Gene 575 (2016) 725–731

Contents lists available at ScienceDirect

Gene

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

Research paper

Molecular cloning and expression analysis of neuregulin 1 (Nrg1) in the hypothalamus of Huoyan goose during different stages of the egg-laying cycle

Zhongzan Cao [a], Dawei Liu [a], Mei Liu [a], Ming Gao [a], Zimo Chen [a], Zhe Xing [a], Xiaoying Zhang [b], Yunhou Yin [c], Xinhong Luan [a,*]

[a] College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, China
[b] Liaoning Province Livestock and Poultry Genetic Resources Conservation and Utilization Center, Liaoyang 111000, China
[c] Guizhou Minzu University, Guiyang 550025, China

ARTICLE INFO

Article history:
Received 10 June 2015
Received in revised form 14 August 2015
Accepted 21 September 2015
Available online 25 September 2015

Keywords:
Huoyan goose
Nrg1
Hypothalamus
Race
Real-time PCR

ABSTRACT

Neuregulin 1 (Nrg1) is one of the most active members of the epidermal growth factor (EGF)-like family, which bind to the ErbB tyrosine kinase receptor and play many roles in modulation of synaptic activity, synaptogenesis, GABAergic neurotransmission, neurotransmitter receptor expression and the hormonal control of neuroendocrine reproductive development. In this study, we cloned and characterized the cDNA of goose Nrg1 originating from hypothalamus tissues of Huoyan goose using RACE method, investigated the mRNA expression profiles during different stages of the egg-laying cycle by real-time PCR. Multiple alignments and phylogenetic analyses of the deduced amino acid sequence were conducted using bioinformatics tools. We also determined the profiles of blood serum progesterone, estradiol, FSH and LH content during different egg-laying stages using radioimmunoassay. The cDNA of Nrg1 is consisted of 2061 bp open reading frame encoding 686 amino acids. The deduced amino acid sequence of goose Nrg1 contains one EGF domain from amino acid residues 224 to 265 and shows a closer genetic relationship to the avian species than to other mammal species. The expression level of Nrg1 mRNA increased from the pre-laying period to the peak-laying period, reached its peak in the peak-laying period, and then decreased in the ceased period. The concentrations of FSH and estradiol in blood serum have the similar changing trend. These results might suggest a potential correlation between Nrg1/ErbB signaling network with the reproductive neuroendocrine of Huoyan goose.

1. Introduction

Neuregulin 1 (Nrg1) is one of the most active members of the epidermal growth factor (EGF)-like family, and a growth factor that plays many roles in central nervous system development and function (Ritch et al., 2005). Nrg1 gene is highly complex and is alternatively spliced into six types of proteins, all of which contain and signal via an epidermal growth factor (EGF) domain. The effects of Nrg1 are mediated by the ErbB tyrosine kinase receptor family, particularly ErbB2, ErbB3 and ErbB4. Nrg1 secreted from either neurons or glial cells bind to the extracellular domains of the receptor tyrosine kinases ErbB3 and ErbB4 to initiate a complex intracellular signaling cascade in which extracellular signal-regulated kinase (ERK), serine/threonine protein kinase (AKT), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase γ (PIK3γ), protein kinase C (PKC), and Janus kinase-signal transducers and activators of transcription (Jak-STAT) are activated (Zhao, 2013). Activation of this signaling pathway results in a wide range of biological effects including modulation of synaptic activity, synaptogenesis, GABAergic neurotransmission, neurotransmitter receptor expression and the hormonal control of neuroendocrine reproductive development (Ozaki et al., 2000; Falls, 2003; Prevot et al., 2003; Harrison and Law, 2006; Woo et al., 2007; Mei and Xiong, 2008).

Nrg1 was detected in many regions of the brain, including the hippocampus (HPC), prefrontal cortex (PFC) and paraventricular nucleus (PVN) of the hypothalamus (Law et al., 2004; Taylor et al., 2011). In rat hypothalamus, Nrg1 receptors were reported to be expressed in hypothalamic astrocytes, where their activation as a result of paracrine Nrg1 stimulation, is essential for stimulating secretion of luteinising hormone-releasing hormone (Prevot et al., 2003). Nrg1 also was reported to be expressed in gonadotroph cells of the pituitary, which may

Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; kDa, kilodalton; mRNA, messenger RNA; ORF, open reading frame; PCR, polymerase chain reaction; PKC, protein kinase C; RACE, rapid-amplification of cDNA ends; rRNA, ribosomal RNA; UTR, Untranslated Regions; qRT-PCR, real-time PCR; EGF, epidermal growth factor; ErbB, neuro/glioblastoma derived oncogene homolog.

* Corresponding author.
E-mail addresses: caozhongzan@163.com (Z. Cao), 2689558571@qq.com (D. Liu), meiliu2014@163.com (M. Liu), minggao2014@163.com (M. Gao), 779594756@qq.com (Z. Chen), 731994204@qq.com (Z. Xing), hyzhang2014@163.com (X. Zhang), yinyunhou@163.com (Y. Yin), xhluan@163.com (X. Luan).

http://dx.doi.org/10.1016/j.gene.2015.09.049
0378-1119/

function as a paracrine/juxtacrine regulator of PRL secretion (Zhao and Ren, 2011; Zhao et al., 2011). In addition, Nrg1 was shown to be expressed in ovarian granulosa cells after the LH surge and may impact luteinization and oocyte maturation (Noma et al., 2011; Kawashima et al., 2014). In poultry, the reproductive endocrine system and the reproductive activity are strictly controlled by the hypothalamic–pituitary–gonadal axis (Padmanabhan et al., 2002). hypothalamus regulate reproduction by releasing neurohormones (gonadotropin-releasing hormones, GnRH) to the pituitary gland, the pituitary gland synthesizes and releases gonadotropins (luteinizing hormone, LH; follicle-stimulating hormone, FSH) which in turn act on the gonads to stimulate gametogenesis (spermatogenesis, oogenesis) and sex steroid hormones secretion (androgens, estrogens, and progesterone). In our previous report, Nrg1 was identified to be differentially expressed in the hypothalamus of Huoyan geese between laying period and ceased period (Luan et al., 2014). In order to investigate the potential role of Nrg1 in the process of geese egg-laying and reproductive neuroendocrine, the current study was undertaken to clone the full-length cDNA of the Huoyan goose Nrg1 by RACE (rapid amplification of cDNA ends) method, and identify its sequence characteristics. Sequently, to determine its mRNA expression profiles in hypothalamus of Huoyan goose during pre-laying, early-laying, peak-laying and ceased period, with the use of real-time PCR (qRT-PCR) technique. Meanwhile, to investigate the profiles of blood serum progesterone, estradiol, FSH and LH content during different egg-laying stages using radioimmunoassay.

2. Materials and methods

2.1. Animal and tissue collection

Thirty-six Huoyan geese were selected randomly from two hundred geese on the Liaoning Huoyan goose stock breeding farm and raised according to the program used at this farm. During the experiment, geese were fed ad libitum with rice grain and were supplemented with green grass or water plants whenever possible. Feed was given during the daytime when the geese were released into an open area outside the house. Huoyan geese become sexually mature at approximately 7 months of age and reach the peak egg-laying stage in the following year. In the current study, goslings were purchased in the fall of the year and become sexually mature during the summer of the following year. Nine geese were killed by exsanguinations at the age of 6 months (pre-laying period), 9 months (early laying period), 12 months (peak-laying period), and 15 months (ceased period). The hypothalamus were quickly dissected, frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction prepared. Blood serum samples were collected and kept at −20 °C until progesterone (P), estradiol (E_2), FSH, and LH determination. All experimental procedures were reviewed and approved by the animal welfare committee of the College of Animal Science and Veterinary Medicine of Shenyang Agricultural University (No. 2,011,036).

2.2. Radioimmunoassays of hormone

Concentrations of P, E_2, FSH and LH in blood serum were determined radioimmunologically by Beijing North Institute of Biological Technology (Beijing, China) using Iodine[^{125}I] Progesterone Radioimmunoassay Kit, Iodine[^{125}I] Estradiol (E_2) Radioimmunoassay Kit, Iodine[^{125}I] FSH Radioimmunoassay Kit, Iodine[^{125}I] LH Radioimmunoassay Kit. The assay's standard range were 0.2–100 ng/ml for P, 5–4000 pg/ml for E_2, 2.5–100 mIU/ml for FSH and 5–200 mIU/ml for LH. The sensitivities of the assays were 0.2 ng/ml for P, 2 pg/ml for E_2, 1.0 mIU/ml for FSH and LH. The intra- and interassay coefficients of variation for P, E_2, FSH and LH were 10% and 15% respectively. Hormone concentration was calculated per milliliter of blood serum.

2.3. Cloning and sequencing of goose Nrg1

Total RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's protocol. The quality of the RNA was determined by agarose gel electrophoresis and NanoDrop 8000 spectrophotometer (NanoDrop, Thermo Scientific). One microgram of RNA was reversely transcribed into cDNA using PrimeScript®RT reagent Kit (TaKaRa, Dalian, China) in a total volume of 20 μl with 4.0 μl of 5 × PrimeScript®Buffer, 1.0 μl of PrimeScript®RT Enzyme Mix, 1.0 μl of Random 6 mers, 1.0 μl of oligo$(dT)_{18}$ Primer and 9.0 μl of RNase Free H_2O. Thermal cycling was performed for 15 min at 37 °C, and then 5 s at 85°C. RT products were stored at 20 °C for the PCR. Specific PCR primer pair used to amplify parts of Nrg1 cDNA sequences was designed using Primer Premier 6.0 (Primer Biosoft International, Palo Alto, California, USA) according to the mRNA sequence of the Anas platyrhynchos Nrg1 gene (XM_005019006). The primer pair was synthesized commercially by Sangon Biotech Co., LTD (Shanghai, China). Information of the primers was listed in Table 1. The 50 μl reaction consisted of 1 μl of cDNA, 8 μl of deoxynucleoside triphosphate Mix (2.5 mmol/L each dATP, dGTP, dCTP and dTTP), 2 μl of each primer (10 μmol/l), 5 μl of 10 × LA PCR Buffer, 0.5 μl of 5 U/μL LA Taq™ (TaKaRa, Dalian, China), and 31.5 μl sterile MilliQ water. PCR condition was 35 cycles consisting of denaturing at 94 °C for 30 s, specific annealing at 55 °C for 30 s, and extension at 72 °C for 90 s with an initial denaturing step at 94 °C for 5 min and a final extension step at 72 °C for 10 min. The PCR product was gel-purified and ligated into pMD-18-T vector (TaKaRa., Dalian, China), transformed into the competent E. coli DH5α competent cell. Positive clones containing the expected-size inserts were screened by colony PCR and then sequenced by Sangon Biotech Co., LTD, and its characteristics were determined using Basic Local Alignment Search Tool (nBLAST) at http://www.ncbi.nlm.nih.gov.

Based on the partial cDNA sequence of Nrg1 obtained from the above RT-PCR reaction, specific primers were designed to amplify the full-length cDNA sequence of goose Nrg1 (primers shown in Table 1) using the SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories, CA, USA) according to the manufacturer's instructions. The 3′- and 5′-end cDNA templates were synthesized using the 3′-CDS Primer A and 5′-CDS Primer A provided in the kit. Nested PCR was used in the 3′-RACE analysis. The first-round PCR was performed in a total volume of 50 μl that contained 2.5 μl of the first strand 3′- end cDNA template, 5.0 μl of 10× Advantage 2 PCR buffer, 1.0 μl of 10 mM dNTP Mix, 1.0 μl of 10 μM gene-specific primer GSP3, 5.0 μl of 10 × Universal Primer Mix (UPM; Clontech, USA), 34.5 μl of sterile deionized water, and 1.0 μl of 50 × Advantage 2 Polymerase Mix (Clontech, USA). Then, 1 μl PCR

Table 1
Primers used in this study.

Primers purpose	Primer name	Primer sequence (5′-3′)
RT-PCR		
	Nrg1-F	CAGCTTGACCGTCTCCAT
	Nrg1-R	GCAACTCTTCTGGTGTGG
3′-RACE		
	Nrg1-GSP3	TGAGAGCGAAACAGAGGATGAAAGAA
	Nrg1-NGSP3	CACCATTTCTGAGCATACAAAACCCC
5′-RACE		
	Nrg1-GSP5	TGCTGAAGGTGGTTGTCG
	Nrg1-NGSP5	TGGGGTCACCAGGAGCAG
RACE		
	UPM-Long	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT
	UPM-Short	CTAATACGACTCACTATAGGGC
Real-time PCR		
	Nrg1-S	GATTTGCTGGGTTAGTCCTG
	Nrg1-A	CACAAGCTCTGAGAGCAC
Internal control		
	18 s RNA-S	CGGACAGGATTGACAGATTGAG
	18 s RNA-A	GCCAGAGTCTCGTTCGTTATC