Neurodevelopmental effects of natural and synthetic ligands of estrogen and progesterone receptors in zebrafish eleutheroembryos

Olivier Kah, François Brion, Elisabeth Pellegrini, Colette Vaillant, Marie-Madeleine Gueguen, Justyne Feat, Thierry D Charlier, Pascal Coumailleau

Neurodevelopmental effects of natural and synthetic ligands of estrogen and progesterone receptors in zebrafish eleutheroembryos

Colette Vaillant[a], Marie-Madeleine Gueguen[a], Justyne Feat[a,1], Thierry D. Charlier[a], Pascal Coumailleau[a], Olivier Kah[a], François Brion[b], Elisabeth Pellegrini[a,*]

[a] Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France.

[b] Institut National de l'Environnement Industriel et des Risques INERIS, Unité d'Ecotoxicologie, 60550, Verneuil-en-Halatte, France.

* corresponding author
elisabeth.pellegrini@univ-rennes1.fr

Irset - Inserm UMR_S 1085
9 avenue du Prof. Léon Bernard
35000 RENNES
FRANCE

[1]Present address: Laboratoire de RadioPathologie, INSERM U1274, Bât 05/RDC/H006, 92265 Fontenay aux Roses Cedex.

Abstract

Natural and synthetic estrogens and progestins are widely used in human and veterinary medicine and are detected in waste and surface waters. Our previous studies have clearly shown that a number of these substances targets the brain to induce the estrogen-regulated brain aromatase expression but the consequences on brain development remain virtually unexplored. The aim of the present study was therefore to investigate the effect of estradiol (E2), progesterone (P4) and norethindrone (NOR), a 19-nortestosterone progestin, on zebrafish larval neurogenesis. We first demonstrated using real-time quantitative PCR that nuclear estrogen and progesterone receptor brain expression is impacted by E2, P4 and NOR. We brought evidence that brain proliferative and apoptotic activities were differentially affected depending on the steroidal hormone studied, the concentration of steroids and the region investigated. Our findings demonstrate for the first time that steroid compounds released in aquatic environment have the capacity to disrupt key cellular events involved in brain development in zebrafish embryos further questioning the short- and long-term consequences of this disruption on the physiology and behavior of organisms.

Keywords

Zebrafish larval neurodevelopment
Neuro-endocrine disruption
Progesterone
Estradiol
Norethindrone

1. Introduction

Sex steroids are hormones mostly studied for their roles in reproduction. However, a wealth of data also points out complex actions in the brain of vertebrates during development and in adulthood (Duarte-Guterman et al., 2015b; Heberden, 2017). Many experimental results highlighted the effects of estradiol (E2) and progesterone (P4) on neuronal plasticity through different processes such as synaptogenesis (Fester et al., 2012; Sager et al., 2017; Sakamoto et al., 2001, 2001) stem cell proliferation or differentiation, migration, and neuron survival (Azcoitia et al., 2001; Brinton et al., 2008; Brock et al., 2010; Diotel et al., 2018; Mazzucco et al., 2006; Murashov et al., 2004; Tsutsui, 2008; Wang et al., 2003; Zhang et al., 2010).

Pharmaceuticals derived from sex steroids have long been used in human and veterinary medicine. Synthetic estrogens and progestins structurally related to either testosterone or progesterone were developed and used in contraceptive pills but also in different therapeutic applications such as hormone replacement in women undergoing menopause, endometriosis treatment or cancer therapy. Natural and synthetic steroidal pharmaceuticals originating from human and animal excretion and medical applications are now detected in wastewater and aquatic environment at concentrations up to several hundred ng/l (Al-Odaini et al., 2010; Fent, 2015; Liu et al., 2014). The impacts of synthetic estrogens on reproduction of aquatic organisms have been thoroughly documented (Kidd et al., 2007; Mitra et al., 2003; Pawlowski et al., 2004; Runnalls et al., 2015; Soffker and Tyler, 2012). However, synthetic progestins are less studied although several authors pointed out the risks associated with their presence in surface waters (Fent, 2015; Gunnarsson et al., 2019). Up to now, studies on the consequences of exposure to progestins on aquatic organism physiology mainly focused on peripheral organs such as gonads or liver and reproductive health of fish (Kumar et al., 2015) highlighting the fact that progestins can act at low levels.

The brain of fish also represents a prime target for steroids as both nuclear progesterone (PR) and estrogen receptors (ER) are widely expressed in different brain regions both in adult fish and during early development (Forlano et al., 2005; Hanna et al., 2010; Menuet et al., 2002; Mouriec et al., 2009). In the zebrafish brain, ER expression is closely associated to the distribution of radial glial cells (RGC) expressing aromatase B (brain aromatase), the enzyme converting testosterone to estradiol and encoded by the *cyp19a1b* gene (Coumailleau et al., 2015; Menuet et

al., 2002). It is now recognized that aromatase B-RGC display progenitor properties in teleost and support neurogenesis during development but also in adults (Adolf et al., 2006; Pellegrini et al., 2007; Strobl-Mazzulla et al., 2010). A widespread distribution of PR has been also reported in the brain of zebrafish at different developmental stages, both in neurons and RGC, suggesting that progesterone and synthetic progestins could affect neurogenesis supported by RGC and neuronal activity during the development but also later during life (Diotel et al., 2011; Hanna et al., 2010). Estrogen and progesterone effects in the brain could also be mediated through membrane steroid receptors (Hanna and Zhu, 2009; Liu et al., 2009; Mangiamele et al., 2017; Zhang et al., 2016; Zhu et al., 2003). Membrane progesterone receptors (mPRs) are abundantly expressed in different areas of the brain of fish including zebrafish (Hanna and Zhu, 2009; Zhang et al., 2016; Zhu et al., 2003) and GPER is described in olfactory bulbs, telencephalon, optic tectum, hypothalamus, cerebellum and medulla oblongata (Liu et al., 2009).

In fish, the ability of synthetic progestins to activate progesterone receptors is not well characterized and interferences with other nuclear steroid signaling pathways have been described (Bain et al., 2015; Ellestad et al., 2014; Kumar et al., 2015). Using the EASZY assay, we showed that most of the synthetic progestins derived from 19-nortestosterone can activate, in a similar way to estradiol, the estrogen-sensitive *cyp19a1b* gene in radial glial progenitors, as visualized in *cyp19a1b*-GFP transgenic zebrafish larvae (Brion et al., 2012; Cano-Nicolau et al., 2016). These inductions are ER-dependent but require first their metabolization into estrogenic metabolites (Brion et al., 2012; Cano-Nicolau et al., 2016). Given that ER and aromatase B are fully functional at early stages of zebrafish development (Mouriec et al., 2009) and that estradiol affects zebrafish neurogenesis (Diotel et al., 2013), an impairment of neurogenesis processes and brain development through ER pathway can be hypothesized in fish exposed to testosterone-derived progestins.

In this context, we investigated on zebrafish larvae the neurodevelopmental effects of norethindrone (NOR), a 19-nortestosterone synthetic progestin with estrogenic properties. We compared these effects to those induced by the natural ER and PR ligands, estradiol (E2) and progesterone (P4) respectively. We first evaluated the impact of these steroids on the expression of ER and PR involved in brain development and then on the brain proliferative and apoptotic activities of exposed larvae.

2. Material and Methods

2.1. Chemicals

17β-estradiol (E2), progesterone (P4), norethindrone (NOR) and 5-Bromo-2′-deoxyuridine (BrdU) were purchased from Sigma-Aldrich Chemical Co (St.Louis, MO). Stock solutions of steroids were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. Final dilutions were prepared fresh before each experiment. BrdU was directly diluted in the water used for fish incubation at a final concentration of 1mM.

2.2. Animals treatments

Zebrafish were handled and euthanized in agreement with the guidelines for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. The protocols used in this study were approved by the local animal care and ethics committee (Comité Rennais d'Ethique en matière d'Expérimentation Animale, Rennes, France, under the number EEA B-35-040). Adults used for spawning were housed in our facilities (INRA LPGP, BIOSIT, Rennes, France, agreement number: B 35-238-6) under standard conditions of photoperiod (14 hours light and 10 hours dark) and temperature (28°C). Eggs obtained from spawning zebrafish are collected immediately after laying and kept during 24 hours in glass petri dishes containing water, in an incubator under standard conditions of temperature and photoperiod. One day post-fertilization (dpf) embryos that had developed normally were randomly distributed into 10 groups: E2 0.1 nM (27.2 ng/L), 1 nM and 10 nM, P4 0.1 nM (31.4 ng/L), 1 nM and 10 nM, NOR 0.1 nM (29.8 ng/L), 1 nM and 10 nM or control group with DMSO alone (0.01 % V/V). Zebrafish were treated for 6 days and the medium was changed every day. The concentrations chosen for NOR and E2 strongly up-regulate *cyp19a1b* expression while P4 had no effect as we showed previously (Cano-Nicolau et al., 2016; Diotel et al., 2013; Menuet et al., 2005; Tong et al., 2009)

To evaluate transcriptional activity of genes and apoptosis, groups of 70 embryos were placed in glass flask containing 50 ml of water. After exposure, 50 larvae (7 dpf) were terminally anesthetized with MS222 (50 mg/L). Larvae were placed into ice-cold water. The heads were

separated from the rest of the body, immediately frozen in liquid nitrogen and stored at -80°C before RNA extraction and quantitative real-time PCR. The remaining larvae were euthanized in MS222 (50 mg/L) and immersed in 4% paraformaldhyde (PAF) in saline phosphate buffer (PBS, pH 7.4) for apoptosis analysis. Six independent experiments were performed using the same protocol.

To quantify the number of proliferative cells in the brain of treated fish, the same exposure protocol as described above was applied but BrdU (1mM) was added to the water during the last 24 hours of steroid treatments. At the end of the experiments, larvae were euthanized in MS222 and immediately fixed on PAF4%-PBS. Again, six independent experiments were performed.

2.3. RNA extraction, Quantitative Real-Time PCR and data analysis

To monitor the expression level of genes encoding ER and PR, we performed quantitative real-time PCR. Frozen heads were sonicated for 15 sec in 250 µl of Trizol Reagent (Invitrogen) and the RNA extractions were then carried out using the Trizol-Chloroform method according to the manufacturer's protocol. Reverse transcription and quantitative Real-Time PCR were performed as previously described (Diotel et al., 2013). Primers used for the analysis are shown in Table 1. For each condition (E2, P4, NOR and DMSO), the RT-PCR quantification was run in triplicate. Melting curve and PCR efficiency analyses were performed to confirm specific amplification. The threshold cycle (Ct) was determined for each gene. The gene *ef1* was used to normalize the expression of other genes. The Delta-Delta CT method was then applied to calculate the relative expression of each gene of interest. The fold induction/inhibition was determined and expressed as a fold change compared to the normalized control condition (DMSO). Data are represented as the mean +/- standard error of the mean (SEM). The difference between control and treated groups were compared with a one-way analysis of variance (ANOVA) and the subsequent mean comparison was carried out with a Fisher LSD post-hoc test. $p < 0.05$ was considered statistically significant.

2.4. Brain sections preparation

To determine the number of proliferative and apoptotic cells in the brain, PAF-fixed larvae were cryoprotected in PBS containing 30 % of sucrose during 24 hours and embedded in Tissue-teck before freezing at -80°C. Thin serial sections (10 μm) of larvae were prepared with a cryostat and collected from the olfactory bulbs to the end of the hindbrain. If more than two brain sections were lost or damaged in a region during cryostat sectioning, the sample was discarded from the quantitative analysis. Brain sections were then processed for immunohistochemistry or TUNEL-labeling.

2.5. Antibodies characteristics

The primary antibodies used for the study were previously characterized for their specificity in zebrafish. Table 2 summarizes the characteristics of commercial primary and secondary antibodies used in this study. The BrdU monoclonal antibody (RRID_AB10013660, Dako Agilent) was validated in previous studies showing expected staining in neurogenic zones lining the ventricles in zebrafish brain (Chapouton et al., 2011; Diotel et al., 2013; Marz et al., 2010; Pellegrini et al., 2007). Anti-acetylated tubulin monoclonal antibody (RRID AB_477585, Sigma-Aldrich) was used to identify neurons. The staining is cytoplasmic as observed in others studies and is never detected in radial glial cells (Chitnis and Kuwada, 1990; Huang and Sato, 1998; Pellegrini et al., 2007). Radial glial cells were identified with a polyclonal rabbit antibody generated in the laboratory and raised against zebrafish aromatase B. The specificity of this antibody was assessed previously by pre-adsorption with the synthetic peptide used to generate the antibody. The aromatase B staining observed with this antibody has also been validated by *in situ* hybridization and co-staining with GFP in the *cyp19a1b*-GFP transgenic zebrafish line (Menuet et al., 2005; Pellegrini et al., 2007; Tong et al., 2009). The serotonin (5-HT) antibody is a rabbit polyclonal antibody developed and kindly provided by Dr Y.Tillet (INRA, UMR6175, Nouzilly, France) (Tillet et al., 1986). This antibody yielded results identical to those described previously in zebrafish (Lillesaar, 2011; Perez et al., 2013).

2.6. BrdU immunohistochemistry

To determine the effects of steroids on proliferative activity in the brain of treated larvae, we implemented a BrdU-labeling protocol. Brain sections were first washed in PBS. An antigen retrieval step was then carried out in a formamide solution (50% formamide/50% 2× SSC) at 65°C during 120 min. Slides were rinsed successively in 2× SSC (10 min at room temperature), 2N HCl buffer (30 min at 37 °C), 0.1 M sodium tetraborate decahydrate pH 8.5 (10 min at room temperature) and PBS triton 0.2% (15 min at room temperature). Non-specific sites were blocked with PBS 0.2% Triton solution containing 1% of milk powder (45 min at room temperature). The mouse anti-BrdU primary antibody was diluted in PBS containing 0.5% of milk powder and applied on sections overnight at room temperature. On the next day, slides were washed three times in 0.2% Triton PBS and incubated with a goat anti-mouse Alexa Fluor 594 (see table 1 for details). Tissue sections were rinsed several times in PBS 0.2% Triton, mounted with Vectashield medium (Vector Laboratories, Inc. Burlingame, CA) and analyzed for BrdU-positives cells quantification.

2.7. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling)

We quantified the number of apoptotic cells in the brain of treated zebrafish using the TUNEL method. Frozen sections were fixed in 4% PBS-PAF for 20 min at room temperature. They were rinsed 30 min in PBS, dipped in the permeabilization solution (0.1% Triton 100X, 0.1% sodium citrate) for 30 min at 80°C and washed for 20 min in PBS. Sections were incubated with the TUNEL labeling mixture (5 µl of Enzyme solution (TdT) + 45 µl of Label solution (fluorescein-dUTP) overnight at 37°C, in a dark and humidified chamber. We performed a positive control by incubating a slide in a solution containing 300 µl of 50 mM Tris-HCl pH 7.5, BSA 1 mg/ml and 10 µl of RQ1 DNAse (1U/µl) (Promega) for 10 min at room temperature and a negative one by placing on a slide 50 µl of Label solution without TdT. The slides were then rinsed for 30 min in PBS and mounted in a Vectashield medium containing DAPI for microscopic observation and quantification (Vector Laboratories, Inc. Burlingame, CA).

2.8. Double staining with TUNEL and neuronal or glial markers

In order to determine the phenotype (neuron or radial glial cells) of the apoptotic cells, a double labeling was carried out as follow. Double staining was performed on 4 control brains and 4 NOR 10 nM treated brains. Sections were treated as previously described except that a primary antibody was added to the TUNEL labeling mixture. We used a rabbit polyclonal antibody raised against zebrafish aromatase B to identify radial glial cells (dilution 1:500). To characterize mature neurons, we used a mouse monoclonal anti-acetylated tubulin or a rabbit polyclonal anti-5HT (dilution 1:5000). The slides were incubated overnight at 37°C in a dark and humidified chamber with the labeling solution containing the primary antibody. Sections were then rinsed twice in 0.1% triton in PBS and neuronal and glial markers detection was performed with appropriate Alexa fluor secondary antibodies (see table 1 for their characteristics). The secondary antibodies were left in contact with the section for 90 min at room temperature. After several washes in PBS, tissues sections were mounted in Vectashield medium with DAPI and visualized and analyzed under a fluorescent microscope and a confocal microscope.

2.9. Microscopy and cell counting

Brain sections of control and treated larvae were examined with an epifluorescence microscope (Olympus Provis, AX70-TRF equipped with de DP71 digital camera) and images were processed with the Olympus Cell software. BrdU-positive cells (proliferative cells) and TUNEL-positive cells (apoptotic cells) were manually quantified by an experimenter blind to the treatment using photographs of all the brain sections (from the olfactory bulbs to the end of the caudal hypothalamus). Six independent experiments were performed. First, we calculated the mean number of BrdU-positive cells or the number of TUNEL-positive cells in the whole brain (total mean number). Then, in order to know if steroids could exert effects in a region-dependent way, we counted the mean number of labeled cells (BrdU or TUNEL) in specific brain areas according to the nomenclature established in zebrafish by Mueller and Wullimann (Mueller and Wullimann, 2005). The regions of interest were located by neuroanatomical structures identification with DAPI labeling.

To identify the phenotype (RGC or neuron) of apoptotic cells (TUNEL-positive cells), observations were carried out with a confocal microscope (Leica, TCS SP8, DMI 6000). Microphotographs were acquired in TIFF format with a Leica (LCS Lite) software and light and contrast were adjusted using Photoshop CS4 before being assembled on plates.

2.10. Statistical analysis

Data are represented as the means +/- standard error of the mean (SEM). For biological reasons (E2 acting on ER, not on PR and P4 acting on PR, not ER), the difference between the control group and each molecule (at the different concentrations) separately was compared with a one-way analysis of variance (ANOVA). The subsequent between-group comparison was carried out with a Fisher LSD post-hoc test. *$p < 0.05$ was considered statistically significant.

3. Results

3.1. Steroid receptor genes expression in the brain of P4, E2 and NOR exposed larvae

We first quantified the mRNA changes induced by the 3 compounds on *nPR*, *esr1*, *esr2b* and *esr2a*. Data on gene expression are summarized in Table 3. We observed a significant effect of P4 treatment on *nPR* ($F_{(3, 17)} = 65.55$) with a significant increase of nPR gene expression when larvae were exposed to P4 0.1 nM and 10 nM compared to control group. P4 also affected significantly *esr2a* expression ($F_{(3, 18)} = 133.05$) but only at the highest concentration. E2 significantly affected nPR expression ($F_{(3, 20)} = 110.40$), with a significant increase of *nPR* mRNA level when larvae were treated with 1 nM and 10 nM. No significant change was detected on *nPR* at the lowest concentration (0.1 nM). E2 also significantly induced the transcriptional activity of *esr1* ($F_{(3, 20)} = 77.39$), *esr2b* ($F_{(3, 20)} = 268.87$) and *esr2a* ($F_{(3, 20)} = 281.65$) genes. This effect of E2 was only observed 1 nM compared to the control group. NOR significantly affected *nPR* gene expression ($F_{(3, 21)} = 59.25$), where exposure at 10 nM significantly increased nPR compared to control group. The expression of ERs was not modified.

3.2. Proliferative activity in the brain of zebrafish larvae exposed to P4, E2 and NOR

To assess the impact of steroids on the proliferative activity of the developing brain, we quantified the number of cells that incorporated BrdU during the last 24h of treatment. We first carried out the quantification on the whole brain, from the olfactory bulbs to the end of the hindbrain. The total number of BrdU-positive cells in the whole brain tends to decrease in P4 0.1 nM ($P = 0.094$), E2 1 nM ($P=0.093$) and E2 10 nM ($P=0.068$) conditions but the decrease did not reach statistical significance (date not shown). This number is not affected in other experimental groups (data not shown). We then further analyzed three specific regions of the forebrain: the first one included the most anterior part the forebrain (olfactory bulbs, telencephalon and preoptic area, OB-Tel-PO), the second one corresponded to the thalamus and the last one to the mediobasal and caudal hypothalamus.

Exposure to NOR had no effect on brain proliferation (Figure 1). In contrast, significant effects of P4 and E2 on the number proliferating cells were observed in the most anterior part of the brain (OB-Tel-PO, $F_{(9, 116)} = 1360.72$) and in the thalamus ($F_{(9, 123)} = 707.22$ (Figure 1). P4 0.1 nM significantly decreased the number of BrdU-labeled cells in the anterior part of the brain (OB-Tel-PO) (Figure 1). In the thalamus and the mediobasal and caudal hypothalamus, P4 did not affect proliferation (Figure 1). E2 1 nM treatment reduced the number of BrdU-labeled cells in OB-Tel-PO region (Figure 1). This inhibitory effect of E2 is illustrated in the Figure 2 showing representative microphotographs of BrdU staining in the telencephalon of a control animal (Figure 2A) and an E2-treated larva (1 nM, Figure 2B). In the thalamus, a clear and significant inhibition of the proliferation was observed at E2 1 nM and 10 nM (Figure 1). The decrease in the number of proliferating cells is illustrated in representative microphotographs of the dorsal thalamus in a control animal (Figure 2C) and in E2-treated larva (Figure 2D). We did not observe any change in the proliferative activity within the hypothalamus (mediobasal and caudal part) independently of E2 concentrations (Figure 1).

3.3. Apoptosis in the brain of zebrafish larvae exposed to P4, E2 and NOR

In order to evaluate the potential impact of E2, P4 and NOR on apoptosis we investigated the number of apoptotic nuclei positively labeled with the TUNEL method in the different

conditions. While proliferative activity occurred very close to the ventricular surface, apoptotic cells were observed throughout the brain, in periventricular areas but also more deeply in the brain parenchyma in control and treated zebrafish. Apoptotic-cells were observed in many regions such as the olfactory bulbs, the telencephalon, the preoptic area, the mediobasal and caudal hypothalamus, the thalamus, the tegmentum and the optic tectum. We first quantified the number of apoptotic cells in the whole brain. As shown in the Figure 3A, neither P4 nor E2 had any effect on apoptosis even if E2 tended to increase the total number of TUNEL-positive cells. In contrast, treatment of embryos with NOR 10 nM and 0.1 nM had a significant effect on the number of TUNEL-labeled nuclei with an increase of 50% for both concentration compared to control group ($F_{(9, 70)} = 464.17$). To evaluate possible region/territory-specific effect of the different steroids, a quantification was realized in the 3 extended territories: the first one included the olfactory bulbs, the telencephalon and the preoptic area (OB-Tel-PO, Figure 3B), the second one corresponded to the thalamus and the tegmentum of the midbrain (Figure 3C) and the last one was composed with the mediobasal and caudal hypothalamus (Figure 3D). Quantification of the signal showed a significant effect of NOR treatment in the most anterior part of the brain ($F_{(9, 75)} = 284.32$) and in the mediobasal and caudal hypothalamus ($F_{(9, 77)} = 302.84$).

At the highest concentration (10 nM), P4 seemed to increase the number of TUNEL positive cells in the three investigated regions, but the difference with control fish was not significant (Figure 3B-D). A similar trend toward an increase was observed when larvae were incubated with 10 nM E2 (Figure 3B-D). When zebrafish embryos were treated with NOR 0.1 nM, the number of TUNEL-labeled nuclei was significantly increased by 74% in the most anterior part of the brain (Figure 3B). NOR treatments at 1 nM and 10 nM tended to increase the number of apoptotic cells in this region albeit the effect was not significant (Figure 3B and Figures 4A-D). No significant effect of NOR was quantified in the thalamus and tegmentum (Figure 3C). More posteriorly, a significant impact of NOR 0.1 nM and 10 nM was observed in the hypothalamus with an increase in the number of apoptotic cells reaching 58% and 68% respectively as compared to control fish (Figure 3D and Figures 4E-H).

3.4. Characterization of apoptotic cells in the hypothalamus of control and NOR treated zebrafish

To characterize apoptotic cells, we applied the TUNEL-staining in combination with antibodies directed against different markers. We focused our investigation in the hypothalamus because a significant increase in the number of apoptotic cells was clearly highlighted in this brain region in NOR-treated animals (10 nM) (Figure 3D). We did not observe aromatase B-positive radial glial cells labeled with TUNEL in control and NOR-treated fish (Figure 5A-B). Many CSF-contacting neurons were labelled with the 5-HT-antibody but no colocalization with TUNEL-labeled nuclei could be highlighted in DMSO or NOR-treated larvae (Figure 5C-D). Finally, we investigated whether apoptotic cells could correspond to mature neurons other than 5-HT neurons. Using acetylated-tubulin antibody, we showed that apoptotic neurons were clearly identified in NOR-exposed zebrafish (Figures 5F-G and corresponding orthogonal projections). In contrast, in our conditions, we did not observe acetylated-tubulin and TUNEL colocalization in DMSO-treated zebrafish (Figure 5E).

4. Discussion

To our knowledge, our study is the first to address the question of the comparative impact of NOR, P4 and E2 on molecular and cellular mechanisms required for the brain development of an aquatic species. We provide data showing that ER and PR expressions, proliferation and apoptosis were differentially affected in the developing brain of exposed zebrafish.

4.1. ER and PR gene expressions are modulated by P4, E2 and NOR

P4 and NOR did not affect ER mRNA levels, except *esr2a* expression which was stimulated by P4 at 10 nM. A significant increase of *esr1, esr2a and esr2b* transcripts occurred when zebrafish were exposed to E2 at 1 nM but no significant effects were observed with the lowest or highest concentrations. Changes in ER mRNA levels have been reported in the brain of mammals according to the stage of the estrous cycle or following E2-treatment, suggesting that circulating E2 can modulate the expression of its own receptor (Bohacek and Daniel, 2009; Foster, 2012;

Martins et al., 2015; Mitterling et al., 2010; Simerly and Young, 1991). Different effects of E2 on ER expression (stimulatory or inhibitory) have been documented depending on the age, the dose used for the treatment and the region analyzed (Bohacek and Daniel, 2009; Foster, 2012). In fish, a wealth of studies were dedicated to the ER auto-regulation in peripheral tissue such as the liver and the gonads (Bowman et al., 2002; Flouriot et al., 1997; Menuet et al., 2004; Nelson and Habibi, 2013; Pakdel et al., 1991). There are few studies related to the regulation of brain ER expression by estrogens or xeno-estrogens. A study performed in adult male goldfish showed that in vivo E2 treatments exert time and region-differential effects on the ER expression at the level of the brain (Marlatt et al., 2008). The impact of E2 treatment on ER expression observed in this work seemed to display a nonmonotonic concentration-response curve within the range of concentrations tested similar to those often reported in response to endocrine disruptors (Birnbaum, 2012; Genovese et al., 2014; Vandenberg et al., 2012; Weber et al., 2015). In our case, the curve obtained was an inverted U-shape with the intermediate E2 concentration inducing an effect which is not highlighted at either 10 nM and 0.1 nM. This biphasic dose-response curve, often reported for the action of hormones or endocrine disruptors (Birnbaum, 2012; Genovese et al., 2014; Vandenberg et al., 2012; Weber et al., 2015), must be confirmed by additional experiments covering wider dose ranges.

Our qPCR data also demonstrated that P4, E2 and NOR stimulate PR expression. The stimulatory effect of E2 on PR has previously been reported in the brain of mammals (Guerra-Araiza et al., 2003; Kudwa et al., 2009, 2004; Mendoza-Garcés et al., 2013; Quadros and Wagner, 2008) and we have shown in a previous study that E2 can induce PR expression at different developmental stages in zebrafish (Diotel et al., 2011). The significant increase in PR transcript levels in response to P4 demonstrated in this work reinforces the observations from Zucchi et al. in the zebrafish larvae (Zucchi et al., 2012). As shown in table 3, PR mRNA responses to E2 or P4 do not show a dose-effect relationship. Similarly, no concentration-response effect has already been described for PR expression regulation by P4 (Zucchi et al., 2012). Several mechanisms can be proposed to explain the lack of relationship between the dose and the response, including activation of multiple versus single signaling pathways (for high concentrations), down-regulation of receptors (for high doses), differential recruitment of co-activators or co-repressors depending on the concentration of the hormone (Vandenberg et al., 2012). In addition, the actual serum and organ concentration of each exogenous steroid and their bioavailability (free or bound to transport protein

such as SHBG) will significantly influence the molecule's biological activity. Finally, there is no information on enzymatic metabolization of natural and synthetic steroids within exposed larvae. Zebrafish embryos are metabolically competent and therefore it is likely that the natural steroids and the synthetic progestin used in our study are subjected to intensive metabolic activities (Le Fol et al., 2017; Verbueken et al., 2018). The metabolic pathways for E2, P4 and NOR are not known for zebrafish embryos and specific experiments should be conducted to determine the actual concentration of the 3 steroids within the brain of zebrafish embryos. The absence of dose-response observed for E2 and P4 on PR transcripts could therefore be the consequence of one or more of these mechanisms.

In the present work, PR transcript levels were also significantly up-regulated in larvae exposed to NOR at 10 nM. In a previous study, NOR was shown to modulate the expression of PR in a concentration and time-dependent manner in zebrafish embryos (Zucchi et al., 2012). The mechanisms leading to the up-regulation of PR by NOR could rely on the capacity of NOR or its estrogenic metabolites to strongly stimulate the aromatase B expression in radial glial cells (present study Brion et al., 2012; Cano-Nicolau et al., 2016), likely leading to local increase of E2 synthesis. Since PR are expressed in aromatase B-positive radial glial cells (Diotel et al., 2011; Hanna et al., 2010), such an increase in E2 could trigger the up-regulation of PR through ER pathway. Another potential signaling pathway could involve estrogenic metabolites generated from NOR directly interacting with ER.

All together, these results showed that P4, E2 and NOR modulate ER and PR transcripts in the brain of zebrafish larvae. Considering that ER and PR are expressed in radial glial progenitor cells in zebrafish (Diotel et al., 2011; Pellegrini et al., 2015) and that E2 and P4 could affect neurogenesis (see the introduction part of the manuscript), an alteration of ER and PR receptor expression was likely to impair the neurogenic activity of these progenitor cells.

4.2. Cell proliferation in the developing brain of zebrafish was affected by E2 and P4 but not by NOR

Studies investigating the impact of P4, E2 or synthetic progestins in the developing brain in fish are scarce. Our data reveals that P4 inhibited cell proliferation in the most anterior region when larvae were treated at the lowest concentration while only a tendency was reported for the

two highest concentrations. Our study thus confirms that P4 target the brain and can affect proliferation of cells which agrees with the down-regulation of expression of two cell cycle regulators, i.e. *cdc20* and *ccnb1* in the brain of female zebrafish exposed to P4 (Zucchi et al., 2013). In contrast to P4, the synthetic progestin NOR does not affect proliferative activity in our conditions. P4 inhibitory effect on proliferation could be mediated trough PR and mPR. Indeed, it has been shown that P4 is able to regulate the proliferation of human glioblastoma cells not only by PR interaction but also through mPRα (Atif et al., 2015, 2015; Gonzalez-Orozco et al., 2018; Gonzalez-Orozco and Camacho-Arroyo, 2019). To date, there is very few data on binding properties of synthetic progestins on mPR but experiments using human breast cancer cells stably transfected with human mPRα showed no detectable binding with NOR suggesting that 19-nortestosterone-derived progestins do not recognize mPR (Kelder et al., 2010; Thomas et al., 2007). The marked difference observed in our study between P4 and NOR on brain proliferative activity could be partially explained by different signaling pathways involving or not mPR.

The most notable effects were observed in embryos exposed to E2 in which impairment of proliferation in the most anterior part of the forebrain and in the thalamus was observed. A wealth of data in vertebrates demonstrated that E2 exerts complex stimulatory and/or inhibitory effects on the brain proliferation, through nuclear or membrane estrogen receptors, depending on many factors such as the species, the timing, the concentration and the region investigated (Bowers et al., 2010; Brock et al., 2010; Diotel et al., 2018; Duarte-Guterman et al., 2015a; Heberden, 2017; Mazzucco et al., 2006; Ormerod et al., 2003; Pawluski et al., 2009; Rossetti et al., 2016; Tanapat et al., 1999). In fish, the data are sparse but it was previously shown that E2 inhibited the proliferation and prevented the migration of new-born cells in male and female adult zebrafish (Diotel et al., 2013; Makantasi and Dermon, 2014). The results obtained herein in embryos exposed to E2 are in line with those acquired in adult zebrafish although the area affected in the larvae are not necessarily the same as in adults (Diotel et al., 2013; Makantasi and Dermon, 2014). While a clear inhibitory effect of E2 on proliferative activity was observed in the hypothalamus of both male and female adults (Diotel et al., 2013; Makantasi and Dermon, 2014), no effect was detected in larvae (this study). One explanation for this difference could rely on the differential expression of ERs in brain in adulthood and during development (Mouriec et al., 2009). An alternative hypothesis relies on the capacity of the different brain regions to metabolize E2. Sulfotransferases (SULT) are enzymes that were shown to display sulfating activity, increasing the water solubility

of compounds and their removal from the body. Many SULT have been characterized in fish, some of them involved in the sulfation of steroid hormone, including E2 (James, 2011; Kurogi et al., 2013). In preliminary unpublished results, we have shown that several SULT are expressed in the brain of zebrafish but further investigations in this field could contribute to determine when and where these enzymes are expressed in the brain and if a region-specific pattern of expression could explain a different biological activity of E2.

4.3. Cell apoptosis in the developing brain of zebrafish was affected by NOR but not by P4 and E2

It is commonly accepted that E2 and P4 promote neuronal survival and prevent cell loss (Deutsch et al., 2013; Sasaki et al., 2006; Wang et al., 2003, 2001; Yao et al., 2005) but apoptotic effects of these hormones have been reported in mammals during brain development and following traumatic brain lesions (Atif et al., 2015; Waters and Simerly, 2009). Less is known about synthetic progestins effects on cell death in the brain but it has been shown that apoptosis is increased in hippocampal samples of levonorgestrel-treated rats (a synthetic progestin) (Liu et al., 2010).

In our experimental conditions, P4 and E2 do not affect the number of apoptotic cells in the brain of exposed larvae. The most significant effect on apoptosis was observed with NOR. We provide here evidence that NOR may adversely affect brain development by increasing the number of apoptotic cells of exposed zebrafish larvae. The stimulatory effect of NOR on apoptosis was observed on specific brain territories, notably in the mediobasal/caudal hypothalamus and the most anterior part of the forebrain. Currently, the apoptotic pathways affected by NOR in the brain of zebrafish larvae are completely unknown. In mammals, ovariectomized adult female rats treated with subcutaneous injection of levonorgestrel (a progestin structurally related to testosterone) exhibited an up-regulation of the Bax/Blc2 ratio in the hippocampus of treated animals, indicating an increase of apoptotic activity (Liu et al., 2010). Numerous publications provide evidence for the neuroprotective and beneficial effects of P4 in injured brains through PR and mPR (Deutsch et al., 2013; Guennoun et al., 2019; Thomas and Pang, 2012). A study designed to assess the efficacy of progestins to protect primary hippocampal neurons against degeneration induced by glutamate excitotoxicity showed that P4 has neuroprotective properties but norethindrone had no effect (Liu et al., 2010). Similarly, to proliferation, apoptosis in the brain is differentially affected by P4 and

NOR, suggesting, once more, very specific signaling pathways for each progestin (PR and mPR for P4 and PR only for NOR). Although designed to interact with PR in mammals, several reports mention that progestins have the capacity to interact with other steroid receptors to alter nuclear-regulated pathways, including androgen receptors and ER (Bain et al., 2015; Cano-Nicolau et al., 2016; Ellestad et al., 2014; Kumar et al., 2015). In mammals, binding experiments showed that progestins can interact with glucocorticoid receptors (Liu et al., 2010; Schoonen et al., 2000). In zebrafish, glucocorticoid and mineralocorticoid receptors are both expressed at early stages of development. Consequently, interaction of NOR with corticoid signaling pathway cannot be ruled out in zebrafish (Alsop and Vijayan, 2008; Schaaf et al., 2008; Takahashi and Sakamoto, 2013). Data using antisense oligonucleotide knock-down of glucocorticoid receptors in zebrafish also highlighted the critical role of this receptor in early molecular events essential for the nervous system development, including cell death (Nesan and Vijayan, 2013). Similarly to mammals, mineralocorticoid receptors are also highly expressed in the brain of teleost fishes (Takahashi and Sakamoto, 2013) and Zucchi et al. have demonstrated that mineralocorticoid receptor transcripts were significantly up-regulated in zebrafish larvae treated with NOR (Zucchi et al., 2012). Recent mineralocorticoid receptor knock-out experiments performed in the medaka provided evidence that this signaling pathway is required for normal motor activity in response to visual stimulation (Sakamoto et al., 2016). Overall, there is a need to further investigate the interaction of NOR through multiple zebrafish nuclear/membranes steroid receptors and to determine to which extent these pathways could explain the effect of this synthetic progestin observed on brain apoptosis.

4.4. NOR triggers apoptosis of hypothalamic neurons

We further explored the identity of apoptotic cells in the hypothalamus, the most significantly affected brain region. First, we verified if TUNEL-labeled cells, in control and NOR conditions, could correspond to aromatase B-expressing RGC or to 5HT-CSF-contacting-neurons, two abundant cell populations in this region (Bosco et al., 2013; Pellegrini et al., 2007; Perez et al., 2013). Apoptotic cells did not correspond to aromatase B-positive RGC or 5HT-CSF-contacting-neurons. However, our results clearly showed that, in NOR 10 nM treated zebrafish, apoptotic cells could correspond to mature neurons labeled with an acetylated-tubulin antibody. Contrary to NOR-treated groups, apoptotic cells in the control group were not mature neurons. These results show

that NOR exposure increases the number of apoptotic mature neurons in the hypothalamus. Such neuron-targeted apoptotic effect of NOR has never been described before and needs to be further examined.

5. Conclusion

The present study investigated for the first time the effects of natural and synthetic steroids (i.e. estradiol, progesterone and norethindrone) on two key brain developmental processes. Our data clearly show that the compounds investigated in this study exert differential effects on brain specific regions. While progesterone and estradiol inhibit proliferation of cells in the brain, norethindrone increases apoptosis. The two progestins were expected to generate similar effects, but the distinct responses observed on brain proliferation and apoptosis could be accounted for their different disrupting potencies due to their mode of action. Indeed, the effects of progesterone are likely related to both nuclear and membrane progesterone receptors activation, while norethindrone impacts probably result from more complex signaling pathways involving nuclear but not membrane progesterone receptors, and probably nuclear estrogen, androgen and corticoids receptors. While norethindrone acts in a similar way to estradiol on aromatase B expression in radial glial progenitors (Cano-Nicolau et al., 2016), the effect of this progestin on zebrafish brain neurodevelopment is very different from that of estradiol, suggesting that its impact on brain proliferation and apoptosis cannot be exclusively predicted through aromatase B expression and ER-dependent signaling pathways. Overall our study highlights the need to further explore the underlying mechanisms mediating such cellular events but also to investigate the short- and long-term consequences on the physiology and behavior of zebrafish. Such information is essential for assessing the risk associated with exposure of fish to steroidal pharmaceuticals notably progestins.

Acknowledgments

This work was supported by the ANR PROOFS "Occurrences and effects of environmental ligands of progesterone receptor on fish reproduction and neuro-development" (ANR-13-CESA-0006-3). The authors acknowledge the staff of zebrafish facility of the SFR Biosit (INRA, LPGP, Rennes, France. We thank Dr Yves Tillet (INRA, PRC, Nouzilly, France) for providing the 5-HT antibody.

We are grateful to Stephanie Dutertre from BIOSIT platform IBiSA MRic-photonics for microscopy platform for her assistance with confocal microscopy.

Legends

Table 1: Oligonucleotides sequences used in real-time polymerase chain reaction experiments.

Table 2: Characteristics of commercial primary and secondary antibodies used in this study.

Table 3: Fold induction of steroid receptors expression in the brain of P4, E2 and NOR-treated zebrafish larvae compared to the control condition (DMSO). Expression of ER and PR (*nPR, esr1, esr2b and esr2a*) in the brain of zebrafish larvae treated with DMSO and 3 different concentrations (0.1 nM, 1 nM, 10 nM) of P4, E2 or NOR during 6 days. The table represents the mean of induction factors +/- SEM obtained from 6 independent experiments. For each independent experiment, 50 heads were pooled for each condition. Asterisks indicate significant differences with the control condition (DMSO): *$p < .05$; **$p < .005$.

Figure 1: Number of BrdU-positive cells in the brain of zebrafish larvae treated with P4, E2 and NOR. The upper part of the figure shows sections representative of the different regions where quantification was done. Representative sections were taken from the "Atlas of early zebrafish brain development" by Mueller and Wullimann (2016). The BrdU-positive cells, lining the ventricle, were quantified in the anterior part of the brain corresponding to the olfactory bulbs, the telencephalon and the preoptic area (OB-Tel-PO). The red lines (neuroanatomical levels 14, 21 and 24 of the atlas) represent the periventricular zones that have been quantified in this anterior part of the brain. Quantification of BrdU-labeled cells was also performed in the thalamus (blue frames at the neuroanatomical levels 33, 37, 41, 43) and in the mediobasal and caudal hypothalamus (green boxes at the same levels). The values in the table represent the mean number of BrdU-positive cells +/- SEM. The number of animals used for the statistical study is indicated in the first column (between brackets). Asterisks indicate significant differences with the control condition (DMSO): *$p < .05$.

Figure 2: BrdU immunohistochemistry staining on transverse sections in the telencephalon (**A and B**) and in the dorsal thalamus (**C and D**) of zebrafish larvae treated with DMSO (**A and C**) or with E2 10 nM (**B and D**). Red dots in the panels E and F indicate the localization of BrdU-positive nuclei in representative sections taken from the "Atlas of early zebrafish brain development" by Mueller and Wullimann (2016) at the level of the telencephalon (cross sections 21 of the atlas) and at the level of the dorsal thalamus (cross section 33). The number of BrdU-positive nuclei lining the ventricles was decreased after E2 treatment (B and D) in both regions. OE: olfactory epithelium; POC: preoptic commissure. Scale bar: 20μm.

Figure 3: Number of TUNEL-positive cells in the brain of zebrafish larvae treated with P4, E2 and NOR (0.1, 1 and 10 nM). The quantification was first performed throughout the brain (**A**, whole brain). Quantifications at different levels of the brain have been carried out in order to evidence potential region-dependent effects of the molecules. The representative sections at the left of the plate were taken from the "Atlas of early zebrafish brain development" (Mueller and Wullimann, 2016). They indicate the areas where quantifications were made: in the most anterior part of the brain (including the olfactory bulbs, the telencephalon and the preoptic area: OB-Tel-PO, red area, **B**), in the region combining the thalamus and the midbrain tegmentum (blue area, **C**), and in the mediobasal and caudal hypothalamus (green area, **D**). The histograms represent the mean number of TUNEL-positive cells +/- SEM. The number of animals used for statistical analysis is indicated between brackets under the different experimental groups (A). Asterisks means a significant difference with the control group (DMSO): *p<.05.

Figure 4: Induction of abnormal cell death program in the brain of larvae treated with NOR. Figures **A, C, E and G** show cell nuclei labeled with DAPI. Figures **B, D, F and H** show TUNEL-positive apoptotic cells (arrows). **B and D**: Apoptotic cells in the olfactory bulbs of a control **(B)** and a larva treated with NOR 10 nM **(D). F and H**: Apoptotic cells in the hypothalamus of a control (**F**) and a larva treated with NOR 10 nM (**H**). The number of TUNEL-positive cells is higher in the olfactory bulbs and in the hypothalamus after NOR exposure. OB: olfactory bulbs; OE: olfactory epithelium; Hi: intermediate hypothalamus; PTv: ventral part of the posterior tuberculum. Scale bar: 40 μm.

Figure 5: Confocal identification of apoptotic cells in the hypothalamus of a control zebrafish (**A, C and E**) and a NOR 10 nM treated animal (**B, D, F and G**). Transverse sections show Tunel/Aromatase B double staining (**A and B**), Tunel/Serotonin (**C and D**) and Tunel/Acetylated Tubulin (**E, F and G**). Orthogonal projections of Z stak (16 optic sections of 0.33 μm) show Tunel staining in the nucleus of two acetylated tubulin-positive neurons in a NOR 10 nM treated larvae (**F, F', F'' and G, G', G''**). Scale bar: B, E, F, F', F'', G, G', G'' = 20 μm; A, C, D = 30 μm.

References

Adolf, B., Chapouton, P., Lam, C.S., Topp, S., Tannhäuser, B., Strähle, U., Götz, M., Bally-Cuif, L., 2006. Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. Dev. Biol. 295, 278–293. https://doi.org/10.1016/j.ydbio.2006.03.023

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., 2010. Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1217, 6791–6806. https://doi.org/10.1016/j.chroma.2010.08.033

Alsop, D., Vijayan, M.M., 2008. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R711-719. https://doi.org/10.1152/ajpregu.00671.2007

Atif, F., Yousuf, S., Stein, D.G., 2015. Anti-tumor effects of progesterone in human glioblastoma multiforme: role of PI3K/Akt/mTOR signaling. J. Steroid Biochem. Mol. Biol. 146, 62–73. https://doi.org/10.1016/j.jsbmb.2014.04.007

Azcoitia, I., Sierra, A., Veiga, S., Honda, S., Harada, N., Garcia-Segura, L.M., 2001. Brain aromatase is neuroprotective. J. Neurobiol. 47, 318–329.

Bain, P.A., Kumar, A., Ogino, Y., Iguchi, T., 2015. Nortestosterone-derived synthetic progestogens do not activate the progestogen receptor of Murray-Darling rainbowfish (Melanotaenia fluviatilis) but are potent agonists of androgen receptors alpha and beta. Aquat. Toxicol. Amst. Neth. 163, 97–101. https://doi.org/10.1016/j.aquatox.2015.03.021

Birnbaum, L.S., 2012. Environmental chemicals: evaluating low-dose effects. Environ. Health Perspect. 120, A143-144. https://doi.org/10.1289/ehp.1205179

Bohacek, J., Daniel, J.M., 2009. The ability of oestradiol administration to regulate protein levels of oestrogen receptor alpha in the hippocampus and prefrontal cortex of middle-aged rats is altered following long-term ovarian hormone deprivation. J. Neuroendocrinol. 21, 640–647. https://doi.org/10.1111/j.1365-2826.2009.01882.x

Bosco, A., Bureau, C., Affaticati, P., Gaspar, P., Bally-Cuif, L., Lillesaar, C., 2013. Development of hypothalamic serotoninergic neurons requires Fgf signalling via the ETS-domain transcription factor Etv5b. Dev. Camb. Engl. 140, 372–384. https://doi.org/10.1242/dev.089094

Bowers, J.M., Waddell, J., McCarthy, M.M., 2010. A developmental sex difference in hippocampal neurogenesis is mediated by endogenous oestradiol. Biol. Sex Differ. 1, 8. https://doi.org/10.1186/2042-6410-1-8

Bowman, C.J., Kroll, K.J., Gross, T.G., Denslow, N.D., 2002. Estradiol-induced gene expression in largemouth bass (Micropterus salmoides). Mol. Cell. Endocrinol. 196, 67–77.

Brinton, R.D., Thompson, R.F., Foy, M.R., Baudry, M., Wang, J., Finch, C.E., Morgan, T.E., Pike, C.J., Mack, W.J., Stanczyk, F.Z., Nilsen, J., 2008. Progesterone receptors: form and function in brain. Front. Neuroendocrinol. 29, 313–339. https://doi.org/10.1016/j.yfrne.2008.02.001

Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.-K., Chung, B., Kah, O., 2012. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. PloS One 7, e36069. https://doi.org/10.1371/journal.pone.0036069

Brock, O., Keller, M., Veyrac, A., Douhard, Q., Bakker, J., 2010. Short term treatment with estradiol decreases the rate of newly generated cells in the subventricular zone and main olfactory bulb of adult female mice. Neuroscience 166, 368–376. https://doi.org/10.1016/j.neuroscience.2009.12.050

Cano-Nicolau, J., Garoche, C., Hinfray, N., Pellegrini, E., Boujrad, N., Pakdel, F., Kah, O., Brion, F., 2016. Several synthetic progestins disrupt the glial cell specific-brain aromatase expression in developing zebra fish. Toxicol. Appl. Pharmacol. 305, 12–21. https://doi.org/10.1016/j.taap.2016.05.019

Chapouton, P., Webb, K.J., Stigloher, C., Alunni, A., Adolf, B., Hesl, B., Topp, S., Kremmer, E., Bally-Cuif, L., 2011. Expression of hairy/enhancer of split genes in neural progenitors and neurogenesis domains of the adult zebrafish brain. J. Comp. Neurol. 519, 1748–1769. https://doi.org/10.1002/cne.22599

Chitnis, A.B., Kuwada, J.Y., 1990. Axonogenesis in the brain of zebrafish embryos. J. Neurosci. Off. J. Soc. Neurosci. 10, 1892–1905.

Coumailleau, P., Pellegrini, E., Adrio, F., Diotel, N., Cano-Nicolau, J., Nasri, A., Vaillant, C., Kah, O., 2015. Aromatase, estrogen receptors and brain development in fish and amphibians. Biochim. Biophys. Acta 1849, 152–162. https://doi.org/10.1016/j.bbagrm.2014.07.002

Deutsch, E.R., Espinoza, T.R., Atif, F., Woodall, E., Kaylor, J., Wright, D.W., 2013. Progesterone's role in neuroprotection, a review of the evidence. Brain Res. 1530, 82–105. https://doi.org/10.1016/j.brainres.2013.07.014

Diotel, N., Charlier, T.D., Lefebvre d'Hellencourt, C., Couret, D., Trudeau, V.L., Nicolau, J.C., Meilhac, O., Kah, O., Pellegrini, E., 2018. Steroid Transport, Local Synthesis, and Signaling within the Brain: Roles in Neurogenesis, Neuroprotection, and Sexual Behaviors. Front. Neurosci. 12, 84. https://doi.org/10.3389/fnins.2018.00084

Diotel, N., Servili, A., Gueguen, M.-M., Mironov, S., Pellegrini, E., Vaillant, C., Zhu, Y., Kah, O., Anglade, I., 2011. Nuclear progesterone receptors are up-regulated by estrogens in neurons and radial glial progenitors in the brain of zebrafish. PloS One 6, e28375. https://doi.org/10.1371/journal.pone.0028375

Diotel, N., Vaillant, C., Gabbero, C., Mironov, S., Fostier, A., Gueguen, M.-M., Anglade, I., Kah, O., Pellegrini, E., 2013. Effects of estradiol in adult neurogenesis and brain repair in zebrafish. Horm. Behav. 63, 193–207. https://doi.org/10.1016/j.yhbeh.2012.04.003

Duarte-Guterman, P., Lieblich, S.E., Chow, C., Galea, L.A.M., 2015a. Estradiol and GPER Activation Differentially Affect Cell Proliferation but Not GPER Expression in the Hippocampus of Adult Female Rats. PloS One 10, e0129880. https://doi.org/10.1371/journal.pone.0129880

Duarte-Guterman, P., Yagi, S., Chow, C., Galea, L.A.M., 2015b. Hippocampal learning, memory, and neurogenesis: Effects of sex and estrogens across the lifespan in adults. Horm. Behav. 74, 37–52. https://doi.org/10.1016/j.yhbeh.2015.05.024

Ellestad, L.E., Cardon, M., Chambers, I.G., Farmer, J.L., Hartig, P., Stevens, K., Villeneuve, D.L., Wilson, V., Orlando, E.F., 2014. Environmental gestagens activate fathead minnow

(Pimephales promelas) nuclear progesterone and androgen receptors in vitro. Environ. Sci. Technol. 48, 8179–8187. https://doi.org/10.1021/es501428u

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: Concentrations, effects and risk assessment. Environ. Int. 84, 115–130. https://doi.org/10.1016/j.envint.2015.06.012

Fester, L., Prange-Kiel, J., Zhou, L., Blittersdorf, B.V., Bohm, J., Jarry, H., Schumacher, M., Rune, G.M., 2012. Estrogen-regulated synaptogenesis in the hippocampus: sexual dimorphism in vivo but not in vitro. J. Steroid Biochem. Mol. Biol. 131, 24–29. https://doi.org/10.1016/j.jsbmb.2011.11.010

Flouriot, G., Pakdel, F., Ducouret, B., Ledrean, Y., Valotaire, Y., 1997. Differential regulation of two genes implicated in fish reproduction: vitellogenin and estrogen receptor genes. Mol. Reprod. Dev. 48, 317–323. https://doi.org/10.1002/(SICI)1098-2795(199711)48:3<317::AID-MRD3>3.0.CO;2-U

Forlano, P.M., Deitcher, D.L., Bass, A.H., 2005. Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. J. Comp. Neurol. 483, 91–113. https://doi.org/10.1002/cne.20397

Foster, T.C., 2012. Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging. Hippocampus 22, 656–669. https://doi.org/10.1002/hipo.20935

Genovese, G., Regueira, M., Da Cuña, R.H., Ferreira, M.F., Varela, M.L., Lo Nostro, F.L., 2014. Nonmonotonic response of vitellogenin and estrogen receptor α gene expression after octylphenol exposure of Cichlasoma dimerus (Perciformes, Cichlidae). Aquat. Toxicol. Amst. Neth. 156, 30–40. https://doi.org/10.1016/j.aquatox.2014.07.019

Gonzalez-Orozco, J.C., Camacho-Arroyo, I., 2019. Progesterone Actions During Central Nervous System Development. Front. Neurosci. 13, 503. https://doi.org/10.3389/fnins.2019.00503

Gonzalez-Orozco, J.C., Hansberg-Pastor, V., Valadez-Cosmes, P., Nicolas-Ortega, W., Bastida-Beristain, Y., Fuente-Granada, M.D.L., Gonzalez-Arenas, A., Camacho-Arroyo, I., 2018. Activation of membrane progesterone receptor-alpha increases proliferation, migration, and invasion of human glioblastoma cells. Mol. Cell. Endocrinol. 477, 81–89. https://doi.org/10.1016/j.mce.2018.06.004

Guennoun, R., Zhu, X., Frechou, M., Gaignard, P., Slama, A., Liere, P., Schumacher, M., 2019. Steroids in Stroke with Special Reference to Progesterone. Cell. Mol. Neurobiol. 39, 551–568. https://doi.org/10.1007/s10571-018-0627-0

Guerra-Araiza, C., Villamar-Cruz, O., González-Arenas, A., Chavira, R., Camacho-Arroyo, I., 2003. Changes in progesterone receptor isoforms content in the rat brain during the oestrous cycle and after oestradiol and progesterone treatments. J. Neuroendocrinol. 15, 984–990.

Gunnarsson, L., Snape, J.R., Verbruggen, B., Owen, S.F., Kristiansson, E., Margiotta-Casaluci, L., Osterlund, T., Hutchinson, K., Leverett, D., Marks, B., Tyler, C.R., 2019. Pharmacology beyond the patient - The environmental risks of human drugs. Environ. Int. 129, 320–332. https://doi.org/10.1016/j.envint.2019.04.075

Hanna, R.N., Daly, S.C.J., Pang, Y., Anglade, I., Kah, O., Thomas, P., Zhu, Y., 2010. Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. Biol. Reprod. 82, 112–122. https://doi.org/10.1095/biolreprod.109.078527

Hanna, R.N., Zhu, Y., 2009. Expression of membrane progestin receptors in zebrafish (Danio rerio) oocytes, testis and pituitary. Gen. Comp. Endocrinol. 161, 153–157. https://doi.org/10.1016/j.ygcen.2008.10.006

Heberden, C., 2017. Sex steroids and neurogenesis. Biochem. Pharmacol. 141, 56–62. https://doi.org/10.1016/j.bcp.2017.05.019

Huang, S., Sato, S., 1998. Progenitor cells in the adult zebrafish nervous system express a Brn-1-related POU gene, tai-ji. Mech. Dev. 71, 23–35.

James, M.O., 2011. Steroid catabolism in marine and freshwater fish. J. Steroid Biochem. Mol. Biol. 127, 167–175. https://doi.org/10.1016/j.jsbmb.2010.10.003

Kelder, J., Azevedo, R., Pang, Y., de Vlieg, J., Dong, J., Thomas, P., 2010. Comparison between steroid binding to membrane progesterone receptor alpha (mPRalpha) and to nuclear progesterone receptor: correlation with physicochemical properties assessed by comparative molecular field analysis and identification of mPRalpha-specific agonists. Steroids 75, 314–322. https://doi.org/10.1016/j.steroids.2010.01.010

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U. S. A. 104, 8897–8901. https://doi.org/10.1073/pnas.0609568104

Kudwa, A.E., Gustafsson, J.-A., Rissman, E.F., 2004. Estrogen receptor beta modulates estradiol induction of progestin receptor immunoreactivity in male, but not in female, mouse medial preoptic area. Endocrinology 145, 4500–4506. https://doi.org/10.1210/en.2003-1708

Kudwa, A.E., Harada, N., Honda, S.-I., Rissman, E.F., 2009. Regulation of progestin receptors in medial amygdala: estradiol, phytoestrogens and sex. Physiol. Behav. 97, 146–150. https://doi.org/10.1016/j.physbeh.2009.02.023

Kumar, V., Johnson, A.C., Trubiroha, A., Tumová, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kroupová, H.K., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49, 2625–2638. https://doi.org/10.1021/es5051343

Kurogi, K., Liu, T.-A., Sakakibara, Y., Suiko, M., Liu, M.-C., 2013. The use of zebrafish as a model system for investigating the role of the SULTs in the metabolism of endogenous compounds and xenobiotics. Drug Metab. Rev. 45, 431–440. https://doi.org/10.3109/03602532.2013.835629

Le Fol, V., Brion, F., Hillenweck, A., Perdu, E., Bruel, S., Ait-Aissa, S., Cravedi, J.-P., Zalko, D., 2017. Comparison of the In Vivo Biotransformation of Two Emerging Estrogenic Contaminants, BP2 and BPS, in Zebrafish Embryos and Adults. Int. J. Mol. Sci. 18. https://doi.org/10.3390/ijms18040704

Lillesaar, C., 2011. The serotonergic system in fish. J. Chem. Neuroanat. 41, 294–308. https://doi.org/10.1016/j.jchemneu.2011.05.009

Liu, L., Zhao, L., She, H., Chen, S., Wang, J.M., Wong, C., McClure, K., Sitruk-Ware, R., Brinton, R.D., 2010. Clinically relevant progestins regulate neurogenic and neuroprotective responses in vitro and in vivo. Endocrinology 151, 5782–5794. https://doi.org/10.1210/en.2010-0005

Liu, S.-S., Ying, G.-G., Liu, S., Lai, H.-J., Chen, Z.-F., Pan, C.-G., Zhao, J.-L., Chen, J., 2014. Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment. Anal. Bioanal. Chem. 406, 7299–7311. https://doi.org/10.1007/s00216-014-8146-4

Liu, X., Zhu, P., Sham, K.W.Y., Yuen, J.M.L., Xie, C., Zhang, Y., Liu, Y., Li, S., Huang, X., Cheng, C.H.K., Lin, H., 2009. Identification of a membrane estrogen receptor in zebrafish with homology to mammalian GPER and its high expression in early germ cells of the testis. Biol. Reprod. 80, 1253–1261. https://doi.org/10.1095/biolreprod.108.070250

Makantasi, P., Dermon, C.R., 2014. Estradiol treatment decreases cell proliferation in the neurogenic zones of adult female zebrafish (Danio rerio) brain. Neuroscience 277, 306–320. https://doi.org/10.1016/j.neuroscience.2014.06.071

Mangiamele, L.A., Gomez, J.R., Curtis, N.J., Thompson, R.R., 2017. GPER/GPR30, a membrane estrogen receptor, is expressed in the brain and retina of a social fish (Carassius auratus) and colocalizes with isotocin. J. Comp. Neurol. 525, 252–270. https://doi.org/10.1002/cne.24056

Marlatt, V.L., Martyniuk, C.J., Zhang, D., Xiong, H., Watt, J., Xia, X., Moon, T., Trudeau, V.L., 2008. Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17beta-estradiol action in the neuroendocrine axis of male goldfish. Mol. Cell. Endocrinol. 283, 38–48. https://doi.org/10.1016/j.mce.2007.10.013

Martins, S.I., Madeira, M.D., Sá, S.I., 2015. Effects of gonadal steroids and of estrogen receptor agonists on the expression of estrogen receptor alpha in the medial preoptic nucleus of female rats. Neuroscience 310, 63–72. https://doi.org/10.1016/j.neuroscience.2015.09.030

Marz, M., Chapouton, P., Diotel, N., Vaillant, C., Hesl, B., Takamiya, M., Lam, C.S., Kah, O., Bally-Cuif, L., Strahle, U., 2010. Heterogeneity in progenitor cell subtypes in the ventricular zone of the zebrafish adult telencephalon. Glia 58, 870–888. https://doi.org/10.1002/glia.20971

Mazzucco, C.A., Lieblich, S.E., Bingham, B.I., Williamson, M.A., Viau, V., Galea, L.A.M., 2006. Both estrogen receptor alpha and estrogen receptor beta agonists enhance cell proliferation in the dentate gyrus of adult female rats. Neuroscience 141, 1793–1800. https://doi.org/10.1016/j.neuroscience.2006.05.032

Mendoza-Garcés, L., Rodríguez-Dorantes, M., Alvarez-Delgado, C., Vázquez-Martínez, E.R., Garcia-Tobilla, P., Cerbón, M.A., 2013. Differential DNA methylation pattern in the A and B promoters of the progesterone receptor is associated with differential mRNA expression in the female rat hypothalamus during proestrus. Brain Res. 1535, 71–77. https://doi.org/10.1016/j.brainres.2013.08.042

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. J. Mol. Endocrinol. 32, 975–986.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881–1892.

Menuet, A., Pellegrini, E., Brion, F., Gueguen, M.-M., Anglade, I., Pakdel, F., Kah, O., 2005. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. J. Comp. Neurol. 485, 304–320. https://doi.org/10.1002/cne.20497

Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashi, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Schaeffer, J.M., McEwen, B.S., Alves, S.E., 2003. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144, 2055–2067. https://doi.org/10.1210/en.2002-221069

Mitterling, K.L., Spencer, J.L., Dziedzic, N., Shenoy, S., McCarthy, K., Waters, E.M., McEwen, B.S., Milner, T.A., 2010. Cellular and subcellular localization of estrogen and progestin receptor immunoreactivities in the mouse hippocampus. J. Comp. Neurol. 518, 2729–2743. https://doi.org/10.1002/cne.22361

Mouriec, K., Lareyre, J.J., Tong, S.K., Le Page, Y., Vaillant, C., Pellegrini, E., Pakdel, F., Chung, B.C., Kah, O., Anglade, I., 2009. Early regulation of brain aromatase (cyp19a1b) by

estrogen receptors during zebrafish development. Dev. Dyn. Off. Publ. Am. Assoc. Anat. 238, 2641–2651. https://doi.org/10.1002/dvdy.22069

Mueller, T., Wullimann, M.F., 2005. Atlas of early zebrafish brain development. Am. J. Physiol. Regul. Integr. Comp. Physiol.

Murashov, A.K., Pak, E.S., Hendricks, W.A., Tatko, L.M., 2004. 17beta-Estradiol enhances neuronal differentiation of mouse embryonic stem cells. FEBS Lett. 569, 165–168. https://doi.org/10.1016/j.febslet.2004.05.042

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24. https://doi.org/10.1016/j.ygcen.2013.03.032

Nesan, D., Vijayan, M.M., 2013. The transcriptomics of glucocorticoid receptor signaling in developing zebrafish. PloS One 8, e80726. https://doi.org/10.1371/journal.pone.0080726

Ormerod, B.K., Lee, T.T.-Y., Galea, L. a. M., 2003. Estradiol initially enhances but subsequently suppresses (via adrenal steroids) granule cell proliferation in the dentate gyrus of adult female rats. J. Neurobiol. 55, 247–260. https://doi.org/10.1002/neu.10181

Pakdel, F., Féon, S., Le Gac, F., Le Menn, F., Valotaire, Y., 1991. In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol. Cell. Endocrinol. 75, 205–212.

Pawlowski, S., van Aerle, R., Tyler, C.R., Braunbeck, T., 2004. Effects of 17alpha-ethinylestradiol in a fathead minnow (Pimephales promelas) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57, 330–345. https://doi.org/10.1016/j.ecoenv.2003.07.019

Pawluski, J.L., Brummelte, S., Barha, C.K., Crozier, T.M., Galea, L.A.M., 2009. Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. Front. Neuroendocrinol. 30, 343–357. https://doi.org/10.1016/j.yfrne.2009.03.007

Pellegrini, E., Coumailleau, P., Kah, O., Diotel, N., 2015. Aromatase and Estrogens: Involvement in Constitutive and Regenerative Neurogenesis in Adult Zebrafish, in: Estrogen Effects on Traumatic Brain Injury: Mechanims of Neuroprotection and Repair. Duncan K., pp. 51–65.

Pellegrini, E., Mouriec, K., Anglade, I., Menuet, A., Le Page, Y., Gueguen, M.-M., Marmignon, M.-H., Brion, F., Pakdel, F., Kah, O., 2007. Identification of aromatase-positive radial glial cells as progenitor cells in the ventricular layer of the forebrain in zebrafish. J. Comp. Neurol. 501, 150–167. https://doi.org/10.1002/cne.21222

Perez, M.R., Pellegrini, E., Cano-Nicolau, J., Gueguen, M.-M., Menouer-Le Guillou, D., Merot, Y., Vaillant, C., Somoza, G.M., Kah, O., 2013. Relationships between radial glial progenitors and 5-HT neurons in the paraventricular organ of adult zebrafish - potential effects of serotonin on adult neurogenesis. Eur. J. Neurosci. 38, 3292–3301. https://doi.org/10.1111/ejn.12348

Quadros, P.S., Wagner, C.K., 2008. Regulation of progesterone receptor expression by estradiol is dependent on age, sex and region in the rat brain. Endocrinology 149, 3054–3061. https://doi.org/10.1210/en.2007-1133

Rossetti, M.F., Cambiasso, M.J., Holschbach, M.A., Cabrera, R., 2016. Oestrogens and Progestagens: Synthesis and Action in the Brain. J. Neuroendocrinol. 28. https://doi.org/10.1111/jne.12402

Runnalls, T.J., Beresford, N., Kugathas, S., Margiotta-Casaluci, L., Scholze, M., Scott, A.P., Sumpter, J.P., 2015. From single chemicals to mixtures--reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow. Aquat. Toxicol. Amst. Neth. 169, 152–167. https://doi.org/10.1016/j.aquatox.2015.10.009

Sager, T., Kashon, M., Krajnak, K., 2017. Estrogen and environmental enrichment differentially affect neurogenesis, dendritic spine immunolabeling and synaptogenesis in the hippocampus of young and reproductively senescent female rats. Neuroendocrinology. https://doi.org/10.1159/000479699

Sakamoto, H., Ukena, K., Tsutsui, K., 2001. Effects of progesterone synthesized de novo in the developing Purkinje cell on its dendritic growth and synaptogenesis. J. Neurosci. Off. J. Soc. Neurosci. 21, 6221–6232.

Sakamoto, T., Yoshiki, M., Takahashi, H., Yoshida, M., Ogino, Y., Ikeuchi, T., Nakamachi, T., Konno, N., Matsuda, K., Sakamoto, H., 2016. Principal function of mineralocorticoid signaling suggested by constitutive knockout of the mineralocorticoid receptor in medaka fish. Sci. Rep. 6, 37991. https://doi.org/10.1038/srep37991

Sasaki, T., Kitagawa, K., Yagita, Y., Sugiura, S., Omura-Matsuoka, E., Tanaka, S., Matsushita, K., Okano, H., Tsujimoto, Y., Hori, M., 2006. Bcl2 enhances survival of newborn neurons in the normal and ischemic hippocampus. J. Neurosci. Res. 84, 1187–1196. https://doi.org/10.1002/jnr.21036

Schaaf, M.J.M., Champagne, D., van Laanen, I.H.C., van Wijk, D.C.W.A., Meijer, A.H., Meijer, O.C., Spaink, H.P., Richardson, M.K., 2008. Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. Endocrinology 149, 1591–1599. https://doi.org/10.1210/en.2007-1364

Schoonen, W.G., Deckers, G.H., de Gooijer, M.E., de Ries, R., Kloosterboer, H.J., 2000. Hormonal properties of norethisterone, 7alpha-methyl-norethisterone and their derivatives. J. Steroid Biochem. Mol. Biol. 74, 213–222.

Simerly, R.B., Young, B.J., 1991. Regulation of estrogen receptor messenger ribonucleic acid in rat hypothalamus by sex steroid hormones. Mol. Endocrinol. Baltim. Md 5, 424–432. https://doi.org/10.1210/mend-5-3-424

Soffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish--a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42, 653–668. https://doi.org/10.3109/10408444.2012.692114

Strobl-Mazzulla, P.H., Nuñez, A., Pellegrini, E., Gueguen, M.-M., Kah, O., Somoza, G.M., 2010. Progenitor radial cells and neurogenesis in pejerrey fish forebrain. Brain. Behav. Evol. 76, 20–31. https://doi.org/10.1159/000316022

Takahashi, H., Sakamoto, T., 2013. The role of "mineralocorticoids" in teleost fish: relative importance of glucocorticoid signaling in the osmoregulation and "central" actions of mineralocorticoid receptor. Gen. Comp. Endocrinol. 181, 223–228. https://doi.org/10.1016/j.ygcen.2012.11.016

Tanapat, P., Hastings, N.B., Reeves, A.J., Gould, E., 1999. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J. Neurosci. Off. J. Soc. Neurosci. 19, 5792–5801.

Thomas, P., Pang, Y., 2012. Membrane progesterone receptors: evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells. Neuroendocrinology 96, 162–171. https://doi.org/10.1159/000339822

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., Tubbs, C., 2007. Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology 148, 705–718. https://doi.org/10.1210/en.2006-0974

Tillet, Y., Ravault, J.P., Selve, C., Evin, G., Castro, B., Dubois, M.P., 1986. [Conditions for the use of specific antibodies for immunohistochemical visualization of serotonin and melatonin in the pineal gland of sheep]. C. R. Acad. Sci. III 303, 77–82.

Tong, S.-K., Mouriec, K., Kuo, M.-W., Pellegrini, E., Gueguen, M.-M., Brion, F., Kah, O., Chung, B., 2009. A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. Genes. N. Y. N 2000 47, 67–73. https://doi.org/10.1002/dvg.20459

Tsutsui, K., 2008. Progesterone biosynthesis and action in the developing neuron. Endocrinology 149, 2757–2761. https://doi.org/10.1210/en.2007-1592

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.-H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455. https://doi.org/10.1210/er.2011-1050

Verbueken, E., Bars, C., Ball, J.S., Periz-Stanacev, J., Marei, W.F.A., Tochwin, A., Gabriels, I.J., Michiels, E.D.G., Stinckens, E., Vergauwen, L., Knapen, D., Van Ginneken, C.J., Van Cruchten, S.J., 2018. From mRNA Expression of Drug Disposition Genes to In Vivo Assessment of. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19123976

Wang, L., Andersson, S., Warner, M., Gustafsson, J.-A., 2003. Estrogen receptor (ER)beta knockout mice reveal a role for ERbeta in migration of cortical neurons in the developing brain. Proc. Natl. Acad. Sci. U. S. A. 100, 703–708. https://doi.org/10.1073/pnas.242735799

Wang, L., Andersson, S., Warner, M., Gustafsson, J.A., 2001. Morphological abnormalities in the brains of estrogen receptor beta knockout mice. Proc. Natl. Acad. Sci. U. S. A. 98, 2792–2796. https://doi.org/10.1073/pnas.041617498

Waters, E.M., Simerly, R.B., 2009. Estrogen induces caspase-dependent cell death during hypothalamic development. J. Neurosci. Off. J. Soc. Neurosci. 29, 9714–9718. https://doi.org/10.1523/JNEUROSCI.0135-09.2009

Weber, D.N., Hoffmann, R.G., Hoke, E.S., Tanguay, R.L., 2015. Bisphenol A exposure during early development induces sex-specific changes in adult zebrafish social interactions. J. Toxicol. Environ. Health A 78, 50–66. https://doi.org/10.1080/15287394.2015.958419

Yao, X.-L., Liu, J., Lee, E., Ling, G.S.F., McCabe, J.T., 2005. Progesterone differentially regulates pro- and anti-apoptotic gene expression in cerebral cortex following traumatic brain injury in rats. J. Neurotrauma 22, 656–668. https://doi.org/10.1089/neu.2005.22.656

Zhang, Y.T., Liu, D.T., Zhu, Y., Chen, S.X., Hong, W.S., 2016. Cloning and olfactory expression of progestin receptors in the Chinese black sleeper Bostrichthys sinensis. Gen. Comp. Endocrinol. 230–231, 87–102. https://doi.org/10.1016/j.ygcen.2016.03.029

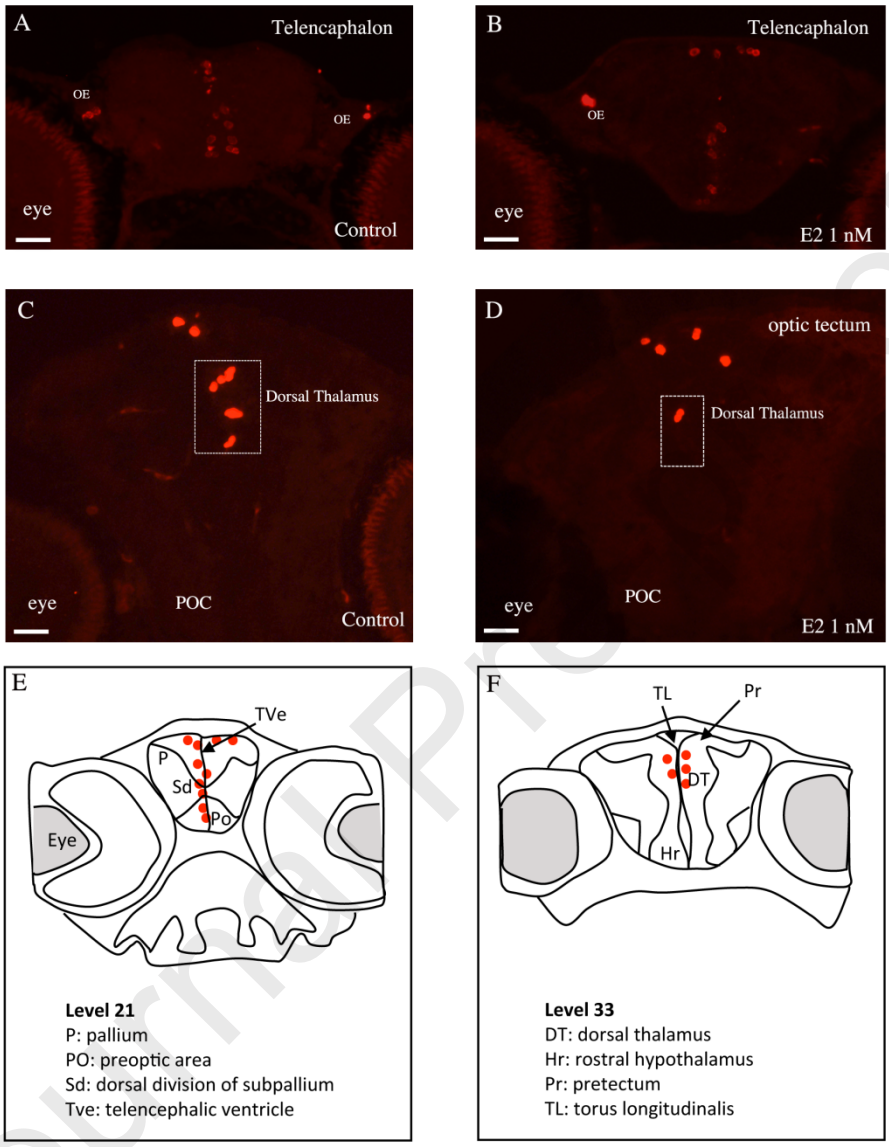
Zhang, Z., Yang, R., Zhou, R., Li, L., Sokabe, M., Chen, L., 2010. Progesterone promotes the survival of newborn neurons in the dentate gyrus of adult male mice. Hippocampus 20, 402–412. https://doi.org/10.1002/hipo.20642

Zhu, Y., Rice, C.D., Pang, Y., Pace, M., Thomas, P., 2003. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. U. S. A. 100, 2231–2236. https://doi.org/10.1073/pnas.0336132100

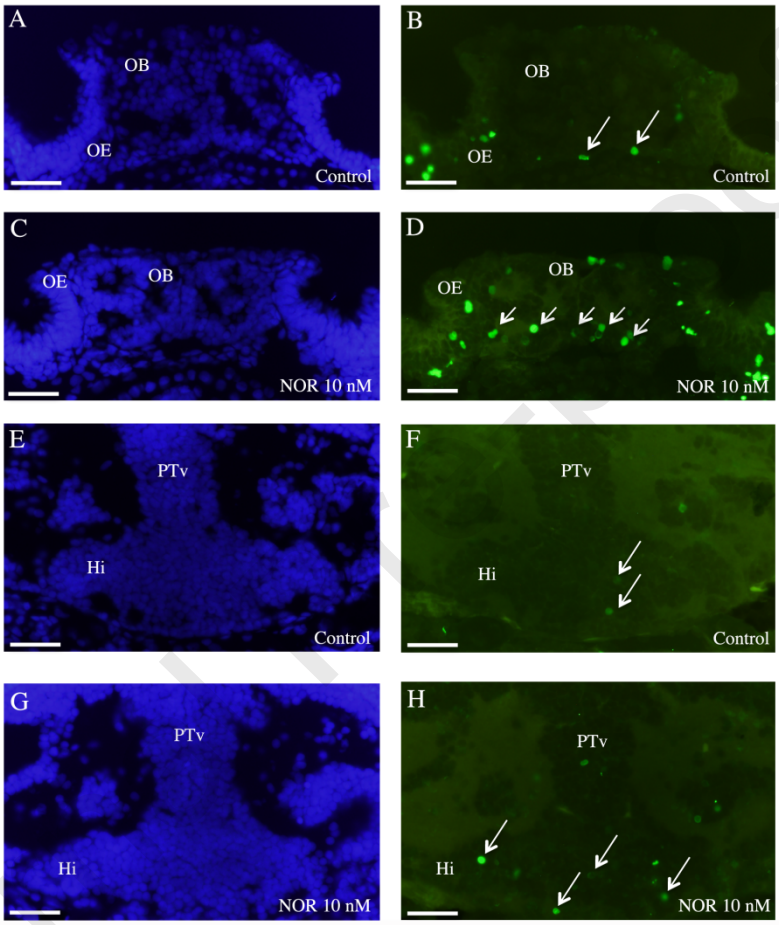
Zucchi, S., Castiglioni, S., Fent, K., 2013. Progesterone alters global transcription profiles at environmental concentrations in brain and ovary of female zebrafish (Danio rerio). Environ. Sci. Technol. 47, 12548–12556. https://doi.org/10.1021/es403800y

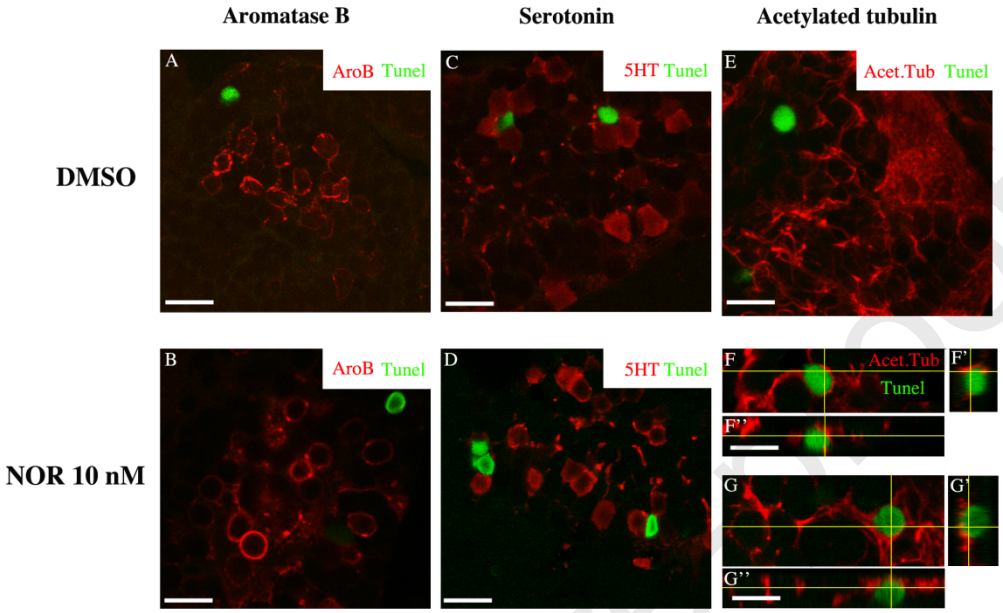
Zucchi, S., Castiglioni, S., Fent, K., 2012. Progestins and antiprogestins affect gene expression in early development in zebrafish (Danio rerio) at environmental concentrations. Environ. Sci. Technol. 46, 5183–5192. https://doi.org/10.1021/es300231y

Brdu-positive cells in the telencephalon and in the dorsal thalamus



Number of apoptotic cells in olfactory bulbs and in hypothalamus





Gene	Accession no.	Sequence
ef1	NM_131263.1	**F:** 5'-AGCAGCAGCTGAGGAGTGAT-3′ **R:** 5'-CCGCATTTGTAGATCAGATGG-3′
cyp19a1b	NM_131642.1	**F:** 5'-TCGGCACGGCGTGCAACTAC-3′ **R:** 5'-CATACCTATGCATTGCAGACC-3′
esr1	NM_152959.1	**F:** 5’-CTGGAGATG CTGGACGCTCA-3’ **R:** 5’-GCTGCAGCTCCTCCTCCTGG-3’
esr2a	NM_180966.2	**F:** 5’-GATCCTCCTGAACTCCAACATG-3’ **R:** 5’-CCAGCAGACACAGCAGCTTGG-3’
esr2b	NM_174862.3	**F:** 5’-GATCCTGCTCAACTCTAATAAC-3’ **R:** 5’-CCAGCAGATTCAGCACCTTCCC-3’
nPR	FJ409244.1	**F:** 5’-GAGCAATGATCAGCTGAGAAGG-3’ **R:** 5’-TCCAGAGGAACAGTGTTGA GG-3’

Primary antibodies			
Antibodies	**Antibody ID**	**Antigen**	**Reference and dilution**
Monoclonal Anti-BrU	RRID AB_10013660	Bromodeoxyuridine conjugated to bovine serum albumin	Clone BU20a Ref MO744 Dako Dilution 1:100
Monoclonal Anti-acetylated tubulin	RRID AB_477585	Acetylated tubulin from the outer arm of sea urchin sperm axonemes	Clone 6-11B-1 Ref T673 Sigma Dilution 1 :100
Secondary antibodies			
Antibodies	**Antibody ID**	**Reference and dilution**	
Goat anti-mouse Alexa Fluor 594	RRID AB_141974	Ref A-11020 In vitrogen Molecular probes Thermo Fisher Scientific Dilution 1:500	
Goat anti-rabbit Alexa Fluor 594	RRID AB_2534095	Ref A-11037 In vitrogen Molecular probes Thermo Fisher Scientific Dilution 1:500	

Fold induction of steroid receptors expression compared to control (DMSO)

	Steroids	P4			E2			NOR		
	Concentration (nM)	0.1	1	10	0.1	1	10	0.1	1	10
Genes of interest	***nPR***	1.9 (+/- 0.23) *	1.5 (+/- 0.06)	2.9 (+/- 0.75) **	0.9 (+/- 0.17)	2.1 (+/- 0.33) **	1.9 (+/- 0.28) *	1.5 (+/- 0.21)	1.3 (+/- 0.26)	2.2 (+/- 0.7) *
	esr1	1.0 (+/- 0.10)	0.8 (+/- 0.12)	1.0 (+/- 0.16)	1.0 (+/- 0.10)	1.9 (+/- 0.55) *	1.4 (+/- 0.22)	0.9 (+/- 0.19)	1.1 (+/- 0.10)	0.9 (+/- 0.10)
	esr2b	1.1 (+/- 0.03)	1.1 (+/- 0.10)	1.0 (+/- 0.08)	1.1 (+/- 0.16)	1.5 (+/- 0.21) *	1.1 (+/- 0.10)	1.1 (+/- 0.10)	1.0 (+/- 0.10)	1.1 (+/- 0.10)
	esr2a	1.2 (+/- 0.10)	1.2 (+/- 0.14)	1.7 (+/- 0.37) *	1.0 (+/- 0.10)	1.6 (+/- 0.21) *	1.4 (+/- 0.20)	0.8 (+/- 0.13)	1.0 (+/- 0.21)	0.8 (+/- 0.10)

nPR: Nuclear Progesterone Receptor; ***esr1***: Nuclear Estrogen Receptor α; ***esr2b***: Nuclear Estrogen Receptor β1; ***esr2a***: Nuclear Estrogen Receptor β2.

*p < .05; **p < .005; compared to control condition.

Highlights

1) Estradiol, progesterone and norethindrone, a 19-nortestosterone synthetic progestin with estrogenic properties, affect brain expression of estrogen and progesterone nuclear receptors in zebrafish larvae.
2) Progesterone inhibits the proliferation in the brain of zebrafish larvae.
3) Estradiol inhibits the proliferation in the brain of zebrafish larvae.
4) Norethindrone increases the number of apoptotic cells in the brain of zebrafish larvae.
5) The effects induced by the three steroids on zebrafish brain development were clearly different.