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Characterization of Gnrh/Gnih elements in the olfacto-retinal system and ovary during zebrafish ovarian maturation

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ABSTRACT

Gonadotropin releasing hormone (GnRH) is one of the key players of brain-pituitary-gonad axis, exerting overall control over vertebrate reproduction. In zebrafish, two variants were characterized and named as Gnrh2 and Gnrh3. In this species, Gnrh3, the hypophysiotropic form, is expressed by neurons of the olfactory-retinal system, where it is related with food detection, intra/interspecific recognition, visual acuity and retinal processing modulation. Previous studies have reported the presence of Gnrh receptors in the zebrafish retina, but not yet in the zebrafish olfactory epithelium. The current study analyzed the presence of *gnrh2* and *gnrh3*, their receptors (*gnrhr 1,2,3* and *4*) and *gnih* (gonadotropin inhibitory hormone) transcripts, as well as the Gnrh3 protein in the olfactory epithelium (OE), olfactory bulb (OB), retina and ovary during zebrafish ovarian maturation. We found an increase of *gnrh* receptors transcripts in the OE at the final stages of ovarian maturation. In the OE, Gnrh3 protein was detected in the olfactory receptor neurons cilia and in the olfactory nerve fibers. Interestingly, in the OB, we found an inverse expression pattern between *gnih* and *gnrh3*. In the retina, *gnrhr4* mRNA was found in the nuclei of amacrine, bipolar, and ganglion cells next to Gnrh3 positive fibers. In the ovary, *gnrh3*, *gnrhr2* and *gnrhr4* transcripts were found in perinucleolar oocytes, while *gnih* in oocytes at the cortical alveolus stage. Our results suggested that Gnrh/Gnih elements are involved in the neuromodulation of the sensorial system particularly at the final stages of maturation, playing also a paracrine role in the ovary.

1. Introduction

In vertebrates, gonadal maturation is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Chen and Fernald, 2008; Sower et al., 2009). Gonadotropin-releasing hormone (GnRH) is a key factor in this axis; it is secreted from the hypothalamus and is responsible to stimulate the synthesis and release of Fsh (Follicle-stimulating hormone) and Lh (Luteinizing hormone) from the pituitary gland, which ultimately control gametogenesis and gonadal steroidogenesis (Chen and Fernald, 2008). Since its discovery in mammalian brains (Amoss et al., 1971; Baba et al., 1971), 18 different forms of GnRH variants have been identified in vertebrates (Roch et al., 2014). In general, GnRH is currently classified into three types according to their location and function: type 1 (GnRH1), type 2 (GnRH2) and type 3 (GnRH3) (White and Fernald, 1998; Guilgur et al., 2007). With respect to their function, GnRH1 is associated with gonadotropin secretion (Schwanzel-Fukuda and Pfaff, 1990); GnRH2 appears to be involved in the regulation of sexual and feeding behavior (Volkoff and Peter, 1999; Temple et al., 2003; Matsuda et al., 2008) and GnRH3 is associated with neural mechanisms of sexual behavior (Eisthen et al., 2000; Biju et al., 2003, 2005). In cyprinids, such as *Danio*

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rerio, two Gnrh forms were described in the brain; Gnrh2 in diencephalon and midbrain, and Gnrh3 in the ventral forebrain (Powell et al., 1996; Abraham et al., 2009).

Several studies have linked GnRH with visual acuity (Maaswinkel and Li, 2003) and olfactory epithelium sensitivity (Kawai et al., 2009) through terminal nerve (NT). The mechanisms by which GnRH modulates olfactory response are not yet defined, but some studies suggested that GnRH is involved on olfactory signal transduction (Kawai et al., 2009), modulating the excitability of the olfactory receptor neurons (ORNs) and increasing its response to odorants (Eisthen et al., 2000; Kawai et al., 2009). In this context, studies in salamanders have localized GnRH receptors in the olfactory epithelium (OE), indicating a possible role of GnRH on the olfactory system (Wirsig-Wiechmann and Jennes, 1993; Zhang and Delay, 2007). Also, in zebrafish, recent data showed visual acuity improvement under different olfactory stimuli (Maaswinkel and Li, 2003; Stephenson et al., 2012). Interestingly, Gnrh3 was detected in TN neurons and GnRH receptors in different layers of the retina of different species (Wirsig-Wiechmann and Wiechmann, 2002; Grens et al., 2005; Servili et al., 2012), reinforcing GnRH as a modulator of the sensorial stimuli. GnRH has also been reported in many extra-hypothalamic mammalian and teleostean tissues including the ovaries (Iwashita and Catt, 1985; Pati and Habibi, 1998; Okubo et al., 1999, 2006; Madigou et al., 2000). In the goldfish ovary, Gnrh is involved in the regulation of follicle development, steroidogenesis, apoptosis and in the maintenance of gonadal synchrony (Andreu-Vieyra and Habibi, 2000).

In the last years, other hypothalamic neuropeptide, gonadotropin inhibitory hormone (GnIH), was discovered. GnIH was originally identified in quails, and is responsible to regulate the synthesis and release of pituitary gonadotropins (Tsutsui et al., 2000). GnIH acts on GnRH neurons and on pituitary cells inhibiting the reproductive system (Tsutsui, 2009; Tsutsui et al., 2012; Parhar et al., 2012; Tsutsui and Ubuka, 2016). In fish, Gnih was found in the olfactory bulb of different species (Ogawa and Parhar, 2014; Biswas et al., 2015; Di Yorio et al., 2016; Paullada-Salmerón et al., 2016) and in the retina (Paullada-Salmerón et al., 2016), however, to the best of our knowledge no reports on Gnih physiological function in these areas have been published. Also, Gnih and its receptors have been described in the gonads of several vertebrates, such as fish, birds, reptiles and mammals (Bentley et al., 2008, 2010; Maddineni et al., 2008; Singh et al., 2008; Qi et al., 2013; Zhang et al., 2010). Also, although not fully explored, recent studies have demonstrated that Gnih inhibited mice follicular development and steroidogenesis (Singh et al., 2011).

In this framework, the aim of this study was to examine the expression and cellular localization of Gnrh (Gnrh2, 3), Gnrh receptors (Gnrh-r1, 2, 3 and 4) and Gnih in the OE, retina, olfactory bulb (OB) and ovary of *D. rerio*. We also evaluated whether Gnrh, Gnrhr and Gnih in the olfacto-retinal system are linked with reproduction by analyzing their expression levels during the different stages of zebrafish ovarian maturation.

2. Material and methods

2.1. Zebrafish stocks

Sexually mature (n = 15) and immature (n = 40) female and male (n = 10) zebrafish (*D. rerio*) between 4 and 12 months of age were used in the present study. Animal housing and experimentation were consistent with the Brazilian national regulations and UNESP Committee for Ethics and Animal Care and Use in Jaboticabal/Botucatu (São Paulo, Brazil) has approved the protocols (2554/15).

2.2. Tissue sampling and ovarian histological analysis

Before sampling, fish were anesthetized (1% benzocaine), and subsequently their organs/tissues (OE, retina, brain, OB and ovary) collected, snap frozen in liquid nitrogen and stored at − 80 °C until RNA isolation. To determine the gonadal maturity and the ovarian stage of each collected animal, part of the ovary was fixed in modified Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]) for at least 24 h. Further, samples were processed, embedded in methyl methacrylate (Technovit 7100-Heraeus Kulzer, Wehrheim, Germany) and 3 μm thickness sections obtained and stained with toluidine blue according to Leal et al. (2009). Ovaries were classified into four maturation stages: primary growth stage (PG), pre-vitellogenic stage (PV), mid-vitellogenic stage (MV) and late vitellogenic stage (LV) (Fig. 1), according to Wang and Ge (2004).

2.3. Expression of gnrh and gnih by RT-PCR and qPCR

To evaluate gene expression, samples were snap frozen in liquid nitrogen and stored at − 80 °C until RNA isolation. OE and OB were removed from 36 females, and grouped according to the gonadal stage (n = 9 per reproductive stage), and pooled (n = 3) for total RNA extraction using PureLink® RNA Mini Kit (Ambion®) following the manufacturer's protocol. For larger organs (brain, retina and gonads), RNA was extracted using Trizol™ (Invitrogen, USA). For both, DNase treatment using DNase I, RNase-free kit (Invitrogen, Carlsbad, CA, USA) was performed, and subsequently cDNA was synthesized using SuperScript® II Reverse Transcriptase kit (Invitrogen ™, Carlsbad, CA, USA) with random hexamers according to standard protocols (Nóbrega et al., 2010). RT-PCR and qPCR reactions were conducted using specific primers for zebrafish *gnrh2*, *gnrh3*, *gnrhr1*, *gnrhr2*, *gnrhr3*, *gnrhr4* and *gnih* (Table S1). Zebrafish

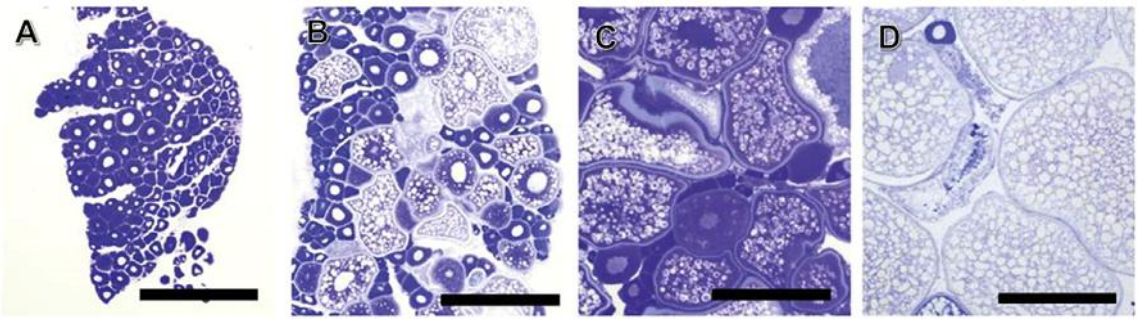

Fig. 1. Characterization of zebrafish ovarian stages. (A) Primary growth stage, PG. (B) Previtellogenic or cortical alveolus stage, PV. (C) Mid-vitellogenesis stage, MV. (D) Late vitellogenesis stage, LV. Scale bars: (A,D) 420 μm; (B) 630 μm; (C) 315 μm.

β-actin (NCBI: AF057040.1) (Table S1) was used as endogenous reference gene for RT-PCR reactions, while elongation factor 1α (*ef1α*) and *β-actin* were used as housekeeping genes for qPCR.

The RT-PCR products were separated on a 1% agarose gel and visualized over a UV transilluminator (Fig. 2). Cq (quantification cycle) values for *gnrh2*, *gnrh3*, *gnrhr1*, *gnrhr2*, *gnrhr3*, *gnrhr4* and *gnih* were determined in a StepOne system (Life Technologies) using SYBR Green (Invitrogen) and specific primers (Table S1). All qPCR reactions (20 μl) used 900 nM for each primer and 300 ng of total RNA. Each reaction was performed in duplicate. Relative gene expression levels were calculated according to the ΔΔCt method as described previously (Vischer et al., 2003). Expression levels for each gene were normalized with two endogenous reference genes (see above), and subsequently calibrated to the Ct average of all samples (ΔΔCt). For comparative analysis per stage, relative expressions were calibrated to the Ct average of all target genes from all samples (Figs. S1-4).

2.4. Localization of gnrh and gnih cellular expression sites by in situ hybridization

The expression sites of *gnrh* and *gnih* in zebrafish OE, retina and gonads were identified by *in situ* hybridization as described previously (Nóbrega et al., 2015). Digoxigenin-labeled sense and antisense cRNA were synthesized from specific PCR product generated with primers containing T3 or T7 RNA polymerase promoter sequences at 5'-ends (Table S1). The expected PCR product was extracted, purified with Zymoclean DNA recovery kit (Synapse D4001) and transcribed using DIG-UTP (digoxigenin) and RNA T7 polymerase (anti-sense) and T3 (sense) Roche kit (Roche 11758888001). The *situ* hybridization was performed with adaptations of Thisse and Thisse. (2008). Briefly, tissues were fixed overnight in 4% phosphate-buffered paraformaldehyde (pH 7.4) in RNase-free conditions, dehydrated, diaphanized, embedded in paraffin (Paraplast®, Sigma) and sectioned with 5 μm thickness. Subsequently, the slides were rehydrated and washed with PBT (Tris buffer phosphate, pH: 7.4) and TrisHCl buffer (0.05 M, pH: 7.5). The material was treated with proteinase K (20μg/ml) at 37 °C for 20 min and incubated with hybridization solution containing either sense or antisense RNA probe at 70 °C overnight. The slides were incubated with anti-DIG-AP primary antibody (anti-digoxigenin-alkaline phosphatase conjugated) diluted 1:2000 in the same blocking solution at 4 °C overnight. Tissues were washed and incubated with staining solution NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) (Thermo Scientific Pierce), and the slides were photographed using

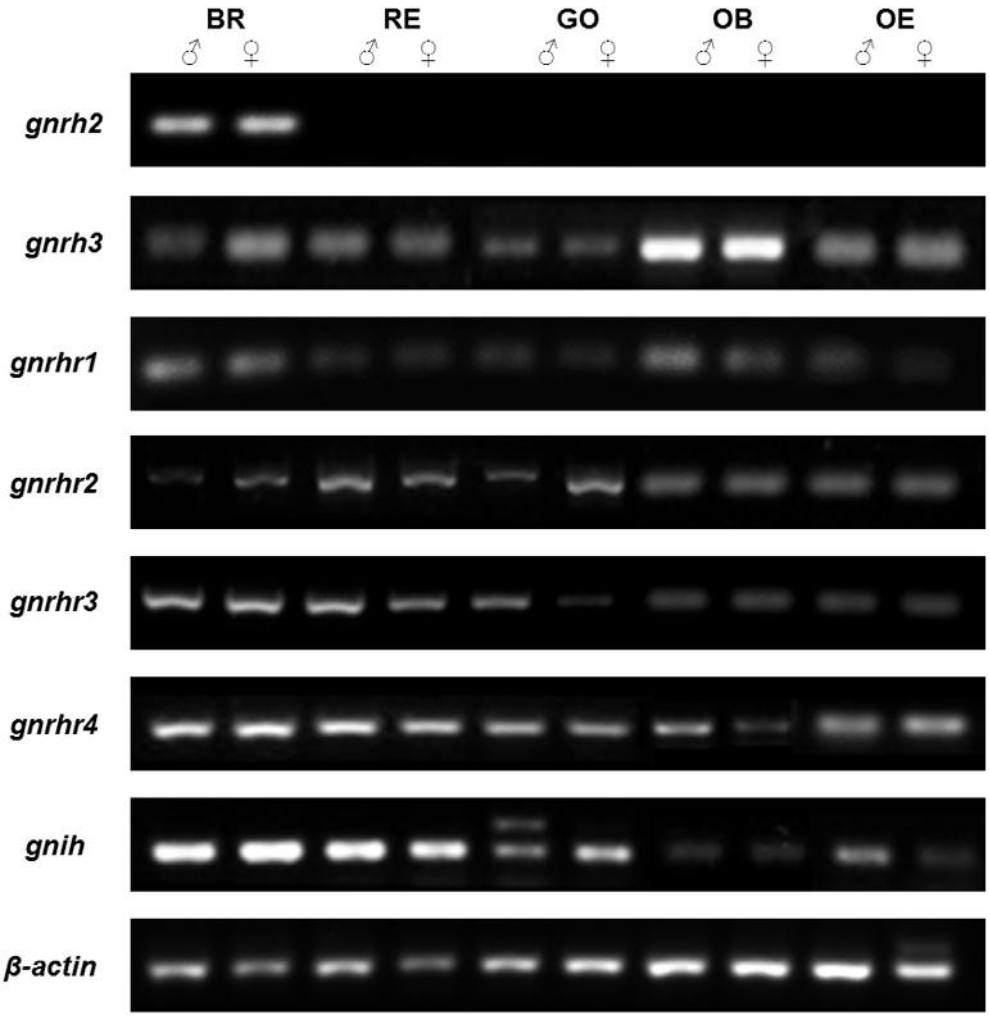

Fig. 2. Expression of *gnrh* (*gnrh2*, *gnrh3*), *ghih* and receptors (*gnrhr1*, *gnrhr2*, *gnrhr3* and *gnrhr4*) in different tissues (BR, brain; RE, retina, GO, gonad; OB, olfactory bulb and OE, olfactory epithelium) of zebrafish adult males and females. β-actin was used as reference endogenous gene.

the microscope Leica DMI 4000B.

2.5. Localization of GnRH3 cellular sites by immunohistochemistry

Immunohistochemistry reactions were performed to identify GnRH3 cellular sites in different organs of zebrafish (OE, retina and gonads), as described previously by Gomes et al. (2013). Tissues were fixed in Bouin solution for 24 h at 4 °C, dehydrated, diaphanized, embedded in paraffin (Paraplast®, Sigma), and sectioned with 5 μm thick. Sections were dewaxed, rehydrated and submitted to hydrogen peroxide 0.3% in PBS (phosphate buffered saline, pH: 7.4) for 5 min to block endogenous peroxidase. After washing with PBS, blocking of nonspecific sites was done with normal goat serum 5%. Subsequently, sections were incubated overnight at 4 °C with primary antibody anti-Gnrh3 (salmon) BB8 (1: 700), kindly provided by Dr Olivier Kah (University of Rennes, France). After the incubation period, sections were washed in PBS and incubated for 30 min in the polymer-HRP EasyLink One (EasyPath). Finally, slides were stained with DAB (3,3-diaminobenzidine, Sigma Chemical) and counterstained with Harris hematoxylin. As negative control, primary antibody was preabsorbed (1:5) with synthetic GnRH analog (1 μM) (CONCEPTAL®, Intervet) diluted in PBS. The primary antibody was omitted in some of the immunohistochemical reactions.

2.6. Statistical analyses

Values were presented as mean ± SEM. Differences between two groups were identified using paired or unpaired Student's t-test, and for three or more groups ANOVA followed by the Student–Newman–Keuls test. Graph Pad Prism 4.0 software (Graph Pad Software, Inc., San Diego, CA, USA, http://www.graphpad.com) was used for all statistical analyzes. Significance level (p) was fixed at 0.05.

3. Results

3.1. Cellular localization of Gnrh, Gnrh receptors and Gnih in the zebrafish olfactory-retinal system and ovary

In the zebrafish OE, immunoreactive-Gnrh3 (ir-Gnrh3) was found in the cytoplasm of the olfactory receptor neurons (Fig. 3A,C), in particular at the apical surface and in the dendritic processes of the neurons (Fig. 3E). Moreover, ir-Gnrh3 fibers were also detected in the connective tissue (lamina propria), located beneath the OE (Fig. 3A). Fascicles of nerve fibers either associated with blood vessels (Fig. 3F) or between fibers towards the OB (Fig. 3G) also exhibited ir-Gnrh3. Control with preabsorbed antibody confirmed the specificity of the immunohistochemistry reactions (Fig. 3B,D). No signals were detected for any of the *in situ* cRNA probes used (*gnrh2, gnrh3, gnrhr1,2,3,4* and *gnih*) (Data not shown).

In the zebrafish retina, ir-Gnrh3 was observed mainly in fibers distributed close to the outer nuclear layer (ONL), in the outer and inner plexiform layers (OPL, IPL) and running along the ganglion cell layer (GCL) (Fig. 4A). No labeling was detected in the preabsorbed control (Fig. 4B). No signals were detected for any of the following *in situ* cRNA probes *gnrh2, gnrh3, gnrhr1,2,3* and *gnih*, with the exception of *gnrhr4*. *gnrhr4* transcripts observed in the photoreceptor cells of the outer nuclear layer (ONL), in bipolar and amacrine cells of the inner nuclear layer (INL), and in the ganglion

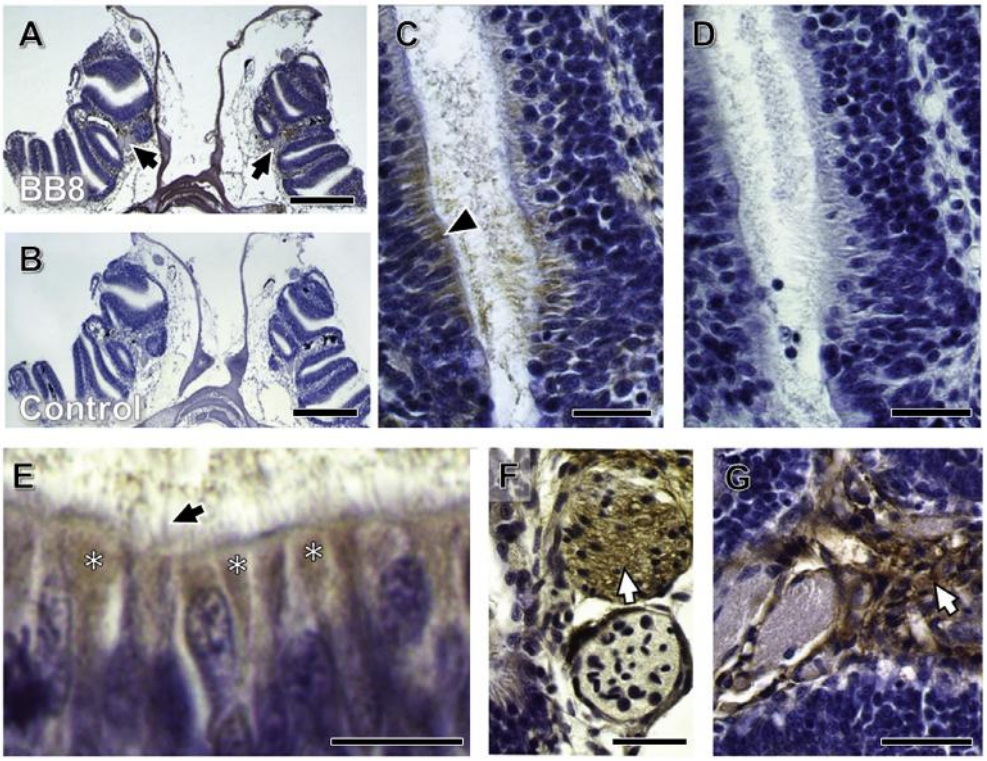

Fig. 3. Immunodetection of GnRH3 in the olfactory epithelium of zebrafish using BB8 antibody. (A) Cross section showing the presence of GnRH3 fibers in the lamina propria of the olfactory lamellae. Note fascicles of positive GnRH3 fibers that will form the olfactory nerve (arrows). (B) Preabsorbed control section showing no GnRH3 signal in the zebrafish olfactory epithelium. (C) Cross section showing GnRH3 immunostaining in the apical region of the sensorial epithelium (arrowhead). (D) Preabsorbed control section. (E) Detail of the olfactory receptor neuron (asterisks indicate the apical region of the olfactory receptor neuron) highlighting the cilia (black arrow). (F) and (G) detail of the positive fascicles of GnRH3 fibers (white arrows) located between the olfactory lamellae. Scale bars: (A-B) 250 μm, (C-D, F-G) 25 μm; (E) 10 μm.

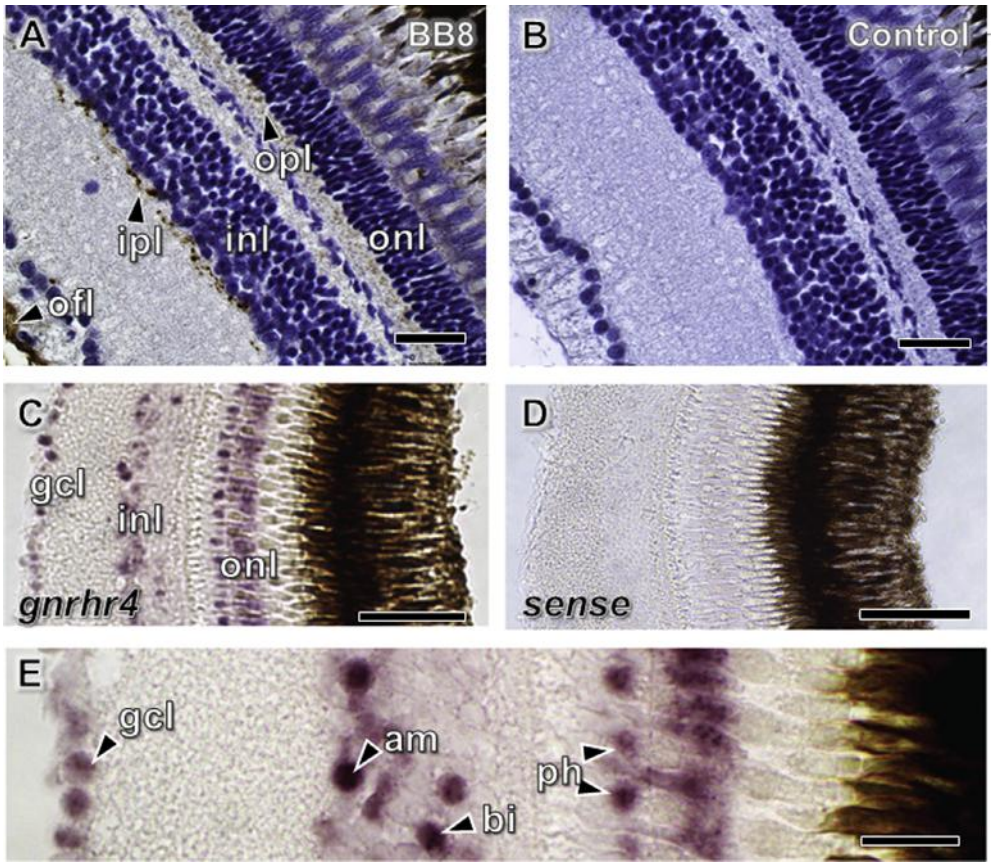

Fig. 4. Immunodetection of GnRH3 and distribution of *gnrhr4* receptor mRNA (*in situ* hybridization - ISH) in the zebrafish retina. (A) GnRH3 immunostaining using BB8 antibody; arrowheads show GnRH3 positive fibers in the inner plexiform layer (ipl), outer plexiform layer (opl) and optic nerve fiber layer (ofl). The inner nuclear layer (inl) is also indicated. (B) Preabsorbed control section. (C) Cross section showing *gnrhr4* mRNA in the outer nuclear layer (onl), inner nuclear layer (inl) and ganglion cell layer (gcl) when tissues were incubated with anti-sense RNA probes (T7). (D) ISH control; tissue was incubated with sense riboprobes (T3). (E) Detail of the *gnrhr4* transcripts expression sites in the nuclei of photoreceptors (ph), amacrine (am), bipolar (bi) and ganglion cell layer (glc). Scale bars: (A-C, F) 25 μm; (D-E) 50 μm.

cells of the ganglion cell layer (GCL) (Fig. 4C, E). No signal was detected when using sense *gnrhr4* cRNA probes (Fig. 4D).

In the zebrafish ovary, primary growth (PG) and previtellogenic (PV) (cortical alveolus stage) oocytes were the only ir-Gnrh3 structures (Fig. 5A and B). Interestingly, ir-Gnrh3 was stronger in the cytoplasm of PG oocytes (perinucleolar oocytes) when compared to PV oocytes (cortical alveolus stage) (Fig. 5A and B). *In situ* hybridization showed Gnrh receptor transcripts, *gnrhr2* and *gnrhr4*, expressed in the cytoplasm and nucleus of PG oocytes (Fig. 5H, I, L). As oocytes reached advanced vitellogenic stages, *in situ* signals for these receptors decreased, being often restricted to the nucleus and small regions of the cytoplasm (Fig. 5H). In addition, *gnrhr4* transcripts were also detected in follicular cells and *zona radiata* of the vitellogenic oocytes (Fig. 4K). With respect to *gnih*, expression sites for this transcript were found in the PV oocytes particularly in the cortical vesicles (cortical alveoli) (Fig. 5D–E). Interestingly, *gnih* was also expressed in the follicular cells as well as in the *zona radiata* of the vitellogenic oocytes (Fig. 5F).

3.2. Expression of gnrh, gnrh receptors and gnih in the olfactory-retinal system and ovary during adult zebrafish ovarian maturation

3.2.1. Olfactory epithelium (OE)

During the different reproductive stages [primary growth stage (PG), pre-vitellogenic stage (PV), mid-vitellogenic stage (MV) and late-vitellogenic stage (LV)], *gnrh2* levels in the OE were significantly higher in the MV stage (Fig. 6A), while *gnrh3* was found to decrease only in the PV stage (Fig. 6B). *gnih* levels remained constant with significantly lower levels in the PV stage (Fig. 6C). All Gnrh receptors (*gnrhr1*, *gnrhr2*, *gnrhr3* and *gnrhr4*) were highly expressed in the OE in later stages of ovarian maturation (MV and LV stages) when compared to earlier ones (PG and PV stages) (Fig. 6D–G). Comparative analysis showed that *gnih* was more abundant than *gnrh2* and *gnrh3* during all stages (Fig. S1), while *gnrhr3* is the most expressed receptor in the OE, with exception in the PV stage (Fig. S1).

3.2.2. Olfactory bulb (OB)

During ovarian maturation, *gnrh2* transcripts progressively increased from early (PV and MV) to late vitellogenesis (LV) (Fig. 7A). On the other hand, *gnrh3* showed an increase in the PV stage (early stage of vitellogenesis) (Fig. 7B). At the same stage, *gnih* was significantly suppressed (Fig. 7C). Analysis of Gnrh receptors showed constant and stable expression along the ovarian cycle, but *gnrhr1* and *gnrhr4* decreased in MV stage and *gnrhr2* and *gnrhr3* in the PV stage (Fig. 7D,G). Comparative analysis showed that *gnih* and *gnrh3* were the most abundant transcripts in the OB (Fig. S2). Interestingly, an inverse correlation is seen between *gnhi* and *gnrh3*; when *gnrh3* levels were high, *gnhi* mRNA was very low (Fig. S3). Among receptors, *gnrhr3* is the most expressed in the OB, followed by *gnrhr4* (Fig. S2).

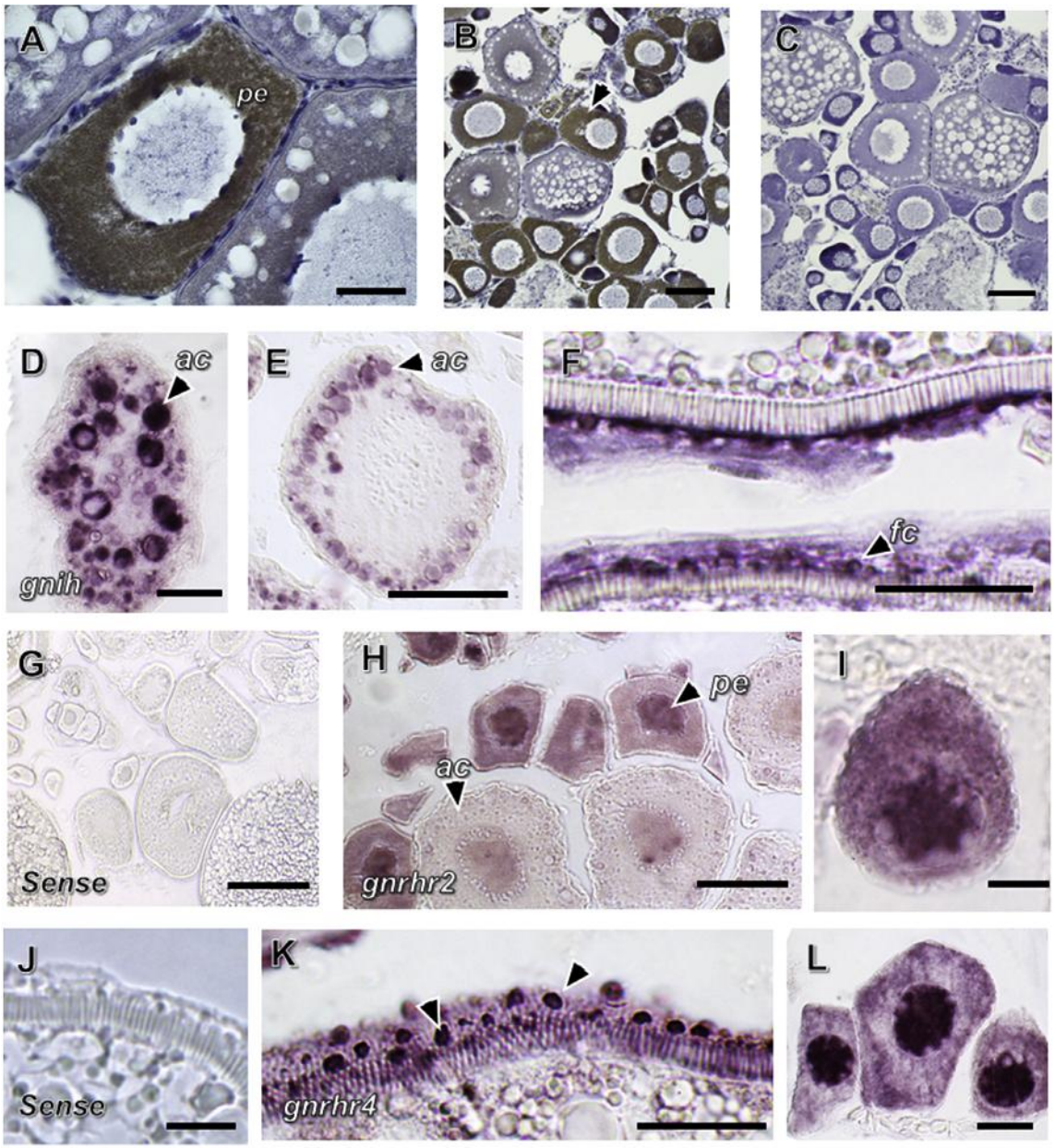

Fig. 5. Immunodetection of GnRH3 and distribution of *gnih, gnrhr2* and *gnrhhr4* transcripts (*in situ* hybridization - ISH) in the zebrafish ovary. (A) Detail of the GnRH3 immunostaining in perinucleolar oocytes (pe) using BB8 antibody. (B) Note an intense GnRH3 immunoreactivity in the perinucleolar oocytes (arrowhead) (pe). GnRH3 immunostaining decreases in cortical alveoli stage. (C) Preabsorbed control section showing no GnRH3 immunostaining in the zebrafish ovary. (D, E) *gnih* expression in cortical alveolus oocytes, mainly within the cortical alveoli (ac). (F) ISH highlighting *gnih* expression sites in the follicular cells (fc) of the granular layer of the vitellogenic oocytes. (G–J) Histological sections incubated with sense probe (T3), showing the specificity of the reaction for the riboprobes used. (H) *gnrhr2* mRNA in perinuclear (pe) and cortical alveoli oocytes (ac). (I) Detail of *gnrhr2* expression sites in the cytoplasm and nucleus of the perinucleolar oocyte. (J) Section incubated with *gnrhr4* sense riboprobe (T3) showing no staining in the follicular cells and radiata zone of vitellogenic oocytes. (K) *gnrhr4* expression in follicular cells (arrowheads) and radiata zone of vitellogenic oocytes. (L) Detail of *gnrhr4* mRNA in the nucleus and cytoplasm of perinucleolar oocytes. Scale bars: (A, D, F, K-L) 25 μm; (B-ck, H) 100 μm; (B-C, E, H) 100 μm; (G) 250 μm; (I) 15 μm.

3.2.3. Retina

gnrh2 and *gnrh3* remained constant in the retina during all ovarian stages (Fig. 8A and B). The same pattern was observed for *gnih*, although their levels were reduced in the LV stage compared to PG (Fig. 8C). No expression changes were detected for *gnrhr2* and *gnrhr3*, while *gnrhr1* and *gnrhr4* showed higher levels at PG stage (Fig. 8D–G). Comparatively, *gnrh3, gnrhr2, gnrhr3* and *gnrhr4* were the most expressed components in the retina during all stages of ovarian maturation (Fig. S3).

3.2.4. Ovary

No significant changes were detected in gene expression levels for ovarian *gnrh2* and *gnrh3* during the different stages of gonadal maturation (Fig. 9A and B). On the other hand, *gnih* was highly expressed in PG stage, but suppressed as ovaries started to mature (PV, MV, LV) (Fig. 9C). For Gnrh receptors, *gnrhr1* showed variable but not significant levels during ovarian maturation (Fig. 9D); *gnrhr2* and *gnrhr3* presented higher expression in PG followed by a significant decrease in later stages (PV, MV LV) (Fig. 9E and F); and

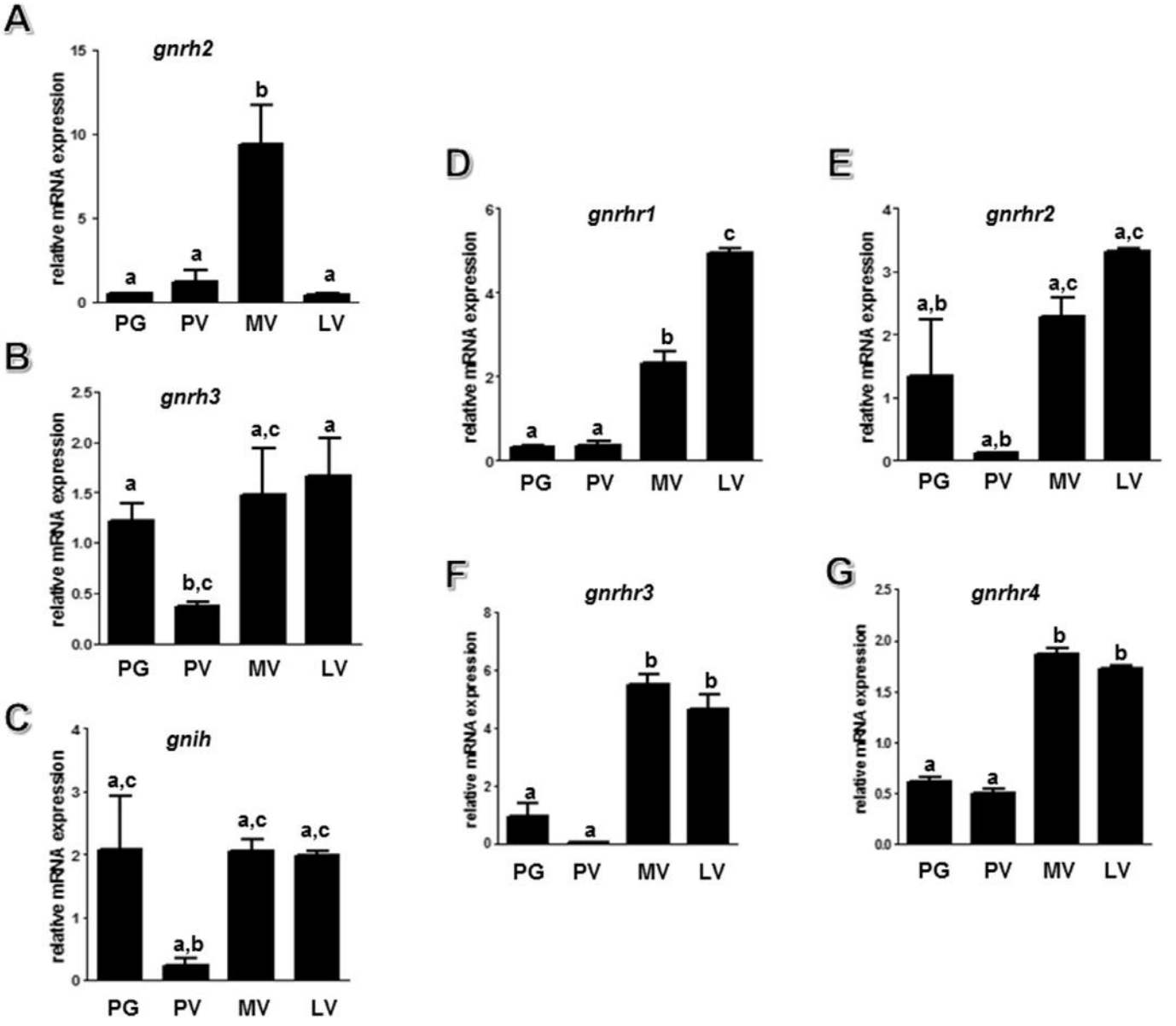

Fig. 6. Expression of *gnrh2* (A), *gnrh3* (B), *gnih* (C) and *gnrh* receptors *1* (D), *2* (E), *3* (F) and *4* (G) in the olfactory epithelium of zebrafish females along the four ovarian maturation stages (PG, primary growth; PV pre-vitellogenesis; MV mid-vitellogenesis and LV late-vitellogenesis) (A-. Different letters indicate significant differences ($p < 0.05$) between stages.

finally, *gnrhr4* which decreased gradually along ovarian maturation (Fig. 9G). *gnih* is more expressed in the ovaries when compared to the other ligands (*gnrh3* and *gnrh2*) (Fig. S4). For the receptors, expression analysis revealed higher levels of *gnrhr2* and *gnrhr3* during all maturational stages (Fig. S4).

4. Discussion

4.1. Olfactory epithelium (OE)

This work characterized Gnrh and Gnih elements in the zebrafish OE. In this organ, ir-Gnrh3 was found in the olfactory receptor neurons (ORN) mainly in the apical cytoplasm and cilia of these cells, and also in the fibers of the olfactory nerve, as already described in other teleost species (Subhedar and Krishna, 1988; Biju et al., 2003, 2005; Kawai et al., 2009). The presence of ir-Gnrh3 in these structures suggested a role of this neuropeptide in zebrafish olfactory signal transduction and transmission. This fact is supported by recent studies showing a potential role of Gnrh on olfactory signal transduction by modulating ORN excitability to specific odorant molecules (Eisthen et al., 2000; Kawai et al., 2009). In this context, we speculated whether Gnrh may integrate and modulate the olfactory-retinal system during zebrafish reproduction. For that, we evaluated OE expression levels of *gnrh*, *gnrh* receptors and *gnih* in different stages of ovarian maturation. Interestingly, both ligands (*gnrh3* and *gnih*) and receptors (*gnrhr1*, *gnrhr2*, *gnrhr3* and *gnrhr4*) showed higher mRNA levels at final stages of ovarian maturation (MV and LV). If we assume that all mRNAs are translated, our data suggested that zebrafish OE is more receptive/sensitive to Gnrh molecules at the final stages of gonadal maturation. In this context, similar studies in salamanders have shown increased Gnrh-responsiveness in ORN during the breeding season (Eisthen et al., 2000; Zhang and Delay, 2007).

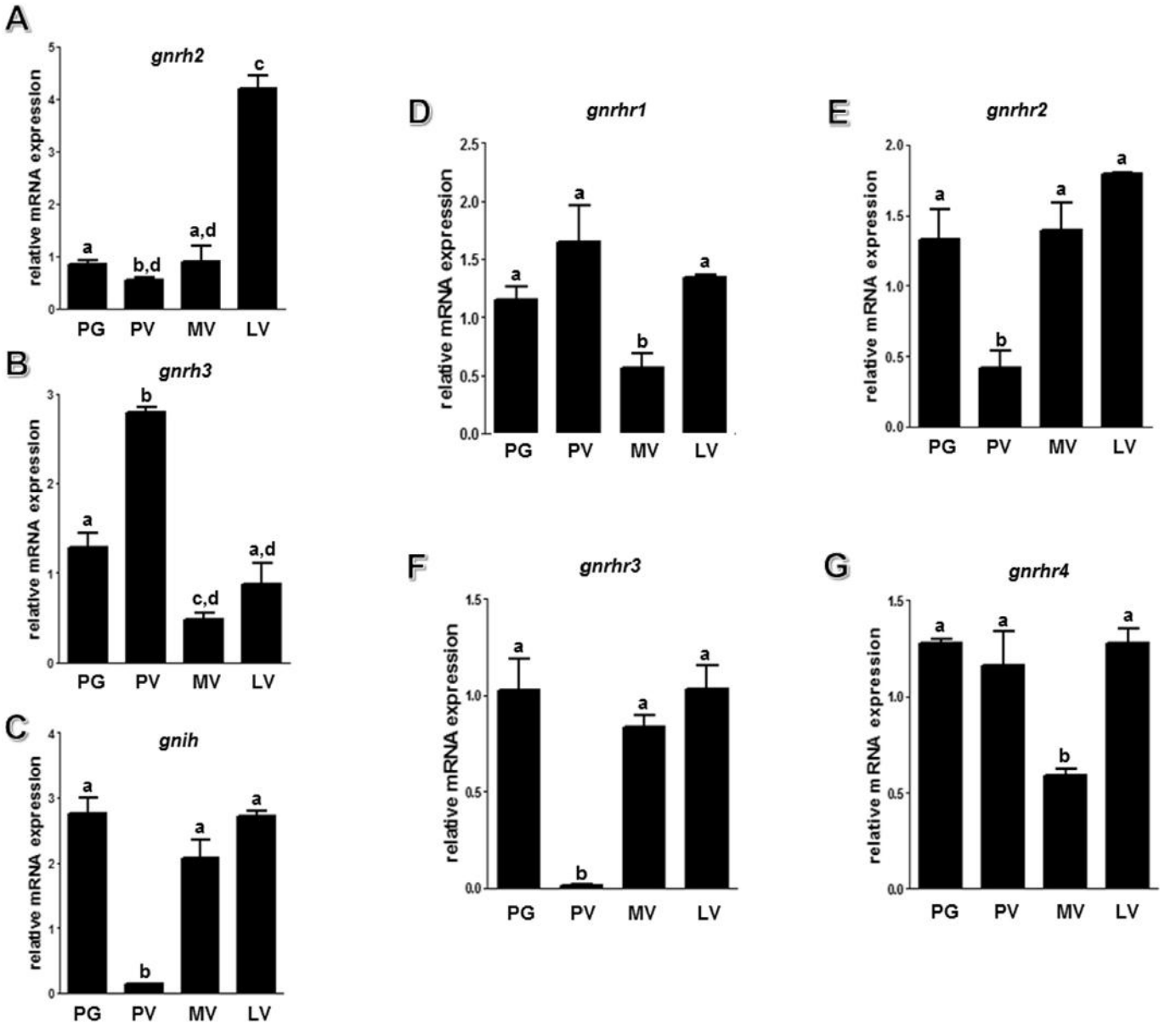

Fig. 7. Expression of *gnrh2* (A), *gnrh3* (B), *gnih* (C) and *gnrh* receptors *1* (D), *2* (E), *3* (F) and *4* (G) in the olfactory bulb of zebrafish females during the ovarian maturation (PG, primary growth; PV pre-vitellogenesis; MV mid-vitellogenesis and LV late-vitellogenesis). Different letters denote significant differences ($p < 0.05$) between stages.

4.2. Olfactory bulb (OB)

In the OB, *gnrh3* and *gnih* were the most expressed transcripts. In this work, we reported an inverse expression pattern between *gnrh3* and *gnih* transcripts in the OB during the zebrafish ovarian maturation. Interestingly, Gnih and Gnrh3 are also neuroanatomically related; Gnih-producing cell bodies are located in the terminal nerve ganglia where Gnrh3 neurons are clustered, as described previously (Sawada et al., 2002; Di Yorio et al., 2016; Paullada-Salmerón et al., 2016). Considering that Gnih might regulate Gnrh secretion in zebrafish (Zhang et al., 2010), it is also possible that Gnih may down-regulate *gnrh3* expression in the zebrafish OB. To assess the functional role of Gnih in the OB and demonstrate a link with the ovarian maturation as reported in this work, a pharmacological administration of recombinant zebrafish (rzf) Gnih in females at PV stage was carried out as described in the Supplemental Material. Our results showed that *gnrh3* expression levels did not change after 8 h *in vivo* injection or 12 h *in vitro* incubation with rzf Gnih (Supplemental Material). Therefore, *gnrh3* is not modulated by Gnih and the inverse expression of *gnrh3* and *gnih* might be attributed to other regulatory mechanisms in the OB.

Although Gnrh2 is the most conserved Gnrh, the role of this variant is not yet totally understood. Different studies have shown that Gnrh2 stimulates sexual behavior in females of mammals (Barnett et al., 2006), birds (Maney et al., 1997) and fish (Volkoff and Peter, 1999). In zebrafish, Gnrh2 is also involved in the inhibition of food intake (Nishiguchi et al., 2012) and Gnrh2 fibers are projected into the olfactory bulbs (Xia et al., 2014). In this study, we found increased mRNA levels for *gnrh2* in the OB of mature females (final stages of ovarian maturation), suggesting that Gnrh2 may play a role in the zebrafish reproduction/breeding at the OB level, but more studies are needed to unveil the role of Gnrh2 in this species.

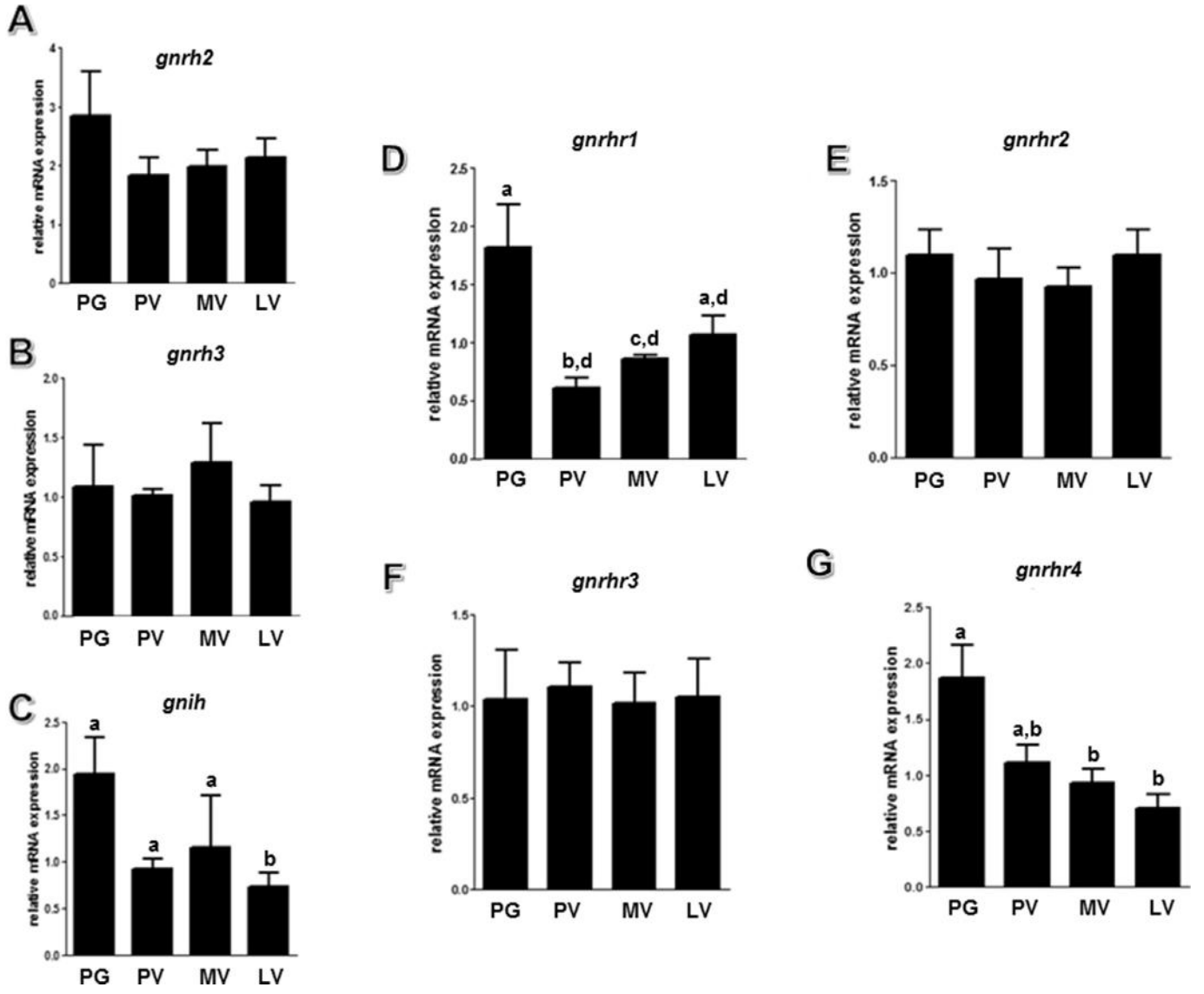

Fig. 8. Expression of *gnrh2* (A), *gnrh3* (B), *gnih* (C) and *gnrh* receptors *1* (D), *2* (E), *3* (F) and *4* (G) in zebrafish (females) retina at four maturation stages (PG, primary growth; PV previtellogenesis; MV mid-vitellogenesis and LV late-vitellogenesis). Different letters indicate significant differences ($p < 0.05$) between stages.

Concerning to the Gnrh receptors, two patterns of expression were observed, one for *gnrhr1* and *gnrhr4* (constant over the stages, decreasing in the MV), and other for *gnrhr2* and *gnrhr3* (constant over the stages, with reduction in PV). Nevertheless, little is known about the role of these receptors in the zebrafish OB, as well their role over gonadal maturation.

4.3. Retina

ir-Gnrh3 fibers were distributed along the three layers of zebrafish retina, as previously described in other teleosts (Münz et al., 1982; Stell et al., 1984; Wirsig-Wiechmann and Wiechmann, 2002; Grens et al., 2005; Maruska and Tricas, 2007). All Gnrh receptors were detected by RT-PCR but only *gnrhr4* expression sites were localized by *in situ* hybridization. In the zebrafish retina, *gnrhr4* is expressed in amacrine, bipolar, and ganglion cells. Interestingly, the *gnrhr4* expression sites are closely located near to Gnrh3 positive fibers. This interaction also occurs in other teleost species as *Astatotilapia burtoni* (Grens et al., 2005), *Chaetodon multicinctus*, *Thalassoma duperrey*, *Abudefduf abdominalis* and *Asterropteryx semipunctata* (Maruska and Tricas, 2007) and *Dicentrarchus labrax* (Servili et al., 2012). During ovarian maturation, *gnrh2* and *gnrh3* remained constant, but *gnih* decreased in the final stage of vitellogenesis. For the receptors, *gnrhr2* and *gnrhr3* transcripts remained constant throughout the stages, while *gnrhr1* and *gnrhr4* decreased at PV and LV. At the moment, we do not know the physiological significance of these observations, as well as the role of Gnih in the retina. However, we do not exclude the role of these neuropeptides as modulators and integrators of the sensorial signal in zebrafish.

4.4. Ovary

In teleosts, Gnrh has been found in the ovaries of various species such as goldfish (Pati and Habibi, 1998), *Anguilla japonica* (Okubo et al., 1999), medaka (Okubo et al., 2006), *Oncorhynchus mykiss* (Madigou et al., 2000), among others. The present study confirmed the expression of two forms of Gnrh (*gnrh2* and *gnrh3*) in zebrafish ovary, and demonstrated that the Gnrh3 protein is specifically located in the cytoplasm of perinucleolar oocytes, as reported in

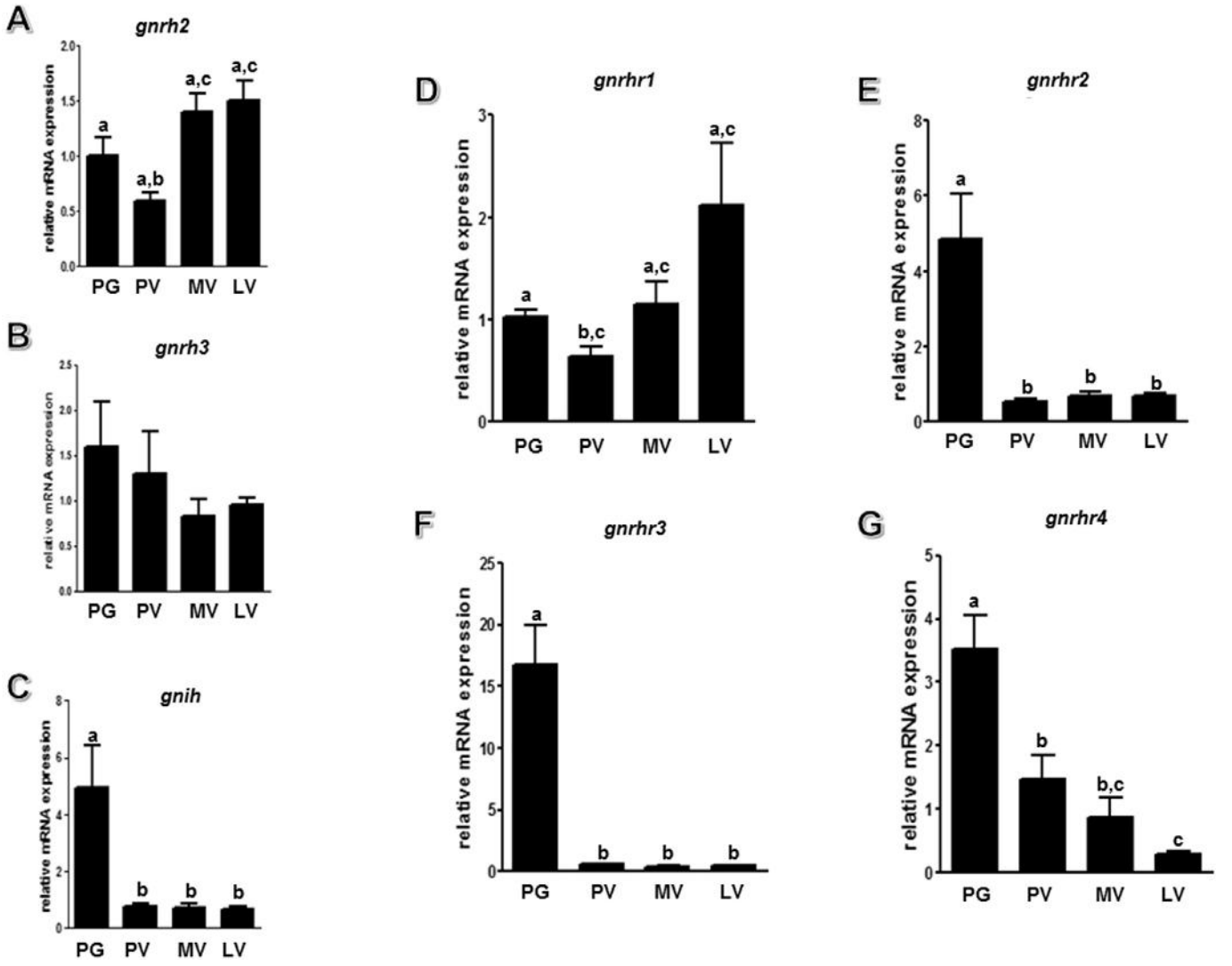

Fig. 9. Expression of *gnrh2* (A), *gnrh3* (B), *gnih* (C) and *gnrh* receptors *1* (D), *2* (E), *3* (F) and *4* (G) in zebrafish ovary during the ovarian maturation (PG, primary growth; PV pre-vitellogenesis; MV mid-vitellogenesis and LV late-vitellogenesis). Different letters indicate significant differences ($p < 0.05$) between stages.

Heteropneustes fossilis (Singh et al., 2007). Moreover, *gnrhr2* and *gnrhr4* mRNA were also observed in zebrafish perinucleolar oocytes. The co-localization of Gnrh with *gnrhr2* and *gnrhr4* receptors in perinucleolar oocytes suggests an autocrine/paracrine role of these molecules in the early stages of ovarian maturation in zebrafish. Interestingly, as vitellogenesis progresses, ir-Gnrh3 and *gnrhr2* and *gnrhr4* mRNA levels decreased. Moreover, *gnrhr4* mRNA was detected in the *zona radiata* and follicular cells of zebrafish vitellogenic oocytes. Similar studies detected *gnrh* mRNA and its receptors in granulosa cells of humans and mice (Cheng et al., 2002; Kang et al., 2003) and also in the goldfish ovary (Pati and Habibi, 2000). In goldfish, GnRH appears to be involved in the regulation of steroidogenesis, in the induction of apoptosis, as well as in the maintenance of gonadal synchrony (Andreu-Vieyra and Habibi, 2000). Although, no significant differences were observed in the expression levels of *gnrh* variants, we showed that *gnrhr2*, *gnrhr3*, *gnrhr4* mRNA decreased significantly during zebrafish vitellogenesis when compared to primary growth oocyte; but *gnrhr1* remained constant. These results are in agreement with the *in situ* hybridization and immunohistochemistry data, which confirmed the expression of Gnrh in the early stages, specifically in perinucleolar oocytes.

The expression of *gnih* was found to be higher in primary growth ovaries, decreasing significantly during zebrafish vitellogenesis. *In situ* hybridization data showed *gnih* transcripts in cortical alveoli stage, specifically in the cortical granules, and also in follicular cells of vitellogenic oocytes. Interestingly, *gnih* expression did not overlap with ir-Gnrh; while Gnrh3 occurs in perinucleolar oocytes and decreased in cortical alveoli of oocytes, *gnih* had an inverse expression pattern. Other studies also described Gnih and its receptor in the gonads of zebrafish (Zhang et al., 2010) as in this study and other fish, birds, reptiles and mammals (Bentley et al., 2008; Maddineni et al., 2008; Singh et al., 2008, 2011; Qi et al., 2013). However, the functional implications of *gnih* expression and its receptor *gnihr* are widely unexplored in teleosts. Recent studies in mice have demonstrated that Gnih inhibited follicular development and steroidogenesis in the ovary (Singh et al., 2011). This observation is in agreement with our expression data showing decrease of *gnih* levels as zebrafish ovarian maturation progresses, indicating a possible inhibitory role of Gnih for follicular development. However, more physiological studies are needed to understand the biological role of Gnih in zebrafish ovary.

In summary, this study characterized Gnrh/Gnih elements in the olfacto-retinal system and ovary, highlighting the following

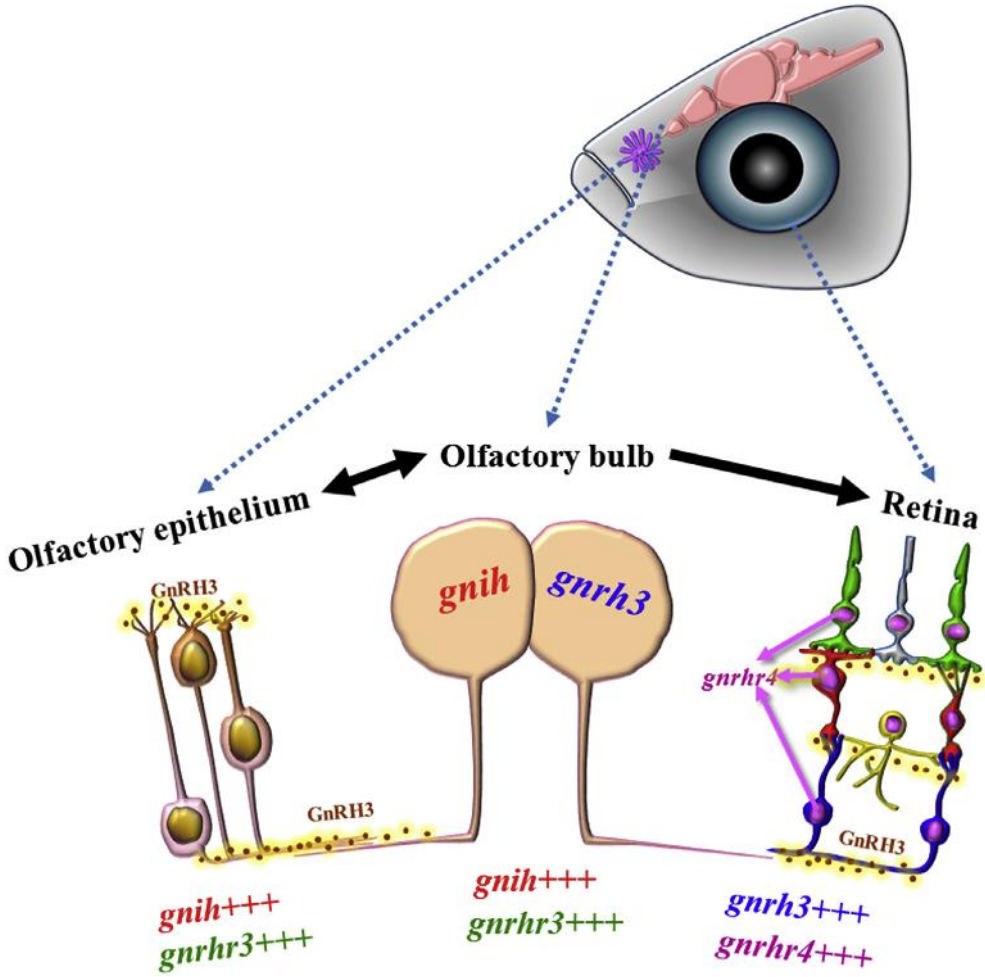

Fig. 10. Schquematic representation summarizing Gnrh/Gnih system in the olfacto-retinal system of zebrafish. Scheme illustrates Gnrh/Gnih expression sites in the olfacto-retinal system of zebrafish. (+++) indicates the most expressed ligand or receptor.

findings (Fig. 10): 1) *gnrh3* and *gnrhr* are highly expressed in the OE at final phases of vitellogenesis; 2) an inverse relationship between *gnih* and *gnrh3* transcripts in the OB; 3) *gnrhr4* expression sites in retina were identified in the nuclei of amacrine, bipolar, and ganglion cells next to ir-Gnrh fibers; 4) Gnrh3 protein is located specifically in the cytoplasm of perinucleolar oocytes. Altogether, these results reinforce Gnrh/Gnih as modulators of the zebrafish sensorial system, establishing a relationship of their expression levels with the reproductive status. Finally, Gnrh/Gnih sites in the zebrafish ovary indicate a local regulation of these peptides, but their role are still not unveiled for zebrafish.

Author contributions

Conceived and designed the experiments: SC ERMM GMS RHN. Performed the experiments: SC ERMM LBD JMBR Analyzed the data: SC ERMM RHN. Contributed reagents/materials/analysis tools: LSON FNV ERMM RHN AJB JMBR. Wrote the manuscript: SC ERMM GMS LBD RHN.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2017.04.002.

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