUR2 being limited to discrete brain areas such as the paraventricular and arcuate (ARC) nuclei (Howard et al., 2000; Raddatz et al., 2000; Brighton et al., 2004). Meanwhile, evidence has revealed that NMS and NMUR2 mRNA are abundant in the central nervous system and reproductive organs, including the hypothalamus and testis (Vigo et al., 2007; Yang et al., 2009). The NMS precursor protein is also found in the male rat hypothalamus and testis (Rucinski et al., 2007), and the central administration of NMS has evoked modest LH secretory responses in rats (Vigo et al., 2007).

Except for mice and rats, the pig is the third most commonly used experimental animal and has an important role in animal husbandry. Therefore, it is necessary to explore the regulatory function in the reproduction of NMS in pigs. The patterns of NMS and NMUR2 mRNA abundance and the function of NMS in the pig hypothalamus-pituitary-ovary axis during the estrous cycle has been studied and it was found that NMS could regulate the reproductive hypothalamic and pituitary hormone secretions of female pigs (Yang et al., 2009). Male pigs as the model to research the potential contribution of NMS to control the reproductive axis. To study the effect of NMS on sexual development and its potential role in male pigs, the male pig NMS and its receptor genes were first cloned and analyzed, and subsequently the relative abundances of NMS and NMUR2 were determined in the male Xiaomeishan pig reproductive axis during postnatal development, including day 3, 30, 60, 90 and 120, using real-time PCR and immunohistochemistry.

2. Materials and methods

2.1. Animals

All male Xiaomeishan pigs were fed according to the breeding standards of the Chinese Local Pigs and National Research Council (NRC). All experiments were performed according to the guidelines of the regional Animal Ethics Committee and the rules for experimental animals of Nanjing Agricultural University.

Male Xiaomeishan pigs ($n = 3$; a local pig breed developed by the Jiangsu Agricultural Academy, China) which were 30 d old and weighed 10 ± 2 kg were used for the gene cloning study. The animals were managed so that amount and type of feed, temperature, and humidity for at least 7 days prior to tissue collection were relatively constant. The pigs were anesthetized by an intraperitoneal injection of urethane (5 mL/kg body weight) and decapitated within 15 min of the time of pig restraint; then the hypothalamus, pituitary and testis were removed. The tissues used for RNA extraction were collected and stored at −70 °C. All animals were free from overt signs of disease at the time of tissue collection.

Fifteen male Xiaomeishan pigs were used for the mRNA study. The pigs were divided into five groups according to the date on which they would be euthanized: postnatal day 3, 30, 60, 90, and 120 ($n = 3$ per group). The animals were housed under constant conditions and killed on the days as previously described. The hypothalamus, pituitary, and testis were removed and frozen at −70 °C, and were used for RNA extraction.

Fifteen male Xiaomeishan pigs were used for the immunohistochemistry study. The animals were classified into five groups according to the date on which they would be euthanized: postnatal day 3, 30, 60, 90, and 120 ($n = 3$ per group). The pigs were anaesthetized by an intraperitoneal injection of urethane (5 mL/kg body weight) and then perfused transcardially via the left ventricle with 1000 mL of 0.9% normal saline followed by 5000 mL of cold 4% paraformaldehyde fixative in 0.1 M phosphate-buffered saline (PBS) (pH 7.2–7.4). The hypothalamus and testis were removed and then the tissues were post-fixed for 4 h (Su et al., 2008).

2.2. RNA purification and RT

The total RNA was extracted using the TRIzol extraction method (TRIzol reagent, Invitrogen, USA), according to the manufacturer's instructions. The RNA was treated with RNase-free DNase I (Promega, USA) to remove any contaminating DNA. The quality and concentration were determined by a photometer (Eppendorf Biophotometer, Germany). The RNA integrity was verified by electrophoresis with ethidium bromide staining.

For each tissue, equal amounts of all RNA samples were reversely transcribed simultaneously using an oligo(deoxythymidine)15 primer and M-MLV reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. All RT reactions were performed at 42 °C and included a negative control, which contained nuclease-free water instead of RNA.