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Morpho-functional features of the gonads of *Danio rerio*: the role of brain derived neurotrophic factor

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Short running page heading: BDNF in the gonads of zebrafish

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ABSTRACT

Zebrafish, a suitable and widely used teleost fish model in basic biomedical research, displays morphophysiological features of adult gonads that share some commonalities with those of mammalian species. In mammals, gametogenesis is regulated, among several factors, by brain derived neurotrophic factor (BDNF). This neurotrophin has a well-established role in the developing and adult nervous system, as well as gonads development and functions in vertebrates pecies. We hypothesize that BDNF has a role also in the gonadal functions of zebrafish. At this purpose, we investigated BDNF and its receptors p75 and TrkB in the ovary and testis of adult zebrafish, kept under laboratory conditions. Our results display: 1) the expression of BDNF mRNA and pro-BDNF protein outside of the nervous system, specifically in the ovary and testis; 2) the presence of pro-BDNF in primary oocytes and follicular layer, and p75 in follicular cells; 3) the localization of pro-BDNF in type B spermatogonia, and Sertoli cells in testis.

Altogether, these data lead us to consider that BDNF is involved in the gonadal function of adult zebrafish, and mainly in the adult ovary.

Keywords: zebrafish, gonads**,** BDNF, p75, TrkB

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INTRODUCTION

The ovary of adult zebrafish is asynchronous, containing follicles of all stages of development, and the eggs are spawned throughout the year, under laboratory conditions. From the perspective of comparative anatomy, the ovarian follicle corresponds to the mammalian primary follicle and the maturation occurs within the homologue of the mammalian primary follicle. The testis of adult zebrafish is composed of two main compartments, the intertubular (or interstitial) and the tubular compartment. The intertubular compartment contains steroidogenic Leydig cells, blood/lymphatic vessels, macrophages and mast cells, neural and connective tissue cells, the latter being continuous with the tunica albuginea (Koulish et al., 2002). The tubular compartment is delineated by a basement membrane and peritubular myoid cells housing somatic Sertoli cells and germ cells, which are found at different stages of development (Schulz et al., 2010). These morphophysiological features prompted us to investigate if this teleost species could be a powerful model for addressing key questions in the biology of reproduction, as well as in other basic research fields (D'Angelo et al., 2016).

Gametogenesis in vertebrates is regulated by a close interplay of cells, hormones and growth factors (Grier et al., 2016). Particularly in mammals, neurotrophins, which include nerve growth factor, brain derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5, are growth factors playing key roles either in folliculogenesis (Streiter et al., 2016) and spermatogenesis (Li and Zhou, 2013). Despite the numerous studies in mammals, in teleost fish only a previous investigation reported that brain derived neurotrophic factor (BDNF) is considered a potential molecular marker of male sperm quality in zebrafish (Guerra et al., 2013). The gene encoding BDNF, highly conserved during vertebrate evolution (Tettamanti et al., 2010; Aid et al., 2007; Heinrich and Pagtakhan, 2004), is composed of multiple alternative exons: 10 in

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human, 8 in mouse, and 6 in fish (Cohen-Cory, 1999). Multiple promoters can modulate the transcription of the BDNF gene (Aid et al., 2007; Rattiner et al., 2004; Tao et al., 2002). BDNF is initially synthesized as pro-peptide (referred to as pro-BDNF), and is then cleaved into a mature form (Tognoli et al., 2010). pro-BDNF binds and activates the p75 pro-apoptotic receptor. However, p75 may contribute to the binding of high-affinity receptor, TrkB (Schecterson and Bothwell, 2010). Mature BDNF preferentially stimulates the TrkB receptor, activating pathways to promote cell survival and proliferation (Lu et al., 2014; Reichardt, 2006). In zebrafish, p75 has been identified as co-receptors associated to Nogo complex, whereas TrkB exists in two isoforms, TrkB1 and TrkB2 (Martin et al., 1995). Both p75 and TrkB are primarily expressed in neuronal cells (Han et al., 2014; Heinrich and Lum, 2000).

BDNF and its receptors, besides the essential role for the differentiation and survival of various neuronal populations in the central (CNS) and peripheral (PNS) nervous systems of vertebrates, are also important in the development and functioning of non-neuronal cells, as suggested by their detection in different organs (Tessarollo, 1998). Previous reports have shown that BDNF is expressed in the ovary of several mammalian species, including humans (Yi et al., 2008; Lee et al., 2007; Harel et al., 2006; Seifer et al., 2006; Kawamura et al., 2005; De Sousa et al., 2004; Paredes et al., 2004; Seifer et al., 2002), and birds (Jensen and Johnson, 2001). In mammalian male reproductive organs, BDNF is essential for testicular development (Tessarollo, 1998), spermatogenesis (Müller et al., 2006; Park et al., 2001; Mutter et al., 1999) and mitochondrial activity in sperm (Li et al., 2012; Moon et al., 2005).

In teleost fishes, BDNF and its specific receptor TrkB have been largely documented in CNS and PNS during development as well as adulthood (Cacialli et al., 2016; Gatta et al., 2016; Montalbano et al., 2016; D'Angelo et al., 2014; De Felice et al., 2014; Gatta et al., 2014;

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Sánchez-Ramos et al., 2013; D'Angelo et al., 2012; Germanà et al., 2010; Dalton et al., 2009; Vissio et al., 2008). p75 is primarily expressed in neuronal cells during zebrafish development (Han et al., 2014).

The present study was undertaken to explore the possible involvement of BDNF in fish gonadal functions, by analyzing the expression of mRNAs levels and protein distribution both in female and male adult zebrafish, kept under standard laboratory conditions. In addition, to better explore the physiological role of BDNF, we also investigated the distribution of the two receptors p75 and TrkB.

MATERIAL AND METHODS

Animal and tissue processing

Adult females and males (6 months) of *Danio rerio* (zebrafish) were obtained from a local furnisher. The experimental protocols were conducted according to the Italian Decree 26/2014, and were approved by the Institutional committee of the University of Naples Federico II. Adult zebrafish were anaesthetized by 0.1% ethyl 3-aminobenzoate, methanesulfonate (Sigma Chemicals Co., St. Louis, MO, USA), and gonads were excised.

RNA extraction

Ovaries and testis were collected from mature males (10 animals) and females (10 animals). Gonads were pooled by sex before mRNA extraction protocol. For eyes, the extraction was carried out on 10 eyes. Tissues samples were collected into Eppendorf tubes and sonicated

(10s, 3 times) in 1 ml of Trizol Reagent (Gibco, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer's protocol. The concentration and purity of the total RNA samples were evaluated using NanoDrop 1000 (Thermo Scientific) before reverse transcription. Two extractions such as this one were carried out.

Reverse Transcription and Quantitative real-time PCR

Reverse transcription was carried out by incubating 0,5 µg total RNA with 1 µg of random primer oligonucleotides, 2.5 mM dNTPs and 50 U MMLV-RT (Promega) in the appropriate buffer for 10 min at 65 °C and 1hour (h) 30 min at 37 °C. Polymerase chain reaction (PCR) experiments were performed in a Cycler thermocycler coupled to the MyiQ detector (Bio-Rad. Hercules, CA, USA) using Q SYBR-Green Supermix (Bio-Rad) according to the manufacturer's protocol. Quantitative PCR was performed according to the following temperature setting: stage 1: 95°C for 4 minutes, stage 2 (40 cycles): 95°C for 30 sec and 58°C for 30 sec. The Melting curve was performed to confirm the specificity of the primers and the correct amplification. The following primers were used: BDNF (GeneBank accession: NM_001308649.1) forward 5'-ATAGTAACGAACAGGATGG-3'; BDNF reverse 5'-GCTCAGTCATGGGAGTCC-3'; GAPDHs (GeneBank accession: NM_213094.2) (Fw) 5'-CGCTGGCATCTCCCTCAA-3', GAPDHs (Rev) 5'-TCAGCAACACGATGGCTGTAG-3'. The qPCR was run in triplicates for each condition. Expression levels of Gapdhs mRNA were used to normalize the expression levels of BDNF. To quantify PCR results, the threshold cycle (Ct) was determined for each reaction. Ct values for BDNF were normalized with the Gapdhs gene, using the ΔΔ Ct method. The expression of BDNF in ovaries and testis was expressed as a fold induction or inhibition compared to normalized values obtained for the eyes.

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Western Blot

For the analysis of BDNF protein, 10 ovaries and 10 testes of zebrafish, were homogenized using a Tissue Lyzer omogenization (Qiagen) in 200 µl of ice-cold lysis buffer (50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.25% deoxicolic acid, 1% Triton X 100) added with 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 (60 µg) were boiled and loaded on bis/acrylamide gel 15% and electrophoresed. The proteins were blotted from the gel onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS, pH 7.5) containing 0.1% Tween 20 (TBS-T) at room temperature (RT), washed with TBS-T, and incubated with antibody (BDNF N-20, sc-546; Santa Cruz Biotechnology), diluted 1:500 in TBS-T. After appropriate washing steps in TBS-T, anti-rabbit peroxidase-conjugated secondary antibodies (Amersham, Gel Health Care) were applied 1 h at RT, at 1:2000 dilution. Finally, the reaction was developed using a chemiluminescent reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to ChemiDoc gel scanner (Bio-Rad Laboratories). As control, a goat-anti β-actin antibody (42 kDa) has been used.

Immunohistochemistry

Gonads were treated for classical morphological investigation and immunohistochemistry following general instruction described by de Girolamo and Lucini (2011).

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In details, the ovaries and testes and/or the entire trunk were collected through a dissection of the abdomen, and were fixed for one day in Bouin's fluid. Subsequently, dehydrated in ethanol, included in paraffin and cut in longitudinal and transverse 7μm sections. Serial sections were stained with Haematoxylin-eosin for morphological observations. Adjacent sections were stained with the following primary antibodies: purified rabbit polyclonal antibody against a peptide mapping within an internal region of BDNF of human origin (BDNF N-20, sc-546; Santa Cruz Biotechnology; 1:500); polyclonal antibody directed against BDNF corresponding to amino acid residues 166-178 of human BDNF (precursor) (Alomone Labs; #ANT-010; 1:200); polyclonal antibody directed against an extracellular epitope of human p75NTR (Alomone Labs; #ANT-007; 1:1200); polyclonal antibody directed against an epitope located at the extracellular domain of rat TrkB receptor (Alomone Labs; #ANT-019; 1:1200).

Aminoacid sequences of antibodies were previously aligned to check the degree of conservation with zebrafish sequences. Following deparaffinization and rehydration, they were incubated with hydrogen peroxide 0.3% for 30 min, followed by 3 washing in PBS; then again pre-incubated in normal goat serum diluted in PBS (1:5) for 30 min, and overnight at 4 C° in a solution containing the pre-incubated solution and the primary antibodies.

DAKO EnVision-System HRP, based on an HRP labeled polymer conjugated to secondary antibodies, was used. The staining was obtained by a 10 min incubation with 3,3'-diaminobenzidine (DAB), a substrate-chromogen which resulted in a brown-colored precipitate at the antigen site.

The specificity of immunohistochemical reactions was checked as follows: 1) substitution of the primary antibody by PBS; 2) incubation of the primary antibody with correlated antigen, the peptide sc-546P Santa Cruz Biotechnology (100 mg/ml in the final dilution).

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RESULTS

Quantitative PCR

To provide quantitative clues about the cumulative expression of *bdnf* in adult ovary and testis of zebrafish, we examined the expression also in the eye, because retina is considered a source of *bdnf* (Sanchez-Ramos et al., 2013). Comparisons between groups were performed using a statistical Student's *t*-test. Error bars correspond to the standard error of the mean (SEM). $P < 0.05$ is considered statistically significant; $*P \leq 0.05$, $**P \leq 0.01$, when compared to eye. *bdnf* was detected in both gonads, with levels significantly lower than in the eye (Fig. 1a). However, we did not detect any significant expression between ovary and testis.

Western blotting

The antibody employed recognized a band of 28 and 37 kDa in ovary homogenates, and only one of 28 kDa in testis (Fig. 1b). β-actin antibody, as control, revealed a band of 42 kDa.

Morphological analysis of ovary and testis

Based on the morphological features described by Lubzens and colleagues (2010) and Leal et al. (2009), the identified cell stages were: for the ovary a) oocytes I, identifiable by a central large nucleus or germinal vesicle, with few peripherally located nucleoli; b) oocytes II, characterized by an increase of nucleus size, related number of nucleoli, cytoplasm enlargement due to the appearance of cortical alveoli marks (yolk vesicles) and vitellin envelope; c) oocytes

III, IV, V with abundant ooplasm, cortical alveoli and drop oil at the periphery and germinal vesicle close to the central position. At these stages, the oocyte volume is increasing and, at periphery, the zona pellucida and follicular cells appear progressively enhanced.

For the testis, where spermatogenesis occurs in cysts that are formed when a single spermatogonium is completely surrounded by the cytoplasmic projections of one or two Sertoli cells, different cysts were detected containing: a) round shape spermatogonia A, with two or three small nucleoli; b) spermatogonia B, with increased amount of heterochromatin in elongated/round nucleus, one or two small nucleoli; c) spermatocytes, whose different phases of meiosis can be identified by nuclear size, chromosome condensation, and meiotic figures of the chromosomes; d) early, intermediate, and final spermatids, with progressive reduction in the cellular volume and nuclear compaction. The number of germ cells per cyst increased dramatically (1 to 1360 cells) from undifferentiated spermatogonia A to early spermatids.

Immunohistochemistry

Ovary

The two antibodies raised against BDNF revealed immunoreactivity in all the perinuclear cytoplasm of oocytes at the stage I (Fig. 2a,b) and at stage II (Fig. 2c). From the stage III to V, weakly immunostaining to BDNF was observed in the residual perinuclear cytoplasm, and more intense in the cells of the follicular layer, distributed all around the oocyte (Fig. 2a-c). p75 immunoreactivity was observed only in the cells of the follicular layer (Fig. 2d,e). Immunostaining against TrkB was not observed in the ovary. In trunk transverse sections, we detected immunopositivity against all the primary antibodies in the pronephric tubules.

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Testis

Immunoreactivity to BDNF was mainly observed in type B spermatogonia (Fig. 3a,b), in the apical cytoplasm of spermatids, and in Sertoli cells (Fig. 3c,d). Weak immunopositivity to BDNF was also detected in type A spermatogonia. Any immunostaining against p75 and TrkB receptors was detected in the testis of zebrafish. In trunk transverse sections, we detected immunopositivity against all the primary antibodies in the pronephric tubules.

DISCUSSION

In this study, for the first time, we report the presence of BDNF and its receptors in the female and male gonads of adult zebrafish, maintained under standard laboratory conditions. Specifically, our findings demonstrate that *bdnf* is expressed, although at low levels, in both gonads. Furthermore, at protein level, only pro-BDNF is expressed in ovary and testis of adult zebrafish, similarly to mammals (Wu et al., 2012; Müller et al., 2006). On the basis of these observations, we hypothesize that in zebrafish proBDNF constitutes the biological active form at gonadal level. It is likely that the processing of neurotrophin precursor can take place in some specific gonadic cells. Interestingly, the antibody we employed in this study, directed against both precursor and mature BDNF, and also used in the brain of zebrafish (Gatta et al., 2016) and *Cichlasoma dimerus* (Vissio et al., 2008) mainly detect the precursor form of the protein, whereas in *N. furzeri* (D'Angelo et al., 2014) and quail oviduct traits (Maruccio et al., 2016) recognizes both precursor and mature form. This result is of great interest from functional perspective: it is well established that $p75^{NTR}$ is more effectively activated by pro-neurotrophin (Hempstead, 2015), and acts as a bifunctional switch to regulate cell death or survival (Chao,

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2003). We therefore analyzed the localization of pro-BDNF and its receptors in the ovary and testis of adult zebrafish to get more insights on their functional interactions.

Recapitulating our results in the ovary, the analysis demonstrated an immunostaining pro-BDNF gradient: the signal is stronger in the perinuclear cytoplasm of oocytes at the stage I, and becomes weaker concomitantly to the oocyte growth. In addition, from stage III, immunopositivity is detected mainly in the residual perinuclear cytoplasm and in the follicular cells, distributed all around the oocyte. These observations reinforce our hypothesis that BDNF, as growth factor, is implicated in the regulation of primary oocyte growth and maturation. Also in mammalian species, several studies documented that BDNF is a physiological regulator of normal follicle maturation (Seifer et al., 2002; Pan and Auersperg, 1998) and may function in both early and later stages of folliculogenesis (Linher-Melville and Li, 2013), although with specific differences in the localization within the follicle. Indeed, in the ovary of humans, BDNF was detected in the follicular fluid (Sadeu et al., 2012; Seifer et al., 2006; Seifer et al., 2002), in the follicular cells, such as granulosa cells (Zhao et al., 2011) and cumulus cells, and in the oocyte (Anderson et al., 2002). Furthermore, BDNF was reported in the granulosa cells of primordial and primary follicles of mouse (Paredes et al., 2004), and in the cumulus and granulosa cells of mature follicles in mouse (Kawamura et al., 2005), cow (Martins da Silva et al., 2005) and pig (Lee et al., 2007). In addition, BDNF promotes *in vitro* maturation of mouse oocytes by affecting key morphological characteristics, namely by improving the configuration of meiotic spindles and the localization and distribution of cortical granules (Zhao et al., 2011). Neurotrophins are known to promote the growth of follicular cells, as well as to induce Follicule Stimulating Hormone Receptor synthesis (Salas et al., 2006; Romero et al., 2002). The occurrence of pro-BDNF in the ovary could contribute also in zebrafish to the oocyte maturation

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and proliferative activity of follicular cells. Of the two receptors investigated, only p75 was detected in cells of the follicular layer, from stage III. Remarkably, in human ovaries (older fetuses and adults), p75 receptor has never been reported (Abir et al., 2005). We argue that, in adult ovary of zebrafish, pro-BDNF can contribute via $p75^{NTR}$ binding to regulate folliculogenesis. Our experiments, conducted on serial sections, revealed that pro-BDNF and p75 labeled cells in the same population, thus we hypothesize an autocrine mode of action of pro-BDNF and p75 in follicular cells.

In adult testis of zebrafish, we observed pro-BDNF immunoreactivity in the cytoplasm of type B spermatogonia. We suspect that pro-BDNF in type B spermatogonia is implicated in the cell proliferation and differentiation of germ cell to proceed to next phase. Furthermore, we observe staining in Sertoli cells around the spermatogenic cysts. In zebrafish, the total number of Sertoli cells per cyst increased gradually and significantly from type A undifferentiated spermatogonium to pachytene spermatocyte cysts (Leal et al., 2009). In addition, a single Sertoli cell is in contact with only one germ cell (Leal et al., 2009), and retains capacity to proliferate also in adult fish, during cysts formation (Schulz et al., 2010). The positivity in the Sertoli cells suggests that these cells can also represent a preferential target for BDNF in the testis of zebrafish. Accumulating evidences in mammals reported BDNF expression and immunolocalization in Leydig and Sertoli cells, during development and adulthood (Müller et al., 2006; Park et al., 2001; Koeva et al., 1999). In our survey, we do not observe immunoreactive Leydig cells. In human, the presence and cellular localization of BDNF in Leydig and Sertoli cells of both fetal and adult testes, suggested functional implications, namely in morphogenesis and spermatogenesis (Müller et al., 2006). The differences with mammalian

pattern of BDNF localisation may be related to the morphological organization of of zebrafish spermatic cysts.

Notably, we did not detect any immunostaining against p75 and TrkB receptors. Accumulating evidences displayed that pro-BDNF is the selective ligand for p75 receptor, triggering p75-dependent apoptosis (Chao, 2003). However, in the light of our results, we speculate that pro-BDNF activates also non canonical mechanisms to regulate spermatogenesis of adult zebrafish.

In conclusion, our study in adult zebrafish demonstrates: 1) the expression of BDNF mRNA and pro-BDNF protein outside of the nervous system, specifically in the ovary and testis; 2) the presence of pro-BDNF in primary oocytes and follicular layer, and p75 in follicular cells; 3) the localization of pro-BDNF in type B spermatogonia, and Sertoli cells in testis. These observations lead us to consider that this neurotrophin is involved in the reproductive system of adult zebrafish. Particularly in the adult ovary, it shares some similarities in the pattern of distribution and physiological action with mammals, including humans.

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FIGURE LEGENDS

Fig. 1. BDNF expression in the gonads of adult zebrafish.

(a) Levels of BDNF mRNAs in ovary and testis are significantly lower than in the eye of adult zebrafish. Expression levels are normalized to Gapdhs. *$P \leq 0.05$, **$P \leq 0.01$. Any significant difference is detected between ovary and testis.

(b) BDNF antiserum recognized a band of 28 kD in ovary and testis, and a weaker band of 37 kDA only in the ovary, both corresponding to proBDNF.

Fig. 2. Immunolocalization of pro-BDNF and its receptor p75 in the ovary of adult zebrafish.

Transverse sections of ovary. (a, b, c) Immunoreactivity to pro-BDNF in cytoplasm of germ cells (I and II), and in follicular cells (III and IV); (d, e) immunoreactivity to p75 in cytoplasm of follicular cells. Arrows indicate follicular cells.

Fig. 3. Immunolocalization of pro-BDNF in the testis of adult zebrafish.

Transverse sections of testis. (a,b) Immunoreactivity to pro-BDNF (a,b,c,d) in cysts of spermatogonia B. Positive cysts are indicated by arrows in **a**, and are circled in **b**; (c,d) in Sertoli cells. Arrows indicate Sertoli cells.

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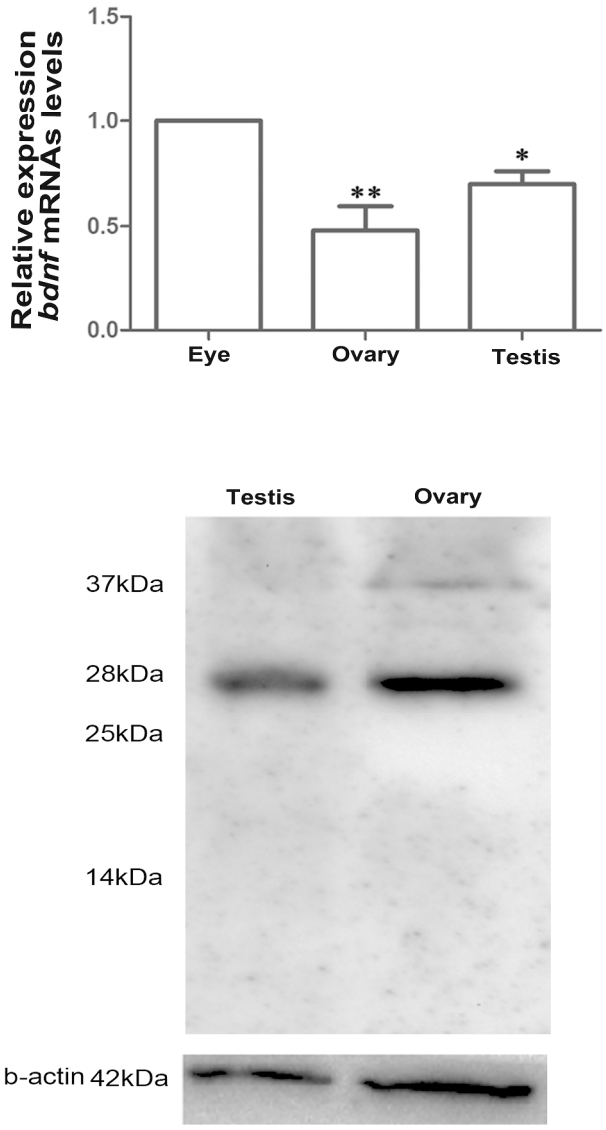


Figure 1. BDNF expression in the gonads of adult zebrafish.
(a) Levels of bdnf mRNAs in ovary and testis are significantly lower than in the eye of adult zebrafish. Expression levels are normalized to Gapdhs. *P ≤ 0.05, **P ≤ 0.01. Any significant difference is detected between ovary and testis.
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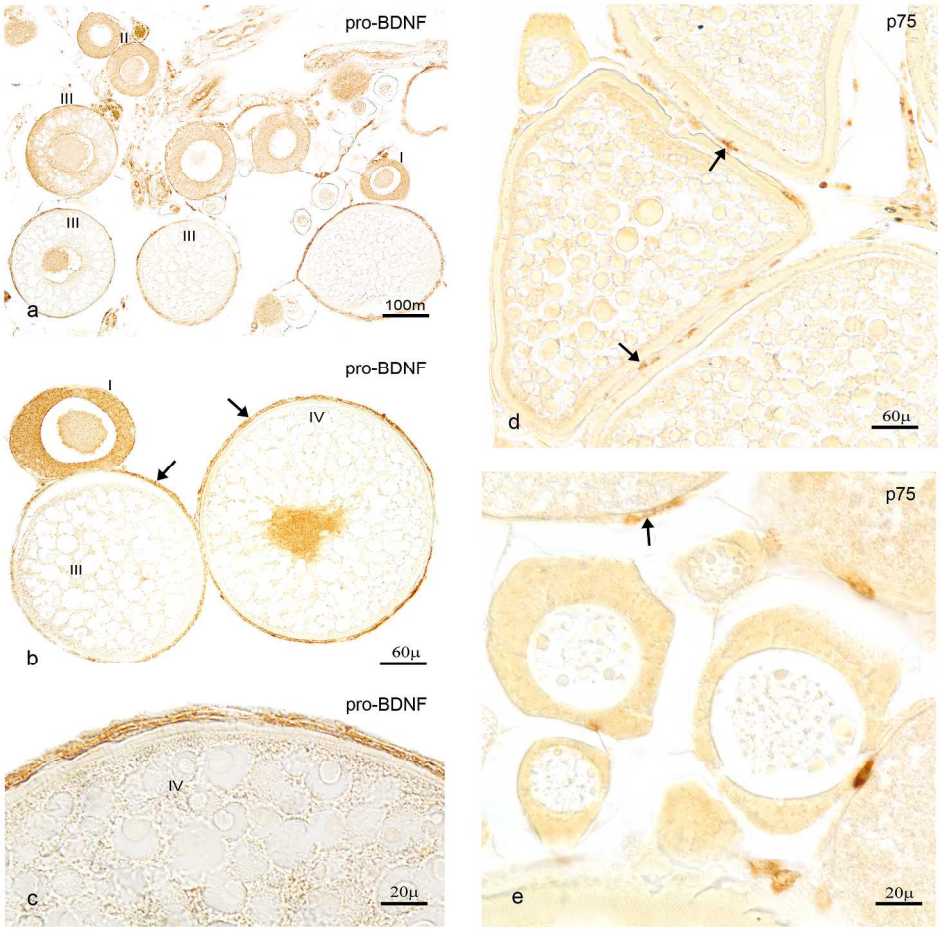


Figure 2. Immunolocalization of pro-BDNF and its receptor p75 in the ovary of adult zebrafish. Transverse sections of ovary. (a, b, c) Immunoreactivity to pro-BDNF in cytoplasm of germ cells (I and II), and in follicular cells (III and IV); (d, e) immunoreactivity to p75 in cytoplasm of follicular cells. Arrows indicate follicular cells.

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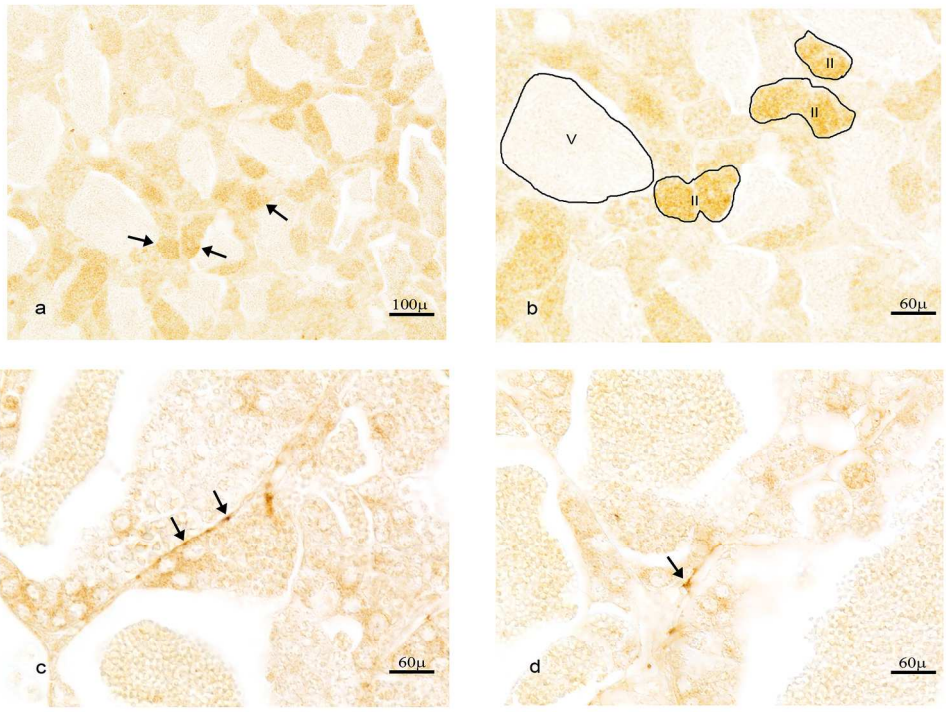


Figure 3. Immunolocalization of pro-BDNF in the testis of adult zebrafish. Transverse sections of testis. (a,b) Immunoreactivity to pro-BDNF (a,b,c,d) in cysts of spermatogonia B. Positive cysts are indicated by arrows in a, and are circled in b; (c,d) in Sertoli cells. Arrows indicate Sertoli cells.

211x160mm (300 x 300 DPI)

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