The Thai Journal of Veterinary Medicine

Volume 49
Issue 3 *September, 2019*

Article 5

9-1-2019

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Original Article

Gene expression profiling of NGF, BDNF, NT-3 and TrkB receptor in the development of prehierarchical follicles of Zi Geese (*Anser cygnoides*)

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Abstract

The expression patterns of neurotrophins (NTs) at the gene and protein level including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and TrkB receptor were investigated in the prehierarchical follicles of Zi Geese by RT-qPCR and Western blotting analysis. The mRNA levels of *NGF*, *BDNF*, *NT-3* and *TrkB* receptor increased significantly in the small white follicles (SWF), and more interesting by *BDNF* and *NT-3* transcripts were not detectable in small yellow follicles (SYF) marked as the last stage of prehierarchical ovarian development before transition into preovulatory follicles. Western blot revealed the presence of all the target neurotrophins and high-affinity receptor at the protein level with varying predominance at each stage of follicle classifications. These results indicate that *NGF*, *BDNF*, *NT-3*, and *TrkB* are implicated in the folliculogenesis and may have an influential effect during the development and growth of the Zi Geese reproductive system.

Keywords: Folliculogenesis, neurotrophins, RT-qPCR, Western blot

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Introduction

Follicle development and growth in poultry is a complicated and well - organized process that includes paracrine, autocrine, and endocrine control factors throughout the developmental stages to reach the maturity stage (Lyu *et al.* 2016). Conversely, most of the ovarian follicles from a resting pool of primordial follicles cannot develop to the ovulatory stage due to the selection mechanism of healthy single follicle per reproduction cycle (Johnson and Lee, 2016). Immediately before and after the transition of primordial follicles into the prehierarchical follicles, a wide variety of cell anatomic and physiological activities transpire, for example, angiogenesis assembly to facilitate the formation of new blood vessels at each cyclic recruitment before ovulation (Retamalesortega *et al.* 2017). Therefore, the relative expression levels of genes encoding neurotrophins at mRNA and protein are prerequisite and retained within the prehierarchical follicles development in a stage-dependent manner. Also, many cell-signaling pathways are implicated in the ovarian follicle development process, in which the neurotrophin signaling pathway has recently been a primary focus of interest (Streiter *et al.* 2015; Alqudah and Aldwairi, 2016).

There are two major families of neurotrophic growth factors: the neurotrophins (NT) and glia-derived neurotrophic factor. The neurotrophins consist of five tight morphological identical peptides namely nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) interacting with tyrosine kinase receptors or p75 neurotrophin receptor and thus NGF binds to TrkA, BDNF, and NT-4/5 bind to TrkB and NT-3 binds to TrkC to enable functional intracellular signaling response (Gatta *et al.* 2016). Neurotrophins maintain growth, development, and regulate apoptosis of neuronal populations in the central and peripheral nervous system and the mammalian productive tract system such as rabbits, guinea pigs, mice, pigs, and horses (Kuhn, 2015; Maranesi *et al.* 2016). Recent studies have shown that neurotrophins and their receptors are extensively expressed in testicular development and spermatogenesis in both rats and humans indicating that they may have a functional role in testicular development (Li and Zhou, 2013). Furthermore, neurotrophins (NGF, BDNF and NT-3) and their specific receptors (TrkA, TrkB and TrkC) have been studied in the oviduct of egg-laying Japanese quails (Maruccio *et al.* 2016). Some previous studies revealed that NTs and their respective receptors play a significant role in folliculogenesis, oogenesis, and ovary development from the initial developmental stage of primordial follicles transiting to secondary follicles observed through cell proliferation and differentiation leading to ovarian maturation (Dissen *et al.*2009; Chaves *et al.* 2013). Other studies have been conducted to evaluate neurotrophins mechanisms in regulating ovarian follicular development and function in humans and other species (Linhermelville and Li, 2013). However, as to whether neurotrophins exert the similar or different roles in controlling geese prehierachical follicular development and the manner in which the neutrophins are expressed at each stage of prehierarchical growth has gained little or no attention. Therefore, the aim of this study was to investigate the mRNA and protein expression levels of *NGF*, *BDNF*, *NT-3* and *TrkB* as high-affinity *BDNF* receptor during the development of prehierarchical follicles of Zi geese. This study will further add information to reaffirm the task unveiled by neurotrophins in ovarian follicle development and maturation.

Materials and Methods

***Animals and sample collection*:** To ensure that Animal welfare control measures were observed, this experiment was authorized by the Animal Health and Care Committee of the College of Animal Science and Technology, Jilin Agricultural University (Approval code GR(J) 18-003). The geese were reared in a deep litter system under semi-intensive production management. The animals were fed ration locally formulated in the farm composed of 17.34% Crude protein, energy, 12.36 ME(MJ/kg), 13.35 DE(MJ/kg), 2.57% Calcium, and 0.26% Phosphorus at *ad libitum* with free access to water and were exposed to natural sunlight and uncontrolled environmental climatic conditions.

Six geese (35-38 weeks old) with a regular laying sequence of 2-3 eggs were randomly selected to take part in the experiment. The birds were anaesthetized with ether and slaughtered via the jugular vein. Before surgery, the abdominal area was treated with tampon alcohol disinfectant. The geese follicles were placed on a plate containing a pre-cooled Phosphate-Buffered Saline (PBS) followed by the removal of the vascular membrane of the outermost layer of the follicles. The anterior follicular size formed the basis for classification of the prehierarchical follicles, which were grouped as primary follicles (PE, < 2mm), small white follicles (SWF, 2-4mm), middle white follicles (MWF, 4-6mm), large white follicles (LWF, 6-10mm), and small yellow follicles (SYF, 10-15mm). After classification, the follicles were ruptured to remove the follicular liquid and the oocyte, thoroughly rinsed with PBS buffer then immediately stored in a -80°C refrigerator for experimental analysis.

***RNA extraction and cDNA synthesis*:** Total RNA from prehierarchical follicle tissues was extracted with Trizol using a commercial kit following the manufacturer's instructions (RNAiso Plus, Code No. 9108, Takara, Kusatsu, Japan). The pre-hierarchical follicles tissues were crushed to a powder using a mortar and pestle and homogenized with 200µl Trizol; the mixture was kept in the deep freeze at -20°C for 5 mins, and then centrifuged at 13,500 rpm for 5 mins at 4°C. Then 200 µl of Chloroform was added to the first aqueous phase. After 5 mins in the refrigerator (-20°C) the solution was centrifuged at 13,500 rpm for 15 mins at 4°C. Isopropanol (500µl) was then added to the separated upper aqueous solution of the three phases and centrifuged at 13,500 rpm for 10 min at 4°C. The resulting pellet was washed with 100µl of 75% ethanol and centrifuged at 7500 rpm for 5 min, air-dried and dissolved in 30 µl of RNase free water. The total RNA

for each sample was treated with DNAse 1 (Ambion/Life Technologies) to avoid genomic DNA contamination. The concentration and quality of total RNA was determined by measuring the absorbance at 260 nm using a spectrophotometer (NanoDrop 2000c ThermoScientific, Bremen, Germany). The quantity and quality of RNA were estimated using spectrophotometric measurements at 260 and 280 nm and 1% agarose gel electrophoresis as shown in Figure 1. The cDNA was synthesized using a reverse transcriptional kit (ReverTra Ace qPCR RT Kit, Code No. FSQ- 101, Toyobo, Osaka, Japan) according to the manufacturer's instructions which consisted of 17.5µl Nuclease-free water, 5µl 5X RT Buffer, 1.25µl of primer mix, 1.25µl and 0.5µl of mRNA. The reverse transcriptional response was followed by moderate centrifugation at 37°C for 15 mins and at 98°C enzyme inactivation for 5 min, the synthesized cDNA was kept at -20°C for further experiment.

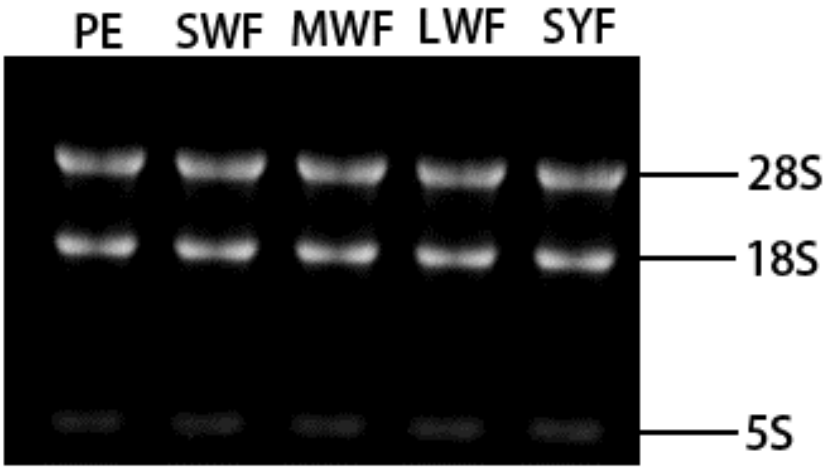

Figure 1 Gel electrophoresis bands of RNA isolated from prehierarchical follicle tissues of geese. In this experiment both 28s and 18s bands were intact showing high molecular weight fractions whereas 5s bands were blurry indicating low molecular weight fractions.

***Primer design, Polymerase Chain Reaction (PCR) and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)*:** Transcriptome sequencing of *NGF* mRNA (K02582), *BDNF* (K04355), *NT-3* (K04356) and *TrkB* mRNA (K03176) were screened with KEGG (Kyoto Encyclopedia of Genes and Genomes) and the *β-actin* sequence was obtained from NCBI (GeneBank: M26111.1). The conventional PCR was performed using specific primers for *NGF*, *BDNF*, *NT-3,* and *TrkB* genes designed by Primer Premier 5.0 software (Primer-E Ltd., Plymouth, UK) which are shown in Table 1. The PCR conditions consisted of 35 cycles; the samples were initially heated at 94°C for 5 min followed by denaturation at 94°C for 30 sec, annealing at 59.0°C for 30 sec and extending at 72°C for 30 sec. The PCR product was visualized under 1% agarose gel electrophoresis which was buffered with Tris base Acetic acid and EDTA (TAE) including 0.15µl Gel Red Nucleic Acid Gel Stain (Biotium Inc, Hayward, CA, USA) under ultraviolet illumination as illustrated in Figure 2. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) was performed using THUNDERBIRD SYRB Green Kit (TOYOBO, Osaka, Japan) for quantitative analysis of the mRNA levels of *NGF*, *BDNF*, *NT-3* and *TrkB* genes in Geese prehierarchical follicles. The RT-qPCR reaction total volume of 20 µl contained 10 µl Thunderbird SYBR qPCR mix, 0.6 µl of both forward and reverse primers, 2µl of cDNA, 6.4µl Dd H_2O RNase free and 0.4 µl 50 x Rox reference dye. The thermal cycle employed was 95°C for 75 sec, 59°C the 60 sec, 95°C for 15 sec with 40 cycles. The multicolored Real-Time PCR Detection System CFX manager (Bio-Rad Laboratories, Mississauga, ON, Canada) was used to calculate the relative expression levels of the candidate genes in the prehierarchical follicles based on the $2^{-\Delta\Delta Ct}$ method. The β-actin was used as the control gene to normalize the mRNA expression levels of the target genes.

Table 1 Primers assembled for RT-qPCR

Primer name	Primer sequence (5′-3′)	Annealing temperature (°C)	Amplicon size (bp)
NGF	F: CACCACAACACACACCTTTG R: GTCTCCCTGACTTCCTGCTAA	59	116
BDNF	F: ATGAAAGCTGCCCCGATGAA R: AAAGTGTCCGCCAGTGATGT	58	143
NT3	F: TGGTGAACAGAACCTCTCG R: CCTGGTGTCCTCTAATGTCAA	59	132
TrkB	F: AACTGCGACTTACCCTCAGC R: AGCACCCAGGACACATTAGG	59	119
β-actin	F: GCATGCCACACCGTGCCCATCTATGAG R: AAGCTTGGCCATCTCCTGCTCGAAGT	59	205

The candidate genes *NGF:* nerve growth factor, *BDNF:* brain-derived neurotrophic factor, *NT-3:* neurotrophin-3, and *TrkB:* tyrosine kinase receptor B encode ngf, bdnf, nt-3, and trkb, respectively; β-actin. F denotes forward primers and R denotes reverse primers

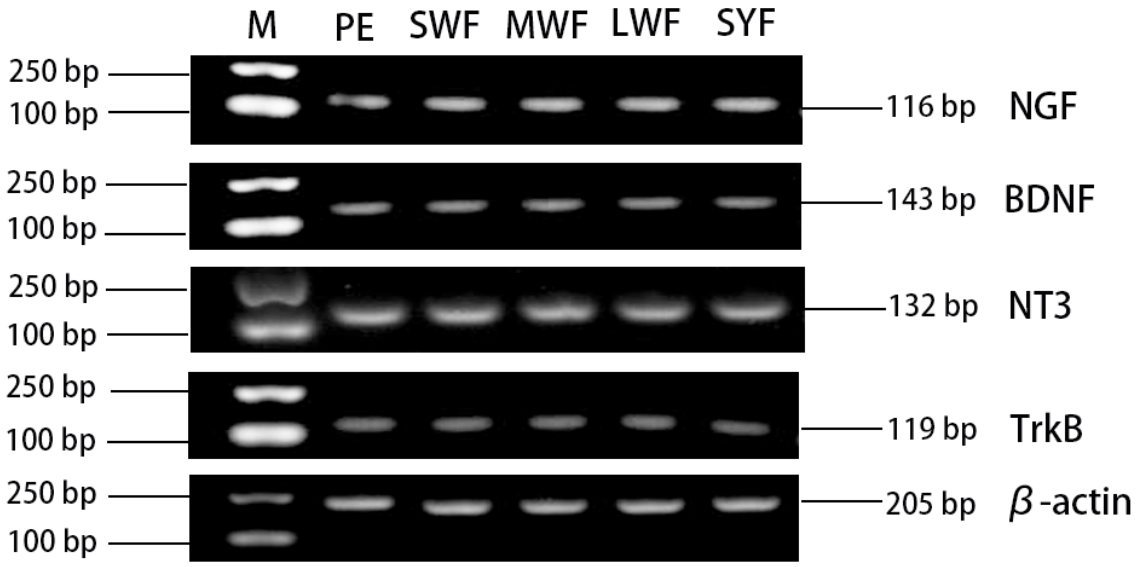

Figure 2 Neurotrophins (NGF, BDNF, NT-3 and TrkB) genes mRNA expression on 1 % electrophoresis gel. Conventional PCR product bands confirmed the desired amplification size indicating the efficiency and quality of the designed primers. M represents the marker.

***Western blot analysis*:** The procedure followed to examine neurotrophin related genes protein expression on the prehierarchical follicles has previously been described by Du *et al.* (2015) with modifications. The BCA method (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to determine the concentration of protein in the samples. The total protein (10 μg) from each sample was loaded onto a 10% SDS-PAGE gel and an electro blotting method performed to transfer the proteins onto a nitrocellulose membrane (Pall, Port Washington, NY, USA). To avoid the binding of non-specific amino acids the membranes were blocked with 5% dry milk at room temperature (about 25°C) for 1 h and subsequently the membranes were incubated at 4 °C overnight immersed with the following primary antibody: anti-NGF (1:2000, sc-365944), anti-BDNF (1:1000, sc-20981), anti-NT-3 (1:1000, sc-80250) and anti-TrkB (1:1000, sc-119) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then thoroughly washed three times for 15 min each with TBST and the membranes were incubated with HRP-conjugated Affinipure Goat anti-Mouse IgG (H+L) for 1 h at room temperature (Wuhan, Hubei, China). The blotted membranes were rigorously washed with TBST and visualized under ECL Western blot detection kit (NC15080; Thermo Fisher Scientific, Waltham, MA, USA). Eventually, the comparative intensities on each protein band were quantified using the ImageJ software and β-actin was used as an internal control.

***Statistical analysis*:** The relative expression levels of target genes in different grades of prehierarchical follicles were analyzed by SPSS23.0 statistical software. Single factor analysis of variance (One-way ANOVA) was used among groups and least square significant difference (LSD) test was used to compare the means. The significant difference of the data was indicated as $P<0.05$ and $P>0.05$ denoted no significant difference.

Results

***The mRNA expression levels of NGF, BDNF, NT-3 and TrkB receptor in prehierarchical follicles*:** Real-time qPCR analysis was performed to determine the mRNA expression levels of the neurotrophins target genes (*NGF*, *BDNF*, *NT-3*, and *TrkB*) in the prehierarchical follicles classified according to size in diameter (PE, SWF, MWF, LWF, and SYF). As presented in Figure 3, the different expression levels of *NGF* and *TrkB* genes were detected in all the prehierarchical stages of the development of the follicles. In contrast, *BDNF* and *NT-3* genes were undetected at the last stage of prehierachical growth (SYF). Moreover, the expression levels of all NTs genes from SWF to LWF revealed a down-regulated pattern with maximum expression in SWF ($P<0.05$). However, there was no statistical difference between PE and MWF for the *BDNF* gene as well as PE and SYF for the *NGF* gene ($P>0.05$). The *BDNF* gene was highly expressed in PE while the *NGF* showed the least expression in PE between the target genes ($P<0.05$).

***Protein expression levels of NGF, BDNF, NT-3 and TrkB receptor in prehierarchical follicles*:** We used the Western blotting technique to study the neurotrophic genes in the prehierarchical follicles samples of Zi geese. The Western blot results in Figure 4, confirmed the quantitative expression of *NGF*, *BDNF*, *NT-3* and *TrkB* receptor genes in all samples of prehierarchical follicles. The *NGF* protein expression was dominant in SWF and significantly different from that in PE, MWF, LWF and SYF ($P<0.05$). There was no significant difference in the expression of *NGF* protein levels in PE, MWF and SYF ($P>0.05$). The highest protein level in *BDNF* was detected in PE followed by SWF, MWF, LWF and SYF ($P<0.05$) in descending order, of which MWF and LWF had no significant difference ($P>0.05$). Interestingly, the protein expression of *NT-3* reflected an upward-regulated pattern from PE to LWF from which there was still no significant difference between

MWF and LWF (P>0.05) respectively. Furthermore, SYF had the least *NT-3* protein, and PE, SWF, MWF, and LWF protein expressions were significantly different (P<0.05). Similar to *BDNF*, the *TrkB* protein expression level was excessively high in PE and least expressed in SYF, and there was a significant difference between PE, SWF, MWF, LWF, and SYF (P<0.05). Although the expression level in LWF was higher than that of MWF, there was no statistical difference between the two (P>0.05).

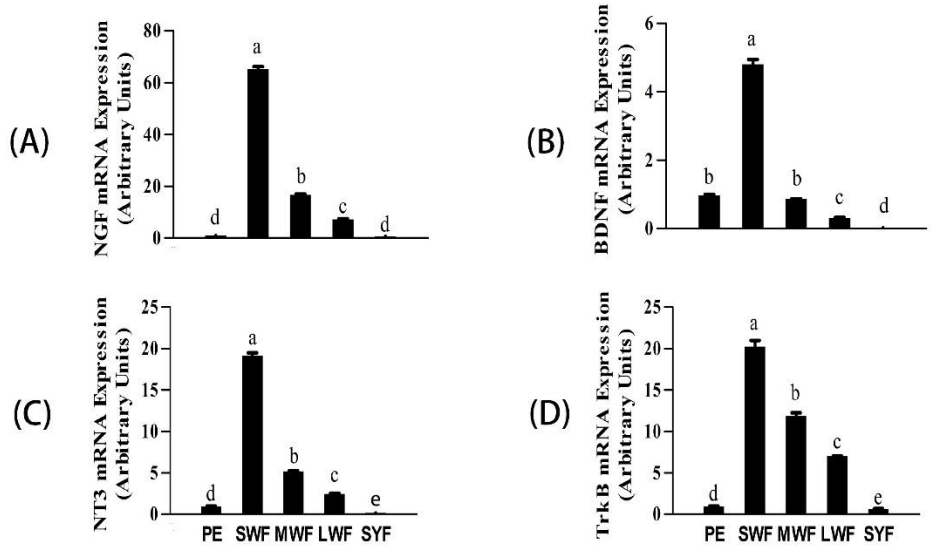

Figure 3 Expression of genes encoding nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and tyrosine kinase receptor B (TrkB) in the prehierarchical follicles of Zi geese**.** The data are represented as means ± SEM; n = 6 geese and the different letters indicate the significant difference of mRNA expression between different sizes of prehierarchical follicles (P<0.05) reported in arbitrary units (AU) normalised with β-actin. The vertical ordinate represents the relative mRNA expression level of each neurotrophin-related gene. The horizontal ordinate indicates the sampled follicle sizes. PE: primary follicles, SWF: small white follicles, MWF: middle white follicles, LWF: large white follicles, and SYF: small yellow follicles.

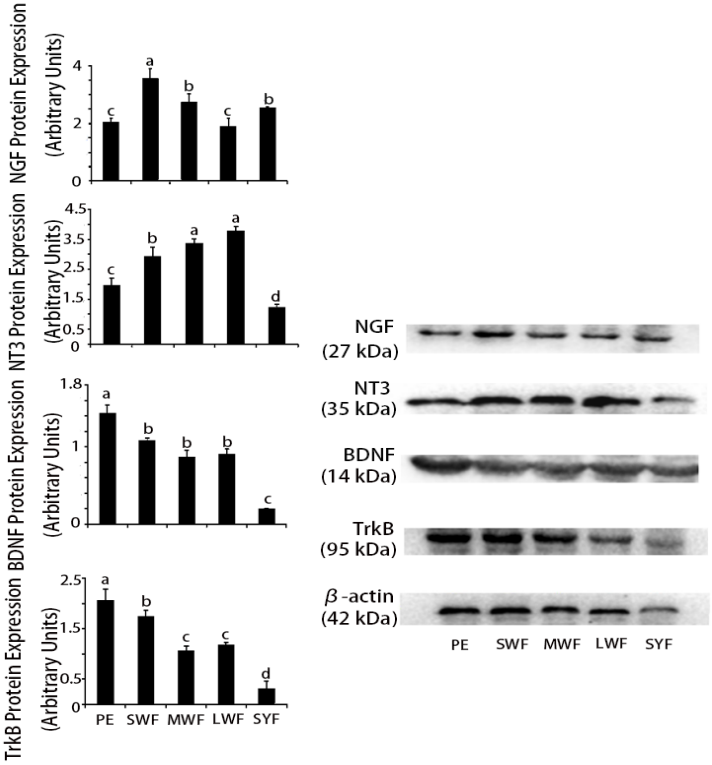

Figure 4 Western blot analysis comparing the densitometric means of the protein bands for *NGF*, *BDNF*, *NT-3*, and *TrkB* in prehierarchical stages of geese follicles (n=6). The upper four panels show the measurable quantities *NGF*, *BDNF*, *NT-3*, and *TrkB*, in prehierarchical follicles at different growth stages. The lower panel indicates the β-actin as an internal control in all samples. Each sample is shown as mean ± SEM of the ratio of the relative density of *NGF*, *BDNF*, *NT-3*, and *TrkB* to β-actin. The different superscripts indicate statistically significant differences (P<0.05). The difference between the five means was statistically significant at (P<0.05). PE: primary follicles, SWF: small white follicles, MWF: middle white follicles, LWF: large white follicles, and SYF: small yellow follicles.

Discussion

The neurotrophin family regulates the important functions in folliculogenesis and ovarian development by maintaining the survival of the nerve cells through paracrine and autocrine mechanisms (Meinel *et al.* 2015). The mRNA levels of nerve growth factor (NGF) were markedly evident in the three stages of prehierarchical follicles development with profound expression in SWF and low quantities were detectable in PE and SYF. At the protein level, *NGF* showed explicit expression at each stage. Conversely, it maintained the highest measure in SWF with no statistical significance between PE and SYF. The findings of this study are consistent with that determined in the sex organs of male rabbits (Maranesi *et al.* 2015) and (Jana *et al.* 2011) in porcine ovaries in which *NGF* also showed irregularity in the expression pattern. The spatial-temporal expression of *NGF* in the prehierarchical follicles implies its possible functions in the control of follicular development in a stage-dependent manner. Studies have previously been conducted on the expression of *BDNF* as a member of the neurotrophin family and its potential biological functions in the ovarian development and oocyte maturation in animals and humans (Anderson *et al.* 2009; Xie *et al.* 2017). In this study, it was also found that *BDNF* transcripts were undetectable in SYF as the follicle transit to the hierarchical stage. This went further to the protein level where significantly low amounts of protein were detected in SYF ($P<0.05$) and the high expression level in PE ($P<0.05$). The results of the present study were concurrent with the findings of Mirshokraei *et al.* (2013) where the undetectable transcripts of *BDNF* were observed in the cervix of non-pregnant ewes and the isthmus of pregnant ewes. The other comparative study conducted by Garcés *et al.* (2014) indicated that *BDNF* protein level decreased towards the end of gestation in both human and rat placenta. The existence of the *BDNF* gene at mRNA and protein level in prehierarchical follicles provides clear evidence of its involvement in follicular development and physiology. We further investigated the presence of *NT-3* gene in goose prehierarchical follicles. It has been indicated that *NT-3* was localized in different ovarian cell types, predominantly in the granulosa cells and oocytes to endorse the transition of primordial follicles into primary follicles (Nilsson *et al.* 2009). Similarly, *NT-3* at mRNA level was present in only four prehierarchical categories and undetectable amounts were identified in SYF to produce quantifiable signals. Wherein *NT-3* protein expression was detectable in all the samples classification even though SYF showed the least quantity of expression. These results suggest that the casual expression pattern of *NT-3* could be associated with the structural development and physiological differences during folliculogenesis. It has been documented that *NT-3* expression was significantly higher in both brain selective breast cancer cell lines compared with the non-brain selective breast cancer cell lines and hence promoting metastatic breast cancer brain growth (Louie *et al.* 2013). It was initially believed that NTs were mainly obligatory for the development of the nervous system; however the abundance of the high-affinity Trk receptors in various non-neural tissues resulted in the decision that the NTs may play a critical role both in anatomic and physiological functions of animal organs and systems such as reproductive, immune, endocrine, and cardiovascular systems (Paredes e*t al.* 2004). In this current research, the expression of *TrkB* receptor was investigated, wherein its presence was determined in the prehierarchical follicles. The *TrkB* mRNA transcripts were predominantly expressed in SWF whereas it was high in PE at the protein level. These findings suggest that the *TrkB* receptor may significantly be involved in regulation in the early stages of ovarian follicular development and may have less functional contributory effects as the prehierarchical follicles reach the preovulatory stage. In ovaries from the human fetus and adults, the expression of *TrkB* was predominant in the granulosa cells of the primordial, primary, and secondary follicles with the highest quantity in granulosa cells of the primordial follicles categorically (Harel *et al.* 2006). Jensen and Johnson, (2001) revealing that *TrkB* mRNA significantly increased in preovulatory follicles as compared to prehierarchical follicle in domestic hens *(Gallus Gallus Domesticus).* It is worth noticing that all the target genes at mRNA level increased significantly at SWF whereas at protein level showed varying significance in their expression pattern levels at different stages of prehierarchical follicle development. These findings suggest that the candidate neurotrophins might have a substantial contributory effect at SWF compared to the other stages of development. Furthermore, the variations of the target genes at protein level may be due to incredible successions of interrelated processes such as the post-transcriptional modification regulation and translation rate modulation (Vogel and Marcotte, 2012).

In conclusion, this research findings provide indications that *NGF*, *BNDF*, *NT-3* and *TrkB* receptors are expressed differently at each prehierarchical stage to enhance the molecular physiological functions in the follicular development of geese. These results also provide the basis for further studies on interactive cell regulatory mechanisms of neurotrophins related genes in ovarian follicle growth and development.

Acknowledgements

This work was supported by the Jilin Province Science and Technology Development Project (20180201034NY, 20150204025NY), the Jilin Province Scientific Activities of Selected Returned Overseas Professionals and the Jilin Agricultural University youth talent support program (2018). We thank the Jilin Agricultural University Goose Farm for providing the experimental animals.

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