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Developmental expression and distribution of nesfatin-1/NUCB2 in the canine digestive system

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ABSTRACT

Nesfatin-1/NUCB2 is a neuropeptide that plays important roles in regulating food intake and energy homeostasis. The distribution of nesfatin-1/NUCB2 protein and mRNA has not been investigated in the canine digestive system. The present study was conducted to evaluate the expression of nesfatin-1/NUCB2 protein and *NUCB2* mRNA in the canine digestive organs (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver and pancreas). The tissues of the digestive system were collected from dogs at different developmental stages (infantile, juvenile, pubertal and adult). Nesfatin-1/NUCB2 protein localization in the organs of adult dogs was detected by immunohistochemistry. The expression of *NUCB2* mRNA at the four developmental stages was analyzed by real-time fluorescence quantitative PCR (qRT-PCR). Nesfatin-1/NUCB2 protein was distributed in the fundic gland region of the stomach, and the islet area and exocrine portions of the pancreas. However, *NUCB2* mRNA was found in all digestive organs, although the expression levels in the pancreas and stomach were higher than those in liver, duodenum and other digestive tract tissues ($P < 0.05$) at the four different developmental stages of the dogs. In this study, nesfatin-1/NUCB2 was found to be present at high levels in the stomach and pancreas at both the protein and mRNA levels; however, *NUCB2* expression was found at lower levels in all of the digestive organs. These findings provide the basis of further investigations to elucidate the functions of nefatin-1 in the canine digestive system.

1. Introduction

Nesfatin-1 is an 82 amino acid polypeptide derived from NUCB2, its 396 amino acid precursor. NUCB2 can be cleaved into three fragments: nesfatin-1 (1–82 residues), nesfatin-2 (85–163 residues) and nesfatin-3 (166–396 residues) (Oh et al., 2006). Studies have shown that food intake in rats is reduced when nesfatin-1 is injected into the third brain ventricle, while nesfatin-2 and nesfatin-3 had no effects (Goebel et al., 2009).

The association of nesfatin-1/NUCB2 with the regulation of food intake is widely distributed among brain nuclei, such as the paraventricular nucleus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC) and the nucleus of the solitary tract (NTS) in rats (Brailoiu et al., 2007; Fort et al., 2008; Garcia-Galiano et al., 2010a; Gonzalez et al., 2010). Studies have shown that *NUCB2* mRNA is also expressed in fat tissue (Oh et al., 2006) and the gastric organs in rats (Goebel et al., 2009; Stengel et al., 2009). In fact, nesfatin-1/NUCB2 concentrations are found to be high in the blood of people with high body mass index, indicating that nesfatin-1/NUCB2 secreted from fat cells can regulate food intake (Ramanjaneya et al., 2010). Nesfatin-1/NUCB2 was first reported to induces satiety and inhibit food intake as an anorexigenic factor in the cerebrospinal fluid and hypothalamus in rats (Oh et al., 2006). Furthermore, there is a high level of homology in primary amino acid sequences of rat and human nesfatin-1/NUCB2 proteins, indicating the importance of this protein in physiological functions(Colmers, 2007; Cowley and Grove, 2006; Myers, 2006).

Previous studies have described the distribution of nesfatin-1/NUCB2 in the gastrointestinal tracts of humans and rats (Stengel et al., 2009). Experimental evidence has demonstrated that nesfatin-1/NUCB2 plays important roles in regulating gut motility, glucose levels, insulin secretion, and whole-body energy homeostasis (Garcia-Galiano et al., 2010a; Gonzalez et al., 2011; Stengel

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and Tache, 2011). Nesfatin-1/NUCB2 expression has been detected in the glandular epithelium of the ileocecal valve and in the glandular epithelium of the gastric fundus of pigs, further indicating the physiological relevance of this protein and providing additional evidence of the role played by nesfatin-1/NUCB2 in the regulation of food intake (Varricchio et al., 2014). Recent studies showed that nesfatin-1/NUCB2 is distributed in the stomach, spleen, thymus, heart, liver, and muscle of mice (Mohan et al., 2014). Nesfatin-1/NUCB2 regulates gastrointestinal motility in dogs, especially in the fasted state (Watanabe et al., 2015); however, the expression and distribution of nesfatin-1/NUCB2 in other digestive organs remains to be classified.

Dogs are now commonly kept as pets and the veterinary medicine industry has experienced significant growth. The aim of the current study was to clarify the distribution of nesfatin-1/NUCB2 protein in the digestive system of adult dogs and to investigate the expression of *NUCB2* mRNA in the canine digestive system at different developmental stages. The results of this study will provide a theoretical reference for further investigation of the role played by nesfatin-1/NUCB2 in regulation of the canine digestive system.

2. Materials and methods

2.1. Animals

Twelve female Chinese rural dogs in good health were obtained from Huachong Pet Market at the following stages of development: infantile (1.5 M), juvenile (4.5 M), pubertal (7 M) and adult (10 M). Each dog was raised in a cage with 70 cm high, 60 cm wide and 80 cm long at room temperature laboratory conditions and fed on standard dog food (Just Born Gold Dog Food, Shanghai Navarch, China), with water available ad libitum. The cages were cleaned daily to reduce the risk of infections, and the mental states and feeding behaviors of the dogs was monitored daily.

All procedures involving animals were approved by the Animal Care and Use Committee of Anhui Agricultural University.

2.2. Collection of samples

All dogs were sacrificed by an overdose of the anesthetic Lumianning-II (Huamu Animal Health Products, Jilin Province, China). Tissue samples were collected from the esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver and pancreas (Fig. 1). Tissue sample (approximately 1 cm^3) was fixed in 4% paraformaldehyde at 4 °C for 48 h, while another sample of the same size was immediately placed into liquid nitrogen and then stored at into −80 °C for RNA isolation.

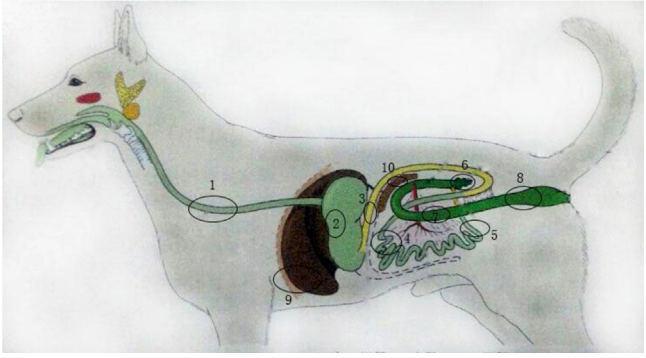

Fig. 1. Picture showing the specific sites of the canine digestive organs. These are: esophagus (1), stomach (2), duodenum (3), jejunum (4), ileum (5), cecum (6), colon (7), rectum (8), liver (9) and pancreas (10).

2.3. Immunohistochemical analysis

After 48 h in 4% paraformaldehyde, the tissue samples were rinsed in running water, dehydrated with alcohol, and embedded in paraffin. Sections (5 μm-thick) were prepared using a microtome (LS-2055+, Shenyang Longshou Electronic Instrument Limited, China). The sections were mounted on slides coated with poly-L-lysine (ZLI-9005, Beijing Zhongshan Golden Bridge Biotechnology, China) for immunohistochemical processing. Serial sections were de-waxed in xylene, dehydrated in graded alcohols and then rinsed under running water. The sections were treated with 0.3% hydrogen peroxidase (H_2O_2) for 10 min at room temperature to block endogenous peroxidase activity and incubated in normal goat serum for 10 min to minimize non-specific background staining. Serial sections were then incubated with sheep anti-mouse/rat nesfatin-1/NUCB2 polyclonal antibody (1:100, CFFX0114111, AF6895, R&D Systems Inc, 614 McKinley Place NE, Minneapolis, MN 55413, USA) at 4 °C overnight. After being rinsed with PBS (pH7.4), the sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (AADR0713071, NL010, R&D Systems) for 30 min at room temperature. Visualization of immunoreactivity was achieved using DAB reagents (ZLI-9017/9018/9019 DAB Kit, Beijing Zhongshanjinqiao-BIO, China). The sections were lightly counterstained with hematoxylin and mounted with neutral gum (BA-7004, Chu Hai Beisuo Biotechnology). The used primary antibodies were polyclonal sheep IgG which shares over 80% aa identity with dog nesfatin-1 in our study. In addition, the pre-absorption control for the nesfatin-1 primary antibody used here was validated by immunohistochemical staining with a well-characterized antiserum.

2.4. RNA isolation and quantitative RT-PCR (qRT-PCR)

2.4.1. RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver and pancreas) using an RNA extraction kit (6934-01 of E.Z.N.A.® Total RNA Kit I/II, 6834-01 of E.Z.N.A.® Total RNA Kit I/II,OMEGA, USA) according to the manufacturer's instructions. The concentration of total RNA was measured using a Bio-drop (μLite Spectrophotometer, UK) and the quality of total RNA was assessed by 1% agarose gel electrophoresis (DYY-6C electrophoresis, Beijing, China). Samples showing three clear electrophoretic bands in addition to A260/280 ratio values between 1.8 and 2.0 were considered suitable for analysis and stored at −80 °C. Samples of total RNA (1 μL) were reversed transcribed using the PrimeScript™ RT reverse transcription kit with gDNA Eraser (Da Lian, China); the resulting cDNA was stored at −20 °C.

2.4.2. Conventional PCR

The conventional PCR system consisted of 10 μL mix (A mixture of buffer, dNTP and DNA Polymerase), 7 μL sterilized double distilled water, 1 μL template cDNA, 1 μL upstream primer and 1 μL downstream primer. The primers were designed for detection of *Canis lupus familiaris NUCB2* mRNA and *β-actin* mRNA. The PCR cycling conditions were as follows: 94 °C for 4 min, followed by 30 cycles of 94 °C for 40 s, 55–60 °C for 30 s, 72 °C for 20 s and a final extension at 72 °C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis to confirm the length of the amplified cDNA.

2.4.3. qRT-PCR

qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) in a reaction system containing the following: 10 μL mix, 8 μL sterilized double distilled water, 1 μL template cDNA (1:5 dilution), 0.4 μL upstream primer, 0.4 μL downstream primer and 0.2 μL BRYT Green