INTRODUCTION

Neurotensin (NT) is a tridecapeptide that is distributed widely in the central nervous system and peripheral tissues of vertebrates and has multiple functions as a neurotransmitter and as a circulating hormone (Reinecke, 1985; St-Gelais et al., 2006; Kalafatakis and Triantafyllou, 2011). In the chicken, NT was initially isolated from the intestine (Carraway and Bhatnagar, 1980). Thereafter, NT immunoreactivity was found in the epithelium and nerve fibers of the smooth muscle layers of the gastrointestinal tract (Atoji et al., 1994, 1995), endocrine cells of the thymus (Atoji et al., 1996), and the hypothalamus in the brain (Esposito et al., 1997). Neurotensin has been shown to decrease gastrointestinal motility (Rawson et al., 1990) and gastric secretion (DeGolier et al., 1997), and to increase pancreatic lipase activity (DeGolier et al., 1999) and hepatic bile acid secretion (Gui et al., 2000). An NT-related hexapeptide known as LANT-6 has also been isolated from chicken intestines (Carraway and Ferris, 1983). The amino acid sequence of LANT-6, Lys-Asn-Pro-Tyr-Ile-Leu, is identical to the carboxy-terminal half of chicken NT except for the substitutions of Lys to Arg and Asn to Arg. Based on an RIA, both NT and LANT-6 were primarily detected in the brain and gastrointestinal tract, with differences in their regional distribution (Carraway et al., 1983). In the rat, chicken NT induces hypotension, hyperglycemia, vascular permeability, and cyanosis, whereas LANT-6 shows only a hypotensive response (Carraway and Ferris, 1983). The functional difference between NT and LANT-6 is not known in the chicken.

The functions of NT are mediated by the NT receptor (NTR). In mammals, 3 types of NTR have been identified: NTR1, NTR2, and NTR3/sortilin (Tanaka et al., 1990; Vita et al., 1993, 1998; Mazella et al., 1996). The NTR1 and NTR2 belong to the G protein-coupled receptor family with 7 transmembrane domains, whereas NTR3 belongs to the Vps10p receptor family with a single transmembrane domain. The NT-related hexapeptide known as LANT-6 has also been isolated from chicken intestines (Carraway and Ferris, 1983). The amino acid sequence of LANT-6, Lys-Asn-Pro-Tyr-Ile-Leu, is identical to the carboxy-terminal half of chicken NT except for the substitutions of Lys to Arg and Asn to Arg. Based on an RIA, both NT and LANT-6 were primarily detected in the brain and gastrointestinal tract, with differences in their regional distribution (Carraway et al., 1983). In the rat, chicken NT induces hypotension, hyperglycemia, vascular permeability, and cyanosis, whereas LANT-6 shows only a hypotensive response (Carraway and Ferris, 1983). The functional difference between NT and LANT-6 is not known in the chicken.

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has the highest affinity for NTR1, which mediates most of the known functions of NT. Our laboratory recently identified chicken NTR1 mRNA by cDNA cloning and characterized its primary structure and distribution in the gastrointestinal tract of adult chickens (Numao et al., 2011). Nucleotide sequences that resemble mammalian NTR2 and NTR3/sortilin are not found in the chicken genome, so the presence of chicken counterparts of mammalian NTR2 and NTR3/sortilin remains unknown. It has been shown that the NT-binding site is highly abundant in the chicken liver (Mitra and Carraway, 1995, 1997), but the physiological functions of NTR in the chicken liver remain unclear. To gain a better understanding of the physiological functions of NT-NTR1 system in the chicken, it is essential to clarify the tissue-expression profiles of NT and NTR1 mRNAs particularly in intestinal tissues and liver.

In the present study, we report the identification of chicken NT precursor mRNA by cDNA cloning and developmental changes in its expression in intestinal tissues, as well as developmental changes in NTR1 mRNA in intestinal tissues and liver.

**MATERIALS AND METHODS**

**Birds**

Fertilized White Leghorn chicken eggs were obtained from Gen Corporation (Gifu, Japan) and incubated under standard conditions. From 1 d after hatching, the female and male chicks were fed a standard diet and grown for 2 wk in an electric brooder, and hens were further grown under standard conditions. Food and water were provided ad libitum. All procedures were performed in accordance with National Institutes of Health guidelines regarding the principles of animal care. After decapitation, tissues were removed immediately and frozen in liquid nitrogen.

**Cloning of Chicken NT Precursor cDNA**

A chicken duodenum cDNA library was prepared by a method previously described (Yamamoto et al., 2008) using the Marathon cDNA amplification kit (Clontech, Mountain View, CA). The NT cDNA was amplified by PCR from the cDNA library with LATaq polymerase (TaKaRa, Tokyo, Japan) using primer-1 (sense) and primer 2 (antisense) designed from the chicken NT cDNA sequence (GenBank accession no. AB570405). Quantitative measurement was performed by establishing a linear amplification curve from serial dilutions of each cDNA.

**Statistical Analysis**

All data were analyzed by one-way ANOVA, and the significance of the F-value obtained was confirmed by Tukey’s post hoc test. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

**RESULTS**

**Cloning of Chicken NT Precursor cDNA**

The nucleotide sequences of the cloned chicken NT precursor cDNA contained 30 bp of the 5'- untranslated region, 495 bp of an open reading frame encoding 164 amino acid residues, and 229 bp of the 3' untranslated region (Figure 1). The sequence of the cDNA was identical to that of the corresponding region of the predicted chicken NT cDNA sequence (XM_416126) in the DNA database. The amino acid sequence of the chicken NT precursor showed significant overall homology with those of human (66.7%; NM_006183), bovine (69.7%; NM_173945), rat (61.8%; NM_001102381), canine (69.1%; M16443), and Xenopus (58.5%; NM_001092681). The 13-amino acid sequence of chicken NT and the 6-amino acid sequence of LANT-6 were observed in tandem near the C-terminal of the NT precursor. A comparison of the chicken NT
precursor cDNA sequence and a genomic sequence in the DNA database revealed the presence of 3 introns at the positions shown in Figure 1.

**Tissue Distribution of NT Precursor mRNA in the Adult Hen**

The expression levels of NT precursor mRNA in peripheral tissues, such as the liver, kidney, pancreas, spleen, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, and colon/rectum of 250-d-old adult female chickens, were examined by real-time PCR (Figure 2). The mRNA was preferentially expressed in the duodenum, jejunum, ileum, and colon/rectum. The expression levels in other tissues were very low.

**Changes in the Expression Levels of NT Precursor mRNA in Intestinal Tissues during Late Embryonic and Early Posthatching Development**

Figure 3 shows the expression profiles of NT precursor mRNA in the duodenum, jejunum, ileum, and colon/rectum during late embryonic and early posthatching development. In the duodenum and jejunum, the expression levels increased from d 14 to d 20 of the embryo development, reached a maximum at d 1 posthatching, gradually decreased until d 5 (duodenum) or d 7 (jejunum) posthatching, and then remained constant at the level equivalent to that of the adult. In the ileum, the expression levels rapidly increased from d 16 of the embryo development to d 3 posthatching, reached the highest on d 7 posthatching, and then rapidly decreased to the level equivalent to that of the d 250 posthatching adult (Figure 2). In the colon/rectum, the expression levels rapidly increased from d 16 of the embryo development to d 3 posthatching, decreased until d 7 posthatching, and then remained constant.

**Figure 1.** The cDNA sequence of chicken neurotensin (NT) precursor. The deduced amino acid sequence is shown below the cDNA sequence. Arrowheads indicate the positions of introns. The amino acid sequences of NT and its related peptide, LANT6, are shown by solid and dotted underlines, respectively. The positions of the sense and antisense primers used for PCR are shown by right- and left-oriented arrows, respectively.

**Figure 2.** Expression levels of neurotensin (NT) precursor mRNA in chicken tissues. The expression levels of NT-precursor mRNA in the liver, kidney, pancreas, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, and colon/rectum of 250-d-old female chickens were determined by real-time PCR. Values were obtained from 4 birds. Each value of NT precursor mRNA represents the mean ± SEM of triplicate measurements. Values with different letters (a–d) are significantly different (P < 0.05).
Changes in the Expression Levels of NTR1 mRNA in Intestinal Tissues and Liver During Late Embryonic and Early Posthatching Development

The expression levels of NTR1 mRNA in intestinal tissues (Figure 4) and liver (Figure 5) during late embryonic and early posthatching development were examined. In the duodenum and jejunum, the expression levels were high during the late embryonic period, decreased after hatching, and then remained at lower levels until d 14 posthatching. In the ileum, the expression levels remained constant throughout the developmental period, with the exception of d 14 of the embryo development when the level was significantly higher than other periods. In the colon/rectum, the expression levels were low on d 14 and 16 of the embryo...
development, tended to increase until d 1 posthatching, and significantly increased at d 3 posthatching, and then remained at a relatively higher level. In the liver, the expression levels remained constant from d 14 of the embryo development until d 1 posthatching, markedly increased at d 3 posthatching, and then gradually decreased from d 7 until d 14 posthatching. The expression level in the liver of adult chickens was as low as the levels during the embryonic stage, but 10-fold higher than that in the colon/rectum.

**DISCUSSION**

The NT precursor mRNA have been identified in mammals such as canine, bovine, and rat by cDNA cloning (Dobner et al., 1987; Kislauskis et al., 1988; Bean et al., 1992) and have been shown to encode neuromedin N (NN) as well as NT in the precursor protein. The nucleotide sequence of chicken NT precursor cDNA has been predicted from the genomic sequence and the amino acid sequence of NT precursor protein.
encoded in the cDNA has been characterized together with those of other vertebrate species (Hwang et al., 2009). Consistent with the observation of the predicted NT precursor cDNA, the chicken NT precursor cDNA cloned in the present study encoded LANT-6 and NT in tandem near the C-terminal of the precursor protein (Figure 1). As found in mammalian NN and NT, chicken LANT-6 and NT are flanked and separated by Lys-Arg sequences that are known to be proteolytic cleavage sites. The amino acid sequence of LANT-6 is very similar to that of the 6-amino acid sequence in the carboxy terminus of NT, as observed in mammalian NN and NT. These findings confirmed that LANT-6 is a chicken counterpart of mammalian NN. In a binding assay using chicken liver membrane, NT was shown to have higher potency than LANT-6 (Mitra and Carraway, 1995). In mammals, the NT precursor undergoes tissue-specific processing by multiple convertases that cleave the dibasic sites (Kitabgi, 2006). In the chicken, tissue-specific processing of the NT precursor has also been observed (Carraway et al., 1993), but the functional relationship between NT and LANT-6 in individual tissue is not known.

It has been reported that the NT protein content is high in the chicken gut (Carraway and Bhatnagar, 1980; Carraway et al., 1982) and that NT-secreting cells are uniformly distributed along the entire intestinal tract (Sundler et al., 1977). In support of these findings, real-time PCR analysis in the present study revealed that NT precursor mRNA is preferentially expressed in the intestinal tract, with the exception of the cecum. The NT release increases in response to lipid perfusion in the chicken duodenum (Gui et al., 2000), and therefore, NT is considered to be involved in the postprandial regulation of lipid digestion (DeGolier et al., 1997). It has been previously demonstrated that NTR1 mRNA is expressed in the gastrointestinal tract and at a markedly higher level in the colon/rectum (Numao et al., 2011). In the present study, we examined the expression levels of the NT precursor and NTR1 mRNA in intestinal tissues during the late embryonic and early posthatching period when nutrient sources changed from lipid-rich yolk to an external low-lipid diet. The expression levels of NT precursor mRNA in the intestinal tissues increased during hatching, with maximum on d 1 posthatching in the duodenum and jejunum and on d 3 posthatching in the ileum and colon/rectum (Figure 3). By contrast, the expression levels of NTR1 mRNA were higher throughout the late embryonic period compared with the posthatching period in the duodenum and jejunum. In the ileum, the expression levels remained constant during hatching and the level in the colon/rectum increased after the hatching (Figure 4). Because NT stimulates growth of the rat intestine (Wood et al., 1988; Chung et al., 1992), NT may possibly participate in the growth of the duodenum and jejunum during the embryonic period as well as in digestive functions during the posthatching period.

It is known that chicken liver contains a large quantity of NTR (Mitra and Carraway, 1995; Mitra and Carraway, 1997). The present study also detected the abundant expression of NTR1 mRNA in the liver of adult chickens (d 250 posthatching). Interestingly, the expression levels in the liver markedly increased during d 3 to 7 posthatch and then gradually decrease to the adult level. As previously mentioned, the expression of NT mRNA in intestinal tissues increases during hatching. Molecular events occurring in intestinal tissues and the liver that are regulated by NT-NTR1 system remain to be elucidated.

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REFERENCES


