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Neurotrophins and specific receptors in the oviduct tracts of Japanese quail (*Coturnix coturnix japonica*)

L. Maruccio[a,*], L. Castaldo[a], L. D'Angelo[a], C. Gatta[a], C. Lucini[a], C. Cotea[b], C. Solcan[b], E.L. Nechita[b]

[a] Department Veterinary Medicine and Animal Productions, University "Federico II" of Naples. Via Veterinaria, 1, 80137 Naples, Italy
[b] Department Normal and Pathological Morphology, University of Agricultural Science and Veterinary Medicine 'Ion Ionescu de la Brad' Iasi, Faculty of Veterinary Medicine, 8 M. Sadoveanu Alley, 700489 Iasi, Romania

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ABSTRACT

Neurotrophins (NGF, BDNF and NT-3) and their specific receptors (TrkA, TrkB and TrkC) were studied in the oviduct of egg laying quails. Neurotrophins (NTs) are mainly involved in the development and maintenance of neuronal populations in the central and peripheral nervous system, but also in reproductive system. In this survey, we first studied the morphological organization of the quail oviduct, distinguished in infundibulum, magnum, isthmus, uterus and vagina, and then we analyzed the expression and localization of NTs and Trks receptors in the whole tracts. By western blotting we detected that the investigated NTs and Trks receptors are expressed in all oviductal tracts. By immunohistochemistry we were able to define the distribution of NTs and Trks. Specifically, NGF, BDNF and NT3 were localized in lining and ductal epithelial cells, and NGF was also detected in secretory cells of tubular glands and in nervous fibers of vessel wall. TrkA and TrkB were present in the lining and ductal epithelium; TrkA and TrkC were present in nervous fibers of vessel wall in all oviductal tracts. Furthermore, we also observed NGF and BDNF co-localized with TrkA and TrkB in cells of the lining and ductal epithelium, suggesting an autocrine mechanism of action.

1. Introduction

Neurotrophins (NTs) are a family of growth factors including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), NT3, NT4. They control the development and maintenance of neuronal populations in the central and peripheral nervous system, but they can also act on non neuronal cells in different organs and systems, such as the mammalian reproductive system (Ibáñez et al., 1991; Dissen et al., 1995, 2002; Anderson et al., 2002; Abir et al., 2005; Seifer et al., 2006).

The neurotrophin sequences are highly conserved in vertebrates (Lanave et al., 2007; Tettamanti et al., 2010). However, in birds, NT4 has not been identified (Hallbook et al., 1995). All neurotrophin genes code glycosylated precursors (31–35 kDa), that are cleaved by convertases to give rise to mature neurotrophins (13.2–15.9 kDa) (Roux and Barker, 2002; Chao, 2003). The action of NTs is mediated by highly specific tyrosine kinase receptors (Trks) (Teng and Hempstead, 2004), and by a pan-neurotrophin receptor, the $p75^{NTR}$ (Barret, 2000), that binds each neurotrophin with low affinity. Among Trk receptors, TrkA is the preferred receptor for NGF, TrkB for BDNF/NT4, and TrkC for NT-3 (Patapoutian and Reichardt, 2001; Deinhardt and Chao, 2014). In addition, TrkA and TrkB receptors can be activated, even if less efficiently, by NT3, as well (Skaper, 2012). The Trk receptors are generally characterized by full-length and truncated isoforms. Full-length kinase isoforms (140–145 kDa) comprise the tyrosine kinase signaling domain, whereas truncated isoforms (50–95 kDa) lack the domain (Klein et al., 1990; Barbacid et al., 1991; Lamballe et al., 1993). It has been hypothesized that the truncated forms of receptors are expressed by non neuronal tissue (Barbacid, 1995).

In the present survey, we investigate the expression and localization of NTs and Trks receptors in the oviduct of quail *Coturnix coturnix japonica*, species used as animal model in reproductive biological studies (Hosseini et al., 2016). The morphology and physiology of oviduct in birds are much more complex than in mammals, making this organ particularly challenging. The left oviduct is the site of the second meiotic division of the oocyte, and sperm storage and oocyte fertilization occur similarly to that in mammals (Buhi, 2002). Here the formation of the different components of the egg (i.e., albumen, shell membranes, shell and cuticle) (Bell

* Corresponding author. Tel.: +390812536120; fax: +390812536097.
E-mail address: lucianna.maruccio@unina.it (L. Maruccio).

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and Freeman, 1971), necessary for embryonic development, takes place. To our knowledge, there are no data on the presence of NTs and Trks in the avian genital tract. Previous studies have been carried out in mammalian species, documenting the involvement in the oocyte transport and fertilization, in spermatozoa capacitation (Weng et al., 2009), and in development of preimplantation embryos (Kawamura et al., 2007; Mirshokraei et al., 2013).

2. Materials and methods

2.1. Animals and tissue preparations

The Japanese quail (*Coturnix coturnix japonica*) used in this study came from the intensive growing system belonging to the University of Agricultural Science and Veterinary Medicine of Iasi. Three month old quails in the period of egg laying were used. All animals were euthanized by an overdose of sodium pentobarbital. Immediately, the genital tract of 6 quails was collected. Oviduct tracts from 3 animals were separately identified (infundibulum, magnum, isthmus, uterus and vagina) and stored at −80 °C for Western blotting (WB) analysis; and the same tracts from 3 animals were fixed in Bouin's fluid for 24 h for histological and immunohistochemical techniques. The fixed samples dehydrated and embedded in paraffin, were serially sectioned at 7 μm and stained with hematoxylin-eosin (HE), for a general histological description, and used for immunohistochemistry.

2.2. Western blotting analysis

For WB analysis of NGF, BDNF and NT-3, three samples of infundibulum isthmus, magnum, uterus and vagina for each antibody, respectively, were homogenized using Tissue Lyzer (Qiagen) in 100 μl of ice-cold lyses buffer (50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.25% deoxicolic acid, 1% Triton X 100) in addition to 20 mM sodium pyrophosphate, 0.1 mg/ml aprotinin, 2 mM phenylmethylsulphony fluoride (PMSF), 10 mM sodium orthovanadate (Na2VO3), and 50 mM NaF. The quantity of total proteins was determined by use of a protein assay kit (Bio-Rad Laboratories). Equal amounts of lysate samples (80 μg) were boiled and loaded on bis/acrylamide gel and electrophoresed. The proteins were blotted from the gel onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in TBS-0.1% Tween buffer (10 mM Tris–HCl, pH 7.4, 165 mM NaCl, 0.1% Tween) at room temperature (RT), washed with TBS-0.1% Tween, and incubated with antibodies (Table 1). Each antibody was diluted 1:200 in TBS-0.1% Tween. After appropriate washing steps in TBS Tween 0.1%, anti-rabbit peroxidase-conjugated secondary antibody (Amersham, Gel Health Care) was applied 1 h at RT at 1:2000 dilution. The blots were stripped and reprobed against mouse anti-actin antibody (CP01, Calbiochem), 1:5000, to ensure equal amounts of proteins for each sample.

For WB analysis of receptors TrkA, TrkB and TrkC, three samples of infundibulum isthmus, magnum, uterus and vagina for each antibody, were homogenized using Tissue LyzerII (Qiagen, Chatsworth, CA) in 100 ml of lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 μg/ml Leupeptid, 1 μg/ml Aprotimin, 1 μg/ml Pepstatin A, 0.25% Sodium deoxycholate, 1 mM NaF) homogenized at 13,200 rpm at 4 °C. The quantity of total proteins was determined by use of a protein assay kit (Bio-Rad Laboratories). Samples containing 50 μg of lysated protein were denatured by incubation at 95 °C in 0.1% sodium dodecyl sulfate (SDS) 1 mM Beta mercaptoethanol in Tris–HCl pH 6.8 and separated on a 15% SDS-polyacrylamide gel electrophoresis (SDS PAGE) gel in a mini-Protean Tetra Cell chamber (BioRad, Hercules, CA) at 4 °C with 90 V for 2 h. Proteins were transferred onto Hybond-ECL (Amersham, Arlington Heights, IL) nitrocellulose membranes using the Mini Protean system (BioRad) for 45 min using 400 mA at 4 °C. Ponceau red was used to assess homogenous transfer. Prestained ladder (Thermo Scientific PageRuler Prestained Protein Ladder) was used. The proteins were blotted from the gel onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in TBS-0.1% Tween buffer (10 mM Tris–HCl, pH 7.4, 165 mM NaCl, 0.1% Tween) at RT, washed with TBS-0.1% Tween, and incubated with antibodies (Table 1). Each antibody was diluted 1:200 in TBS-0.1% Tween. After appropriate washing steps in TBS Tween 0.1%, anti-rabbit peroxidase-conjugated secondary antibodies (Amersham, Gel Health Care) were applied 1 h at RT at a dilution of 1:2000. The blots were stripped and reprobed against mouse anti-actin antibody (CP01, Calbiochem), 1:5000, to ensure equal amounts of proteins for each sample. As positive controls, two mouse submandibular glands were employed for NGF antibody, and PC12 whole cell lysates for BDNF, NT-3, TrkA, TrkB and TrkC. For TrkA and TrkC, the image was adjusted, by re-organizing the lanes according to the anatomical organization of oviductal tracts.

2.2.1. Morphological stainings and single immunohistochemistry

Hematoxilin-eosin staining was carried out on microtome sections adjacent to those utilized for immunohistochemical stainings.

Immunohistochemistry was carried out using the EnVision system + horse radish anti-peroxidase (HRP) (Dako, Santa Barbara, CA,

Table 1
List and features of antibodies.

Antibody	Antigen	Dilution	Code and source
NGF	N-terminus of the mature chain of NGF of human origin	1:500 IHC 1:50 IF 1:200WB	sc-548 S. Cruz Biotecnology, CA, USA
BDNF	Internal region of BDNF of human origin aa.100-150	1:500 IHC 1:50 IF 1:200WB	sc-546 S. Cruz Biotecnology, CA, USA
NT-3	Internal region of NT-3 of human origin	1:500 IHC 1:50 IF 1:200WB	sc-547 S. Cruz Biotecnology, CA, USA
TrkA	Human COO-domain 763-777	1:400 IHC 1:40 IF 1:200WB	sc-118 S. Cruz Biotecnology, CA, USA
TrkB	Human COO-domain 794-808	1:200 IHC 1:20 IF 1:200WB	sc-12 S. Cruz Biotecnology, CA, USA
TrkC	Human COO-domain 798-812	1:200 IHC 1:20 IF 1:200WB	sc-117 S. Cruz Biotecnology, CA, USA

IHC, immunohistochemistry; IF, immunofluorescence; WB, western blotting.

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