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Localization of Nerve Growth Factor (NGF) and TrkA in Zebrafish Gonads: Insights into Non-Neuronal Functions Across Vertebrates

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ABSTRACT

Nerve growth factor (NGF), belonging to the neurotrophin family, functions as a key regulator in various essential processes within both the peripheral and central nervous systems. Concurrently, numerous studies have demonstrated that this neurotrophin is also active in non-neuronal organs, including reproductive tissues. Although extensive research has been performed in mammals, information regarding NGF and its receptor TrkA in the reproductive organs of other vertebrates, such as teleost fish, remains limited. To broaden current understanding of NGF and TrkA in vertebrate gonadal models, this study investigates the expression profiles of *ngf* and *trka* mRNA in the testes and ovaries of adult zebrafish. Through chromogenic and fluorescent in situ hybridization analyses, we reveal that in zebrafish testes, *ngf* and *trka* are predominantly localized in spermatogonia B and spermatocytes. In the ovaries, both genes are detected across various oocyte developmental stages. Collectively, these observations indicate that NGF and its receptor play a conserved role in the reproductive physiology of vertebrates.

Keywords: Zebrafish, NGF, TrkA, ovary, Testis

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Introduction

Nerve growth factor (NGF) was the first identified neurotrophin [1, 2]. Other family members include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), neurotrophin-4/5 (NT4), and neurotrophin-6/7 (NT6/7) [3–6]. All neurotrophins are produced as precursors. Specifically, NGF exists in a pro-form (pro-NGF) that can be secreted extracellularly or converted within the cell into its mature form, both biologically active [7] and forming homodimers [8]. The *ngf* gene, similar to other neurotrophins, exhibits high sequence conservation among vertebrates, suggesting preserved functions throughout evolution [9–13]. NGF interacts mainly with the tyrosine kinase receptor TrkA and/or the p75 neurotrophin receptor [14, 15]. The NGF–TrkA interaction supports neuronal survival and growth in both the peripheral and central nervous systems [16], whereas p75 binding can trigger apoptosis.

In vertebrates, this signaling pair is also crucial in non-neural tissues [17–22], as evidenced by their detection in several organs, including reproductive structures [23, 24]. Studies on humans, rats, mice, wild ground squirrels, and Japanese quail have revealed that NGF and TrkA are essential for maintaining ovarian sympathetic innervation [25–29]. Within ovaries, NGF and TrkA have been localized in oocytes, interstitial cells, theca layers, and granulosa cells [30–32]. Moreover, the NGF/TrkA signaling cascade is known to promote ovulation and early follicle formation [33].

In non-mammalian vertebrates, NGF and TrkA have been identified in *Xenopus* ovaries, where NGF treatment stimulates meiotic maturation in *Xenopus* oocytes overexpressing TrkA [34]. Additionally, this pathway appears to regulate spermatogenesis [35]. In the testes of various mammalian species, NGF has been detected in primary spermatocytes, pachytene cells, and elongated spermatids [36–38], while TrkA localization is restricted to elongated spermatids and Leydig cell membranes. Functional studies suggest that NGF and TrkA enhance testosterone synthesis, as well as Leydig cell growth and differentiation [39, 40]. Decreased *ngf* mRNA levels in sperm have been linked with azoospermia in humans, a finding corroborated in rabbit and mouse models [41], where suppression of NGF/TrkA signaling led to reduced sperm motility. More recent research has proposed that NGF administration may restore spermatogenesis in murine models with severe testicular atrophy [42].

Despite numerous mammalian studies, limited data exist regarding NGF and its receptor in fish reproductive systems. Here, we present the first description of *ngf* and *trka* expression in the gonads of *Danio rerio* (zebrafish). This species, owing to its small size, external fertilization, and rapid embryogenesis, serves as a valuable vertebrate model in biomedical and veterinary sciences. It is widely utilized for mutagenesis research and pharmacological screening and has contributed to investigations in embryology, genetics, oncology, cardiovascular biology, and organ or tissue regeneration [43–46]. In this study, hematoxylin–eosin staining on paraffin-embedded sections was used to distinguish various cell populations in adult zebrafish gonads. Subsequently, chromogenic and fluorescent in situ hybridization demonstrated that *ngf* and *trka* mRNAs are strongly expressed during spermatogenesis and oogenesis, reinforcing their conserved role across vertebrate evolution.

Materials and Methods

Animal handling and gonad sampling

All procedures adhered to the Italian Legislative Decree 26/2014 and received ethical authorization (permit no. 2/2020-PR) from the Animal Care Committee of the University of Naples Federico II. One-year-old male and female *Danio rerio* were anesthetized in 0.3% ethyl 3-aminobenzoate methanesulfonate (MS222; Sigma, St. Louis, MO, USA). Following anesthesia, the gonads were excised, fixed for 24 h in 4% paraformaldehyde, dehydrated through graded alcohols, embedded in paraffin, and sectioned. These sections were prepared for histological examination with hematoxylin–eosin (HE) staining and for in situ hybridization analyses (see Sections 2.2 and 2.3 for methodological specifics).

Histological procedure (hematoxylin–eosin staining)

Testes and ovaries were immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at 4 °C, then rinsed and preserved in 70% ethanol at 4 °C. Samples were subsequently embedded in paraffin wax and sliced using a rotary microtome. Sections were mounted onto glass slides, deparaffinized in xylene, and rehydrated through a descending ethanol series (100%, 95%, 80%, 70%, and 50%). After rehydration, slides were washed three times in distilled water for one minute each. According to morphological criteria described previously [47, 48], hematoxylin and eosin staining were applied to visualize the tissue structure and cellular composition of the gonads.

Chromogenic and fluorescent In situ hybridization (ISH)

Antisense riboprobes labeled with digoxigenin (DIG) were generated following established methodologies [49–51]. The expression of *ngf* and *trka* transcripts was assessed by in situ hybridization (ISH). The riboprobe templates were obtained by linearizing ZeroBlunt or TOPO-TA vectors containing PCR-amplified inserts of *ngf* and *trka*. Plasmids were digested with appropriate restriction enzymes, and antisense or sense riboprobes were transcribed *in vitro* using SP6 or T7 RNA polymerase (Roche Diagnostics, Chicago, IL, USA) in the presence of DIG-labeled nucleotides. To confirm probe specificity, both sense and antisense riboprobes were hybridized to adjacent tissue sections.

The primer pairs used to synthesize *ngf* and *trka* probes were:

• *ngf* F: GGAGCACAGGAGATCTACGC; R: CGTGGAAAAACCCAACTCAT

• *trka* F: AGTTGTTGCTTGCAGGGTGG; R: TGGGTCAATCATGACCTCAG

After probe synthesis, testes and ovaries were dissected and fixed overnight in 4% paraformaldehyde at 4 °C. After fixation, samples were embedded in paraffin, and 10 µm sections were obtained. Sections were deparaffinized twice in xylene for 3 min each and rehydrated in graded ethanol concentrations (100%, 95%, 80%, 70%, and 50%; 3 min per step). The slides were refixed for 20 min in 4% paraformaldehyde and treated with proteinase K (2 mg/mL) for 7 min at room temperature. Following additional fixation for 20 min in paraformaldehyde, sections were washed twice for 10 min in PBS and 2× SSC buffer. Hybridization was carried out at 63 °C for 24 h in a humidified chamber using two µg/mL of probe in hybridization solution containing 5× Denhardt's reagent, 2× SSC, 50% formamide, four mM EDTA, 5% dextran sulfate, and 50 µg/mL yeast tRNA. Post-hybridization washes were performed with 2× SSC, 50% formamide/2× SSC, 0.2× SSC, and 0.1× SSC buffers. Sections were then incubated in Tris-HCl/NaCl buffer (100 mM Tris-HCl, pH 7.5; 150 mM NaCl) and washed again in the same solution supplemented with 0.5% skim milk and 0.1% Triton X-100.

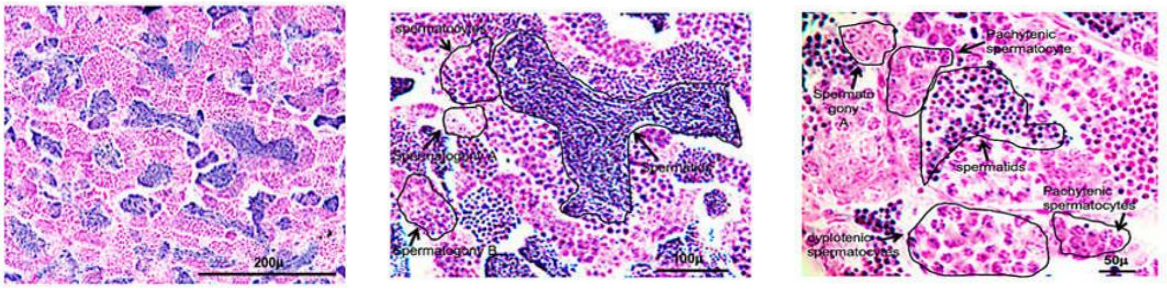
For chromogenic ISH, sections were incubated overnight at room temperature with anti-DIG alkaline phosphatase Fab fragments (1:5000 dilution; Roche Diagnostics, Chicago, IL, USA). After washing with Tris-HCl/NaCl buffer and Tris buffer (pH 8.0) containing 10 mM $MgCl_2$ and 110 mM NaCl, staining was developed using NBT/BCIP buffer (pH 9.5).

For fluorescent ISH**,** sections were incubated at room temperature for 24 h with anti-DIG POD antibody (Roche) diluted 1:200 in blocking solution. Slides were then washed four times in maleic acid buffer (20 min each) and four times in PBS (10 min each). Subsequently, sections were placed in amplification buffer (PerkinElmer Life Sciences, Boston, USA) for one h. Signal development was performed using Cy3 tyramide (TSA Plus Cyanine 3; PerkinElmer Life Sciences, Boston, USA) at a 1:100 dilution and Alexa Fluor 488 tyramide reagent (Invitrogen™, Boston, MA, USA). After several washes (10 min per step), slides were counterstained with DAPI, mounted, and observed under a Nikon Eclipse 90i confocal microscope. Digital images were captured and analyzed with NIS-Elements software version 4.2 (Nikon, Milan, Italy).

Results and Discussion

Morphological assessment of adult zebrafish testes

As described in previous studies, spermatogenesis in zebrafish is organized within cystic units **(Figures 1a–1c)**.



a) b) c)

Figure 1. Hematoxylin–eosin staining of adult zebrafish testes.
(a) General histological structure of the testis. (b) Identification of spermatogonia A–B, spermatocytes, and spermatids. (c) High magnification showing spermatogonia A, spermatocytes at various meiotic phases, and spermatids. Scale bars: 200 µm (a); 100 µm (b); 50 µm (c).

Each cyst is composed of Sertoli cells encasing developing germ cells. Multiple cysts at different maturation stages can be distinguished. Spermatogonia A are round cells with one or several nucleoli; spermatogonia B exhibit denser heterochromatin and smaller, round or slightly elongated nuclei, occasionally containing tiny nucleoli. Spermatocytes at different meiotic phases are recognized by nuclear enlargement and chromatin condensation patterns. Early, mid, and late spermatids progressively reduce in cell and nuclear size as development proceeds **(Figures 1a–1c)**.

Presence of ngf *and trka transcripts in adult zebrafish testes*

Chromogenic and fluorescent in situ hybridization techniques were carried out to determine where *ngf* and its receptor, *trka*, are transcribed within the testes of adult *Danio rerio*.

Signals corresponding to *ngf* were predominantly observed in spermatogony B and diplotene-stage spermatocytes **(Figures 2a–2f)**. High-resolution confocal microscopy further validated these localization patterns **(Figure 3a and 3b)**.

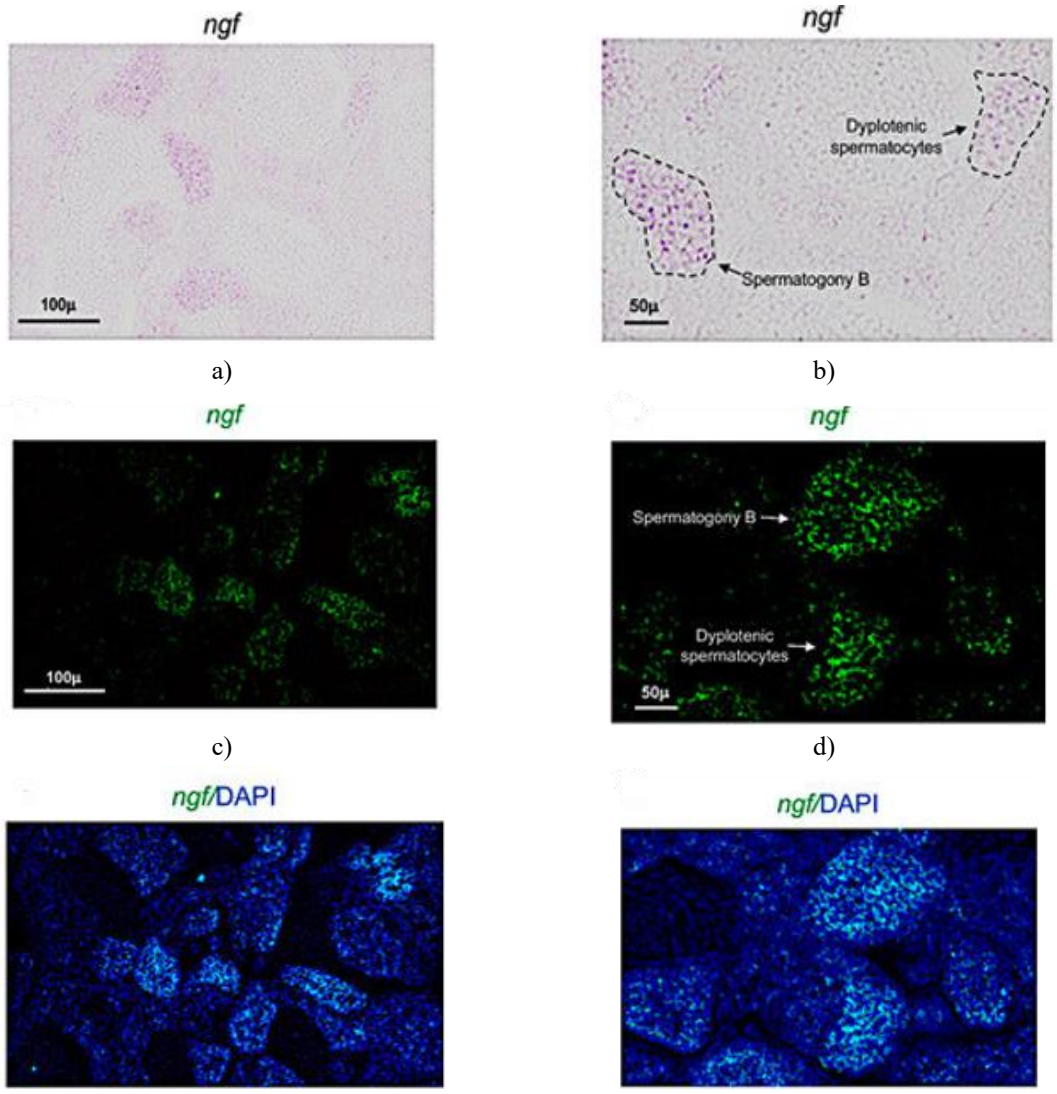


Figure 2. Distribution of *ngf* mRNA in testes of adult zebrafish. (a,b) Chromogenic ISH visualization of *ngf*. (c,d) Fluorescent ISH illustrating *ngf* labeling. (e,f) Dual labeling of *ngf* and nuclear staining with DAPI. Scale bars: 100 µ (a,c,e); 50 µ (b,d,f).

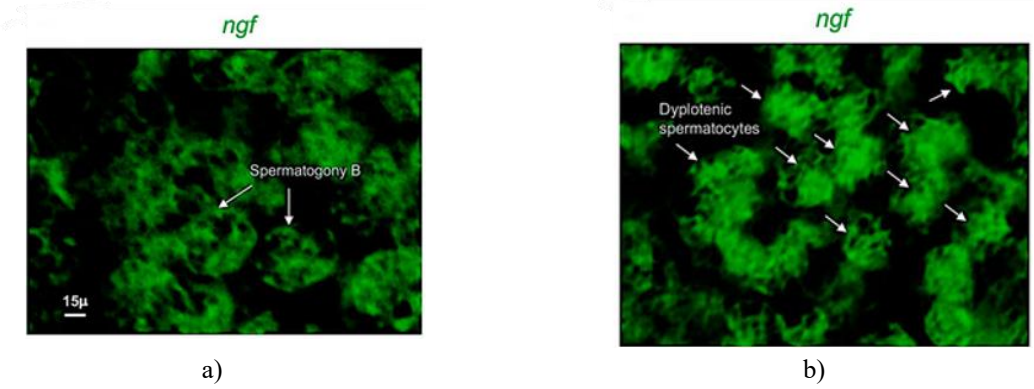


Figure 3. *Ngf* is confined to spermatogony B and diplotene spermatocytes. Enlarged fluorescent ISH views showing *ngf* in (a) spermatogony B and (b) diplotene spermatocytes in adult testes. Scale bar: 15 µ (a,b).

Expression of the receptor *trka* appeared in three germ-cell categories—spermatogony types A and B, along with diplotene spermatocytes **(Figures 4a–4f)**. Confocal microscopy at higher magnification corroborated these findings **(Figures 5a–5c)**.

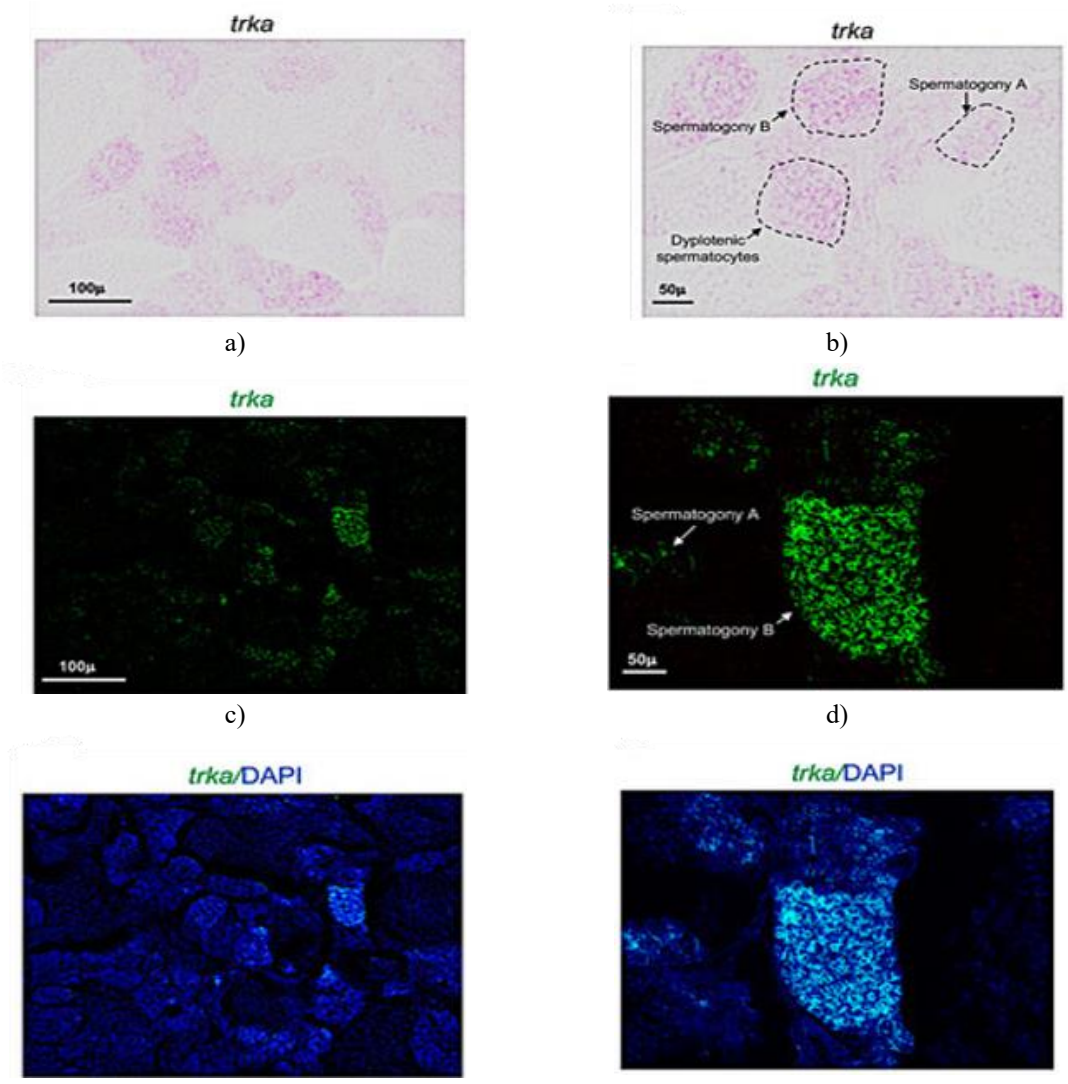


Figure 4. *Trka* transcript localization in testes of adult zebrafish. (a,b) Chromogenic ISH micrographs of *trka*. (c,d) Fluorescent ISH showing *trka* distribution. (e,f) Combined *trka* and DAPI nuclear fluorescence. Scale bars: 100 µ (a,c,e); 50 µ (b,d,f).

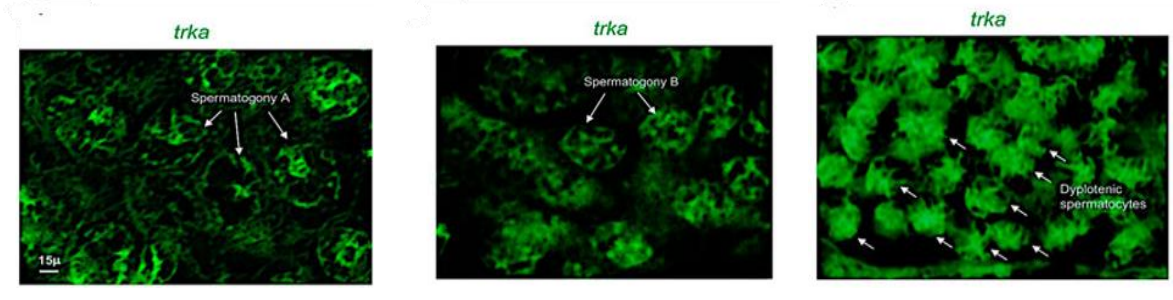


Figure 5. *Trka* was detected in spermatogony A, B, and diplotene spermatocytes. High-magnification fluorescent ISH of (a) spermatogony A, (b) spermatogony B, and (c) diplotene spermatocytes. Scale bar: 15 µ (a–c).

Ovarian morphology in zebrafish

Consistent with earlier histological descriptions, the ovary of adult zebrafish exhibits five distinct developmental phases of oocyte maturation **(Figures 6a–6h)**.

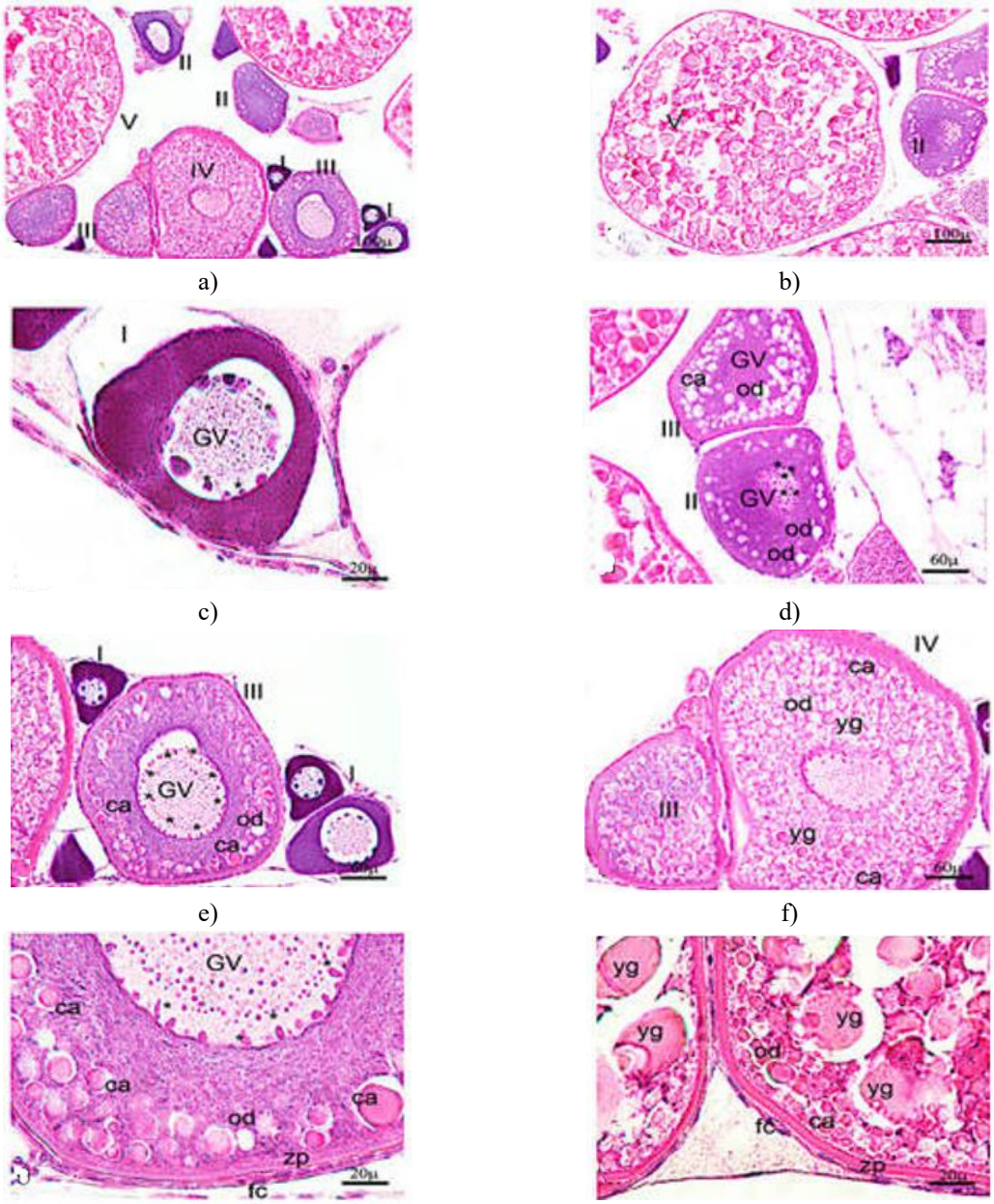


Figure 6. Hematoxylin–eosin–stained ovarian sections from adult zebrafish. (a) General view of ovarian tissue showing oocyte stages I–II–III–IV–V. (b) Oocytes at stages II and V. (c) Oogonia with large euchromatic germinal vesicles (GV) containing numerous peripheral nucleoli. (d) Oocytes in early growth (stages II–III) showing multiple nucleoli and oil droplets (od) in the cytoplasm near the GV. (e) Stage III oocytes with abundant nucleoli along the GV edge. (f) Cells displaying oil droplets and cortical alveoli (ca). (g) Stage IV oocytes enveloped by a zona pellucida (zp) and a single follicular-cell layer (fc). (h) Stage V oocytes with extensive yolk globules (yg), a thickened zona pellucida, and evident thecal tissue. Scale bars: 100 µ (a,b); 60 µ (d–f); 20 µ (c,g,h).

Localization of ngf and trka transcripts in the ovarian tissue of adult zebrafish

Chromogenic and fluorescence in situ hybridization procedures were next applied to reveal the spatial expression profiles of *ngf* and *trka* within mature zebrafish ovaries.

Signals for *ngf* were concentrated in the perinuclear cytoplasm of oocytes from stages I–III **(Figures 7a and 8b)**. In more advanced oocytes (stages IV and V), *ngf* labeling shifted to follicular cells surrounding the oocyte **(Figures 7c and 7d)**. Expression of *trka* was strongest in the perinuclear region of stage II–III oocytes **(Figure 8a and 8b)** and, similar to *ngf*, appeared in follicular cells of stage V oocytes **(Figures 8c and 8d)**.

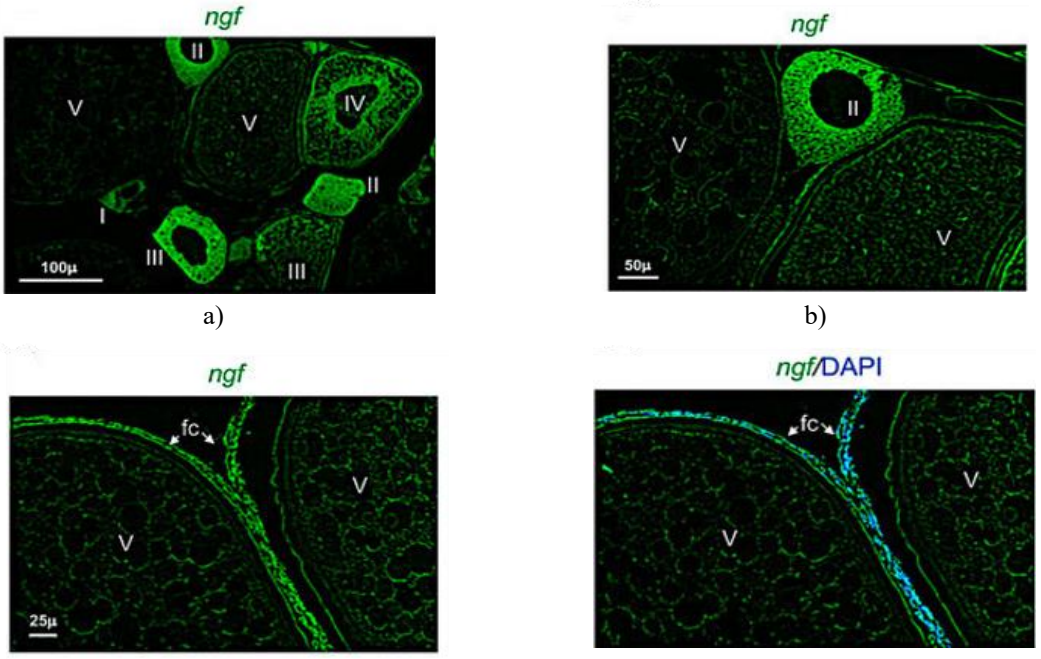


Figure 7. *Ngf* transcript distribution in adult zebrafish ovary. (a) Fluorescent ISH displaying *ngf* in oocytes at stages II, III, IV, and in follicular cells of stage V. (b) Expression of *ngf* in oocytes at stages II and V. (c) Enlarged image showing *ngf* signals in follicular cells of stage V. (d) Overlay of *ngf* and DAPI fluorescence in the same region. Scale bars: 100 µ (a); 50 µ (b); 25 µ (c,d).

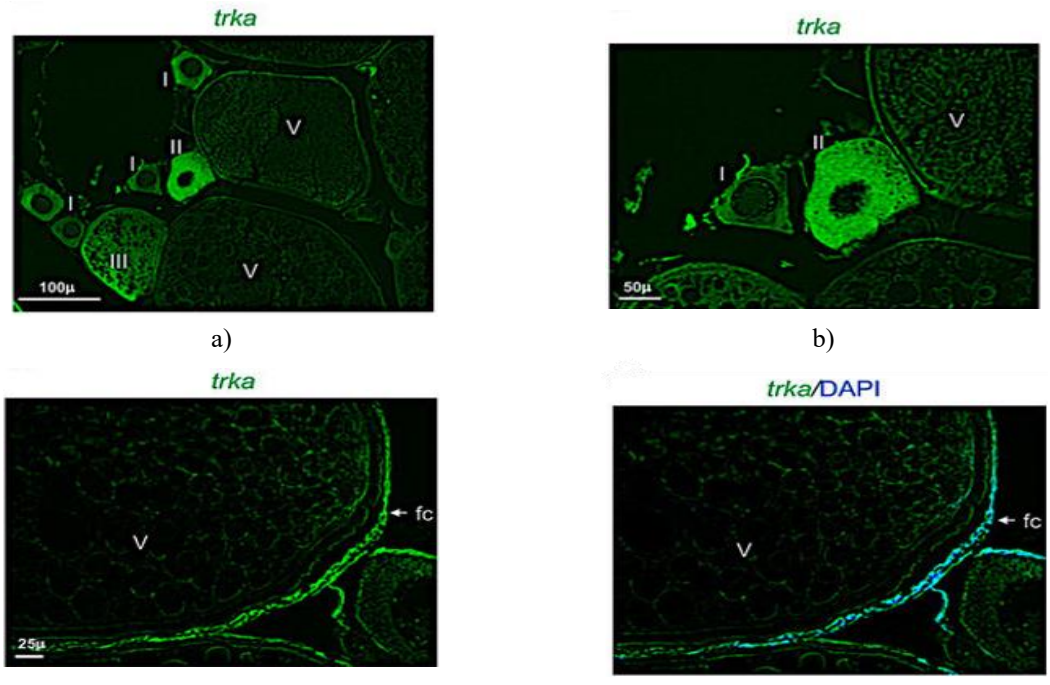


Figure 8. *Trka* transcript localization in adult zebrafish ovary. (a) Fluorescent ISH showing *trka* labeling in oocytes at stages II, III, and in follicular cells at stage V. (b) *Trka* presence in oocytes at stages I, II, and V.

(c) Enlarged follicular cell view with *trka* labeling at stage V. (d) Co-localization of *trka* and nuclear (DAPI) signals in stage V follicular cells. Scale bars: 100 µ (a); 50 µ (b); 25 µ (c,d).

This paper offers, for the first time, a detailed account of how *ngf* and its receptor, *trka*, are distributed within the reproductive organs of adult zebrafish. The analysis demonstrated that *ngf* and *trka* occur specifically in spermatogonia types A and B as well as in diplotene spermatocytes, suggesting their involvement in the regulation of spermatogenesis in mature zebrafish. Earlier experiments in rodents, including rats and mice, indicated NGF presence within primary spermatocytes, pachytene, and elongated spermatids, whereas TrkA localization was largely confined to Leydig cell membranes and elongated spermatids [52–54]. Further evidence has shown that the NGF–TrkA axis enhances sperm motility and activates acrosomal reactions. Moreover, NGF contributes to testosterone synthesis and promotes both proliferation and maturation of Leydig cells [55, 56]. Comparable patterns have been documented in humans, monkeys, and rabbits. By contrast, in adult alpacas, NGF protein was found mainly surrounding the nucleus of stromal, Sertoli, and germ cells [57].

Given the localization of NGF and TrkA within both germ and endocrine cell types, it has been proposed that NGF may function through local autocrine or paracrine mechanisms during testicular differentiation and sperm formation. Reports have indicated that Leydig cells produce NGF, which then binds to TrkA receptors in germ cells—including spermatocytes and spermatids—to regulate various spermatogenic phases in rodents such as rats and ground squirrels. Notably, men suffering from azoospermia display significantly diminished NGF concentrations [58–60]. Experimental suppression of NGF/TrkA signaling in rabbits and mice resulted in reduced sperm motility [61]. More recently, external administration of NGF has been shown to reinstate spermatogenic activity in mouse models with advanced testicular atrophy and germ cell loss.

Regarding the ovarian tissue of adult zebrafish, *ngf* and *trka* expression was detected across several oocyte developmental stages, notably in oocytes at stages II–III, and within follicular cells at stages IV–V. Investigations across different species revealed that NGF and TrkA are critical in the early formation of ovarian follicles, influencing the shift from primordial to secondary stages. The NGF/TrkA cascade drives proliferation of granulosa and theca cells while enhancing the expression of follicle-stimulating hormone (FSH) receptors in granulosa cells. During the ovulatory phase, this signaling system regulates prostaglandin E2 (PGE2) production, restricts gap-junction formation, and increases theca cell growth [62–64]. Elevated NGF levels have been measured in goats and wild ground squirrels. In humans [65], NGF has been identified within oocytes and granulosa cells of preantral follicles ranging from primordial to secondary stages [66]. Additional studies have shown that fetal oocytes exhibit strong cytoplasmic staining for NGF, whereas adult oocytes demonstrate only partial nuclear and cytoplasmic labeling. In vitro work in mice further revealed that NGF exposure can suppress apoptosis by downregulating pro-apoptotic genes. The dynamic interplay between NGF and TrkA has also been associated with the luteotropic influence of ovulation-inducing factors in bovine ovaries [24], a finding likewise observed in cows and pigs [67]. Interestingly, the same pattern has been reported in non-mammalian vertebrates such as *Xenopus*, in which NGF and TrkA are expressed during early oocyte maturation [34]. Functional studies confirmed that the addition of NGF stimulates meiotic progression in *Xenopus* oocytes that express TrkA.

Conclusion

This research offers the first comprehensive depiction of *ngf* and *trka* gene expression within the reproductive system of an adult teleost, the zebrafish. The observations show that both genes are mainly active in spermatogonia and spermatocytes, aligning with findings reported for various mammalian models. Additionally, strong expression of *ngf* and *trka* was observed in early oocyte stages and in follicular cells at stage V. Overall, the evidence suggests that NGF and its receptor are crucial components of reproductive regulation and that their roles are evolutionarily conserved among vertebrate taxa.

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