Dopamine Inhibits Reproduction in Female Zebrafish (*Danio rerio*) via Three Pituitary D2 Receptor Subtypes

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In many teleosts, the stimulatory control of gonadotrope axis by GnRH is opposed by an inhibitory control by dopamine (DA). The functional importance of this inhibitory pathway differs widely from one teleostean species to another. The zebrafish (*Danio rerio*) is a teleost fish that has become increasingly popular as an experimental vertebrate model. However, the role of DA in the neuroendocrine control of its reproduction has never been studied. Here the authors evaluated in sexually regressed female zebrafish the effects of in vivo treatments with a DA D2 receptor (D2-R) antagonist domperidone, or a GnRH agonist, alone and in combination, on the pituitary level of FSHβ and LHβ transcripts, the gonadosomatic index, and the ovarian histology. Only the double treatment with GnRH agonist and domperidone could induce an increase in the expression of LHβ, in the gonadosomatic index, and a stimulation of ovarian vitellogenesis, indicating that removal of dopaminergic inhibition is required for the stimulatory action of GnRH and reactivation of ovarian function to occur. Using double immunofluorescent staining on pituitary, the authors showed in this species the innervation of LH cells by tyrosine-hydroxylase immunoreactive fibers. Finally, using in situ hybridization and immunofluorescence, the authors showed that the three subtypes of zebrafish DA D2-R (D2a, D2b, and D2c) were expressed in LH-producing cells, suggesting that they all may be involved in mediating this inhibition. These results show for the first time that, in zebrafish, DA has a direct and potent inhibitory action capable of opposing the stimulatory effect of GnRH in the neuroendocrine control of reproduction. **(*Endocrinology* 154: 807–818, 2013)**

In fish, as in all vertebrates, the brain–pituitary–gonad axis regulates the reproductive function. External and internal signals are integrated in the brain primarily by preoptic-hypothalamic neurons producing the GnRHs that stimulate in the pituitary the synthesis and release of gonadotropins, FSH, and LH, which in turn stimulate gonadal activity (steroidogenesis and gametogenesis).

In teleost fish, in addition to the GnRH stimulatory system, neurons secreting dopamine (DA) have been identified as an inhibitory system over the reproductive axis (1–3). Such a dual neuroendocrine control of reproduction by GnRH and DA has been demonstrated in several adult teleosts (for review, see Ref. 4). DA may inhibit both basal and GnRH-stimulated LH secretion and thereby regulate the final stages of gametogenesis (oocyte maturation, ovulation in the female, and spermiation in male). The intensity of the dopaminergic inhibitory tone at the time of spawning may vary according to teleost species, from a drastic barrier in the grey mullet (5) to a milder control in salmonids (6). In some species, for instance the Atlantic

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Abbreviations: DA, dopamine; Domp, domperidone; FISH, fluorescent in situ hybridization; GnRHa, GnRH agonist; GSI, gonadosomatic index; ir, immunoreactive; PBST, phosphate buffered saline with Tween; PI, pars intermedia; PPD, proximalis pars distalis; RPD, rostral pars distalis; SSC, saline sodium citrate buffer; TH, tyrosine hydroxylase.

croaker, no dopaminergic inhibition could be seen (7). The DA inhibitory control of gonadotrope function also could be active in juveniles of some species and thus participate in the control of puberty, such as in the European eel (8, 9) and grey mullet (5).

Neuroanatomical investigations in goldfish (3, 10–12), trout (13), and European eel (9, 14) have shown that the DA neurons responsible for the inhibitory control of reproduction originate in a specific nucleus of the preoptic area (the nucleus preopticus anteroventralis) and project directly to the pituitary region (proximalis pars distalis [PPD]), where gonadotrope cells are located (3, 10).

Pharmacological studies have demonstrated that the inhibitory effects of DA on gonadotropin production are exerted directly at the pituitary level (2, 15) and mediated by D2-type dopaminergic receptors (D2-Rs), in all studied teleosts (for review, see Ref. 4). In mammals and birds, D2-Rs are encoded by a single gene, and alternative splicing gives rise to two proteic isoforms D2l (long) and D2s (short) (16). By contrast, in some teleosts, the coexistence of several genes encoding D2-R subtypes has been demonstrated: two in European eel (17) and three in goldfish (18) and zebrafish (19).

The zebrafish (*Danio rerio*), a member of the Cyprinid group of teleosts, is a small tropical freshwater fish, native to India and Burma. It has become a very popular laboratory animal over the past 15 years. Its major application in scientific research has been initially in developmental biology. Rapidly, however, due to the availability of the genome sequence and of the many hundreds of mutants and transgenic lines, zebrafish appeared as a vertebrate model with an impressive range of possible applications. It is now extensively used for research in physiology (20), cancer (21), drug discovery (22), toxicology (23), ecotoxicology (24), endocrine disruption by chemicals (25, 26), endocrinology (27), behavioral studies (28), for high-throughput screening of drug libraries (29), and for reproduction studies (30–34).

Although the main components of the mammalian hypothalamo-pituitary-gonadal axis have been shown to be present in zebrafish, and GnRH is known to play a conserved stimulating role on the gonadotrope cells (35), the potential role of DA in the neuroendocrine control of zebrafish reproduction is still unknown.

The present study aimed at investigating, in zebrafish, the existence of a DA inhibitory tone on the endocrine reproductive axis. The effects of in vivo treatments with a D2-R antagonist domperidone (Domp), or a GnRH agonist (GnRHa), alone and in combination, were evaluated in old sexually regressed females, by analyzing the pituitary transcript levels of gonadotropins (FSHβ, and LHβ), the gonadosomatic index (GSI), and the ovarian histology. Along with the in vivo actions of DA, the morphological basis of this control was studied in sexually regressed and adult cycling females. We looked for dopaminergic fibers innervating gonadotrope cells in zebrafish pituitary. Subsequently, we sought to identify the pituitary D2-Rs potentially involved in the inhibitory control of gonadotrope function by DA. To this aim, we first investigated whether the three subtypes existing in the zebrafish (D2a, D2b, or D2c) were expressed in the pituitary of sexually regressed and adult cycling females. In addition, we searched which subtype(s) of D2-R was (were) expressed by LH cells.

Materials and Methods

Animals

AB strain zebrafish (*Danio rerio*) were maintained at 26 °C on a 13-hour light, 11-hour dark cycle and fed three times daily. Both 24-month-old sexually regressed females (ie, had ceased cycling) and 4-month-old cycling females were used. Animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

In vivo experiments

A batch of 36 female 24-month-old zebrafish were randomly distributed into four experimental groups of nine fish, each receiving two perivisceral injections (1 μl) at 30-h intervals with 1) a long-acting GnRHa ([des-Gly^{10}, D-Ala^{6}]-LH-RH ethylamide (Sigma, St. Louis, MO); GnRHa, 0.2 mg/kg), or 2) with a DA D2 receptor antagonist, Domp (Sigma, 10 mg/kg, according to the protocol of Refs. 36, 37) alone, 3) both in combination, or 4) diluent (NaCl 0.7% containing 0.1% sodium metabisulfite) for the control group. Fish were killed by rapid immersion in ice water 18 h after the second injection and weighed. The pituitary glands were collected under a dissecting microscope with fine forceps and immersed immediately in ice-cold Trizol (Invitrogen, Life Technologies, St. Aubin, France). The ovaries were removed, weighed for calculation of the GSI (gonad weight/body weight $\times$ 100), and immersed in Bouin's fixative.

Quantification of fshβ and lhβ mRNAs by real-time quantitative RT-PCR

RNA extraction and cDNA synthesis

Total RNA was extracted from the zebrafish pituitaries using Trizol according to the manufacturer's instructions. Samples were then treated with deoxyribonuclease (DNase) I (DNase Free kit; Ambion Inc., Life Technologies), before being suspended in 20 μl of RNA storage solution (Ambion Inc.) and stored at −80°C. First strand cDNA synthesis was performed as described previously (in Ref. 38).

Primers and reference gene

Pituitary mRNA levels of FSH β subunit (Zf FSHβ; GenBank accession no. AY424303), LH β subunit (Zf LHβ; GenBank accession no. AY424304), and the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (Zf Gapdh, accession

no. AY818346) used as a reference gene, were assayed by real-time quantitative PCR, using primers designed previously (see Ref. 39) and purchased from MWG-Biotech AG (Ebersburg, Germany).

SYBR green assay

The quantitative assays of the pituitary expression of the various zebrafish genes were set up using the Light Cycler system with SYBR Green I-sequence unspecific detection (Roche Diagnostics, Meylan, France), as described previously (see Ref. 38). Serial dilutions of a pool from all individual pituitary cDNA samples were used as the standard curve for each gene (gapdh, fshβ, lhβ). Relative expression level of RNA for unknown target gene was quantified in duplicate, by comparing Ct (threshold cycles) values to the specific standard run. The levels of *lhβ* and *fshβ* mRNA were normalized to those of *gapdh* in each corresponding sample and expressed as arbitrary units.

Ovarian histology

Analysis of oocyte stage was conducted on histological paraffin sections (6-7 μm thick) stained with hematoxylin and eosin and examined under a light microscope. Sections from at least three female fish from each treatment group were examined.

Immunofluorescent staining of tyrosine hydroxylase (TH) and LHβ

Tissue preparation

Female zebrafish were killed by rapid immersion in ice water. The skull was opened to facilitate fixation (48 h) of the brain and pituitary by paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania) 4% in phosphate buffered saline with Tween (PBST: PBS, 0.1%; Tween-20, pH 7.3). Brains with attached pituitary were collected with a small piece of the underlying bone. They subsequently were incubated for 15 days at 4°C with EDTA (20%) in PBST, then embedded in 3% agarose and cut (60-μm sagittal sections) with a VT1000S vibratome (Leica, Germany).

Double immunofluorescent staining

Sections were incubated for 2 hours at room temperature in a blocking solution (normal goat serum 4%, dimethylsulfoxide 1%, Triton 0.3% in PBST) and subsequently for 48 hours at 4°C with 1) a mouse monoclonal antibody raised against rat TH, previously validated in zebrafish (see Ref. 40); 1/500; from Eurobio/Abcys, les Ulis, France) and 2) a rabbit antibody directed against the β subunit of carp LH, that shares 74.3% amino acid identity with that of zebrafish (1/2,000; see Ref. 41, in the blocking solution). The secondary antibodies used were 1) a goat antimouse IgG coupled to Alexa-488 and 2) a goat antirabbit IgG coupled to Alexa-633 (Invitrogen). After the sections were washed in PBST, they were postfixed in 4% paraformaldehyde at 4°C, washed again, and mounted in Vectashield H-1000 (Vector, Eurobio/Abcys).

Fluorescent in situ hybridization (FISH)

Tissue preparation

After fixation (as described above), the pituitary glands were collected under a dissecting microscope, dehydrated through an ascending series of ethanol solutions, transferred to 100% methanol and stored at −20°C until use. In some females, the brain was removed, dehydrated, transferred to 100% methanol and stored at −20°C until use. Brains subsequently were rehydrated, embedded in 3% agarose, and cut (60-μm frontal sections) with a VT1000S vibratome.

cRNA probes synthesis

The three zebrafish D2-R genes have been cloned into pCR II Vector (Invitrogen) after PCR amplification of the transcripts, using specific primers according to Boehmler et al (19). Please see Supplemental Tables 1 and 2 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. Antisense and sense cRNA probes were synthesized by in vitro transcription by T7 or Sp6 RNA polymerase (Promega, Madison, Wisconsin) and labeled with fluorescein-12-UTP for D2a, digoxigenin-11-UTP for D2c (Roche Diagnostics), or dinitrophenol-11-UTP (Perkin Elmer, Waltham, Massachusetts) for D2b. Probes were purified using Nucleospin RNA clean-UP kit (Macherey-Nagel, Hoerdt, France); their sizes were analyzed by gel electrophoresis.

In situ hybridization

FISH was performed as described previously (see Ref. 42), with modifications. Briefly, fixed samples (whole-mount pituitaries or floating brain sections) were rehydrated through a descending series of ethanol solutions, permeabilized by a 8-minute treatment with proteinase K (10 μg/ml; P6556, Sigma). Samples were prehybridized in hybridization buffer (42) for 4 hours at 65°C. Hybridization was performed at 65°C for 18 h in hybridization buffer containing a mixture of probes at a concentration of 2 ng/μl, each. Samples were washed (50% formamide/50% 2× SSC [saline sodium citrate buffer]; 2× SSC; 0.2× SSC; PBST), treated for 30 minutes with H_2O_2 3% to inactivate endogenous peroxidases, and washed again in PBST. Probe detection was carried out as follows: 1) incubation with hapten-specific antibodies conjugated to peroxidase, 2) incubation in H_2O_2 0.001% with the suitable fluorescent tyramide, and 3) peroxidase inactivation, by H_2O_2 2%. Fluorescein-labeled D2a probe was recognized by an antibody antifluorescein peroxidase conjugated (Roche Diagnostics) and revealed by a home-made fluorescein-conjugated tyramide (protocol available on Xenbase: http://www.xenbase.org/other/static/methods/FISH.jsp). For digoxigenin-labeled D2c probe, an anti-digoxigenin-peroxidase conjugated antibody (Roche Diagnostics), and a home-made Cy3-conjugated tyramide (protocol available on Xenbase) were used. For dinitrophenyl-labeled D2b probe, an anti-dinitrophenyl-peroxidase conjugated antibody (Perkin Elmer), and a Cy5-conjugated tyramide (Perkin Elmer) were used. After extensive washes, samples were postmounted on SuperfrostPlus slides (VWR International S.A.S., Fontenay-sous-Bois, France) in Vectashield H-1000 Mounting Medium (Vector, Eurobio/Abcys).

Combined FISH and immunofluorescence

To determine which subtype of D2-R was expressed by LH-producing cells, single-color FISH for one subtype of D2-R transcript was followed by immunolabeling for LHß. FISH was performed on pituitaries as described above, and revealed using fluorescein-conjugated tyramide. Pituitaries were then incubated for 48 hours at 4°C with the rabbit antibody directed

against the β subunit of carp LH (see above paragraph *Double immunofluorescent staining*), in a blocking solution (goat serum, 4% dimethylsulfoxide 0.5% Triton 0.3% in PBST 0.1%). The secondary antibody used was a goat antirabbit IgG coupled to DyLight 549 (Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, England).

Image acquisition and processing

Fluorescent images were acquired using a Zeiss LSM 700 laser scanning confocal microscope. For fluorescent tyramides used in three-color FISH and for fluorophore-coupled secondary antibodies used in immunofluorescence studies, lasers with a wavelength of 488 (FITC; Alexa-488), 555 (Cy3; DyLight 549) and 633 (Cy5; Alexa-633) nm, respectively, were used. Channels were acquired sequentially to avoid cross talk between the different filters. The focal planes were recorded with a 20 × 0.8 Numerical Aperture Plan Apochromat lens or a 63 × 1.4 Numerical Aperture Plan Apochromat lens (see Figure 3, A and B, bottom panels), and using the Zeiss ZEN 2009 software. Z-projections were obtained using Image J software (http://rsbweb.nih.gov/ij/). Composites were assembled using Adobe Illustrator (Adobe Systems, San Francisco, California).

Statistical analysis

Data are presented as means ± SEM. Statistical differences among experimental groups were analyzed using one-way ANOVA, followed by a Tukey's multiple comparison test (GraphPad Software Inc., San Diego, California). Differences were considered statistically significant at $P < .05$.

Results

1. Effects of in vivo treatment with D2-R antagonist or GnRH agonist, alone and in combination

1.1. Pituitary level of LH and FSH β-subunit transcripts

The amounts of LH and FSH β subunit (*lhβ*, *fshβ*) transcripts were measured in the pituitary by real-time quantitative PCR, 18 h after the end of the different treatments. The amounts of gonadotropin subunits were normalized to the amount of transcripts of a reference gene, zebrafish *Gapdh*, the expression of which (Figure 1A) was not affected by treatments.

Treatment of sexually regressed female zebrafish with a GnRHa alone or Domp (a DA D2-R antagonist) alone did not significantly modify *lhβ* transcript levels compared with the control group. By contrast, the combined treatment (GnRHa + Domp) induced a 2.5-fold increase in the pituitary *lhβ* transcript levels as compared with controls ($P < .01$; Figure 1A). On the other hand, *fshβ* transcript levels were not significantly affected by any treatment (Figure 1A).

In an experiment performed on 4-month-old adult cycling females, we similarly observed a 4-fold increase in the level of *lhβ* transcripts ($P < .001$, as compared with controls), only in fish who received the same combined treatment (Supplemental Figure 1).

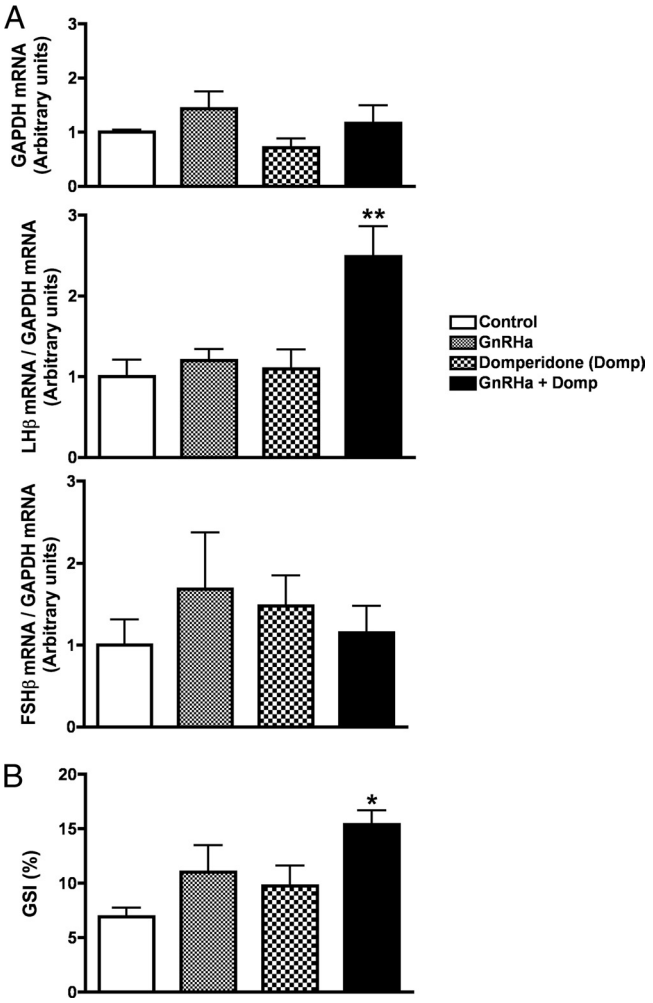

Figure 1. A, Pituitary transcript levels of gonadotropin β-subunits in sexually regressed females after in vivo treatment with diluent (control), a GnRHa, a DA D2-R antagonist Domp, or a combination of both (GnRHa + Domp). The amounts of Lhβ and Fshβ mRNAs in each sample were quantified by real-time quantitative RT-PCR and normalized to the amount of Gapdh (reference gene) mRNAs contained in the same sample. The mean control values were set to 100% (or 1), and each treated animal value has been calculated as a percentage of the control value. B, GSI in sexually regressed females after in vivo treatment with diluent (control), GnRHa, Domp, or a combination of both (GnRHa + Domp). All data presented are expressed as means ± SEM (n = 7-9). Asterisks indicate a significant difference from control group (*$P < .05$; **$P < .01$, ANOVA followed by Tukey's multiple comparison test).

1.2. The GSI

Treatment of sexually regressed female zebrafish with a GnRHa alone or Domp alone did not significantly affect the GSI, with respect to controls (Figure 1B). In contrast, the combined treatment (GnRHa + Domp) markedly increased the ovarian weight (from 0.033 ± 0.004 g to 0.083 ± 0.004 g), with no change in the body weight

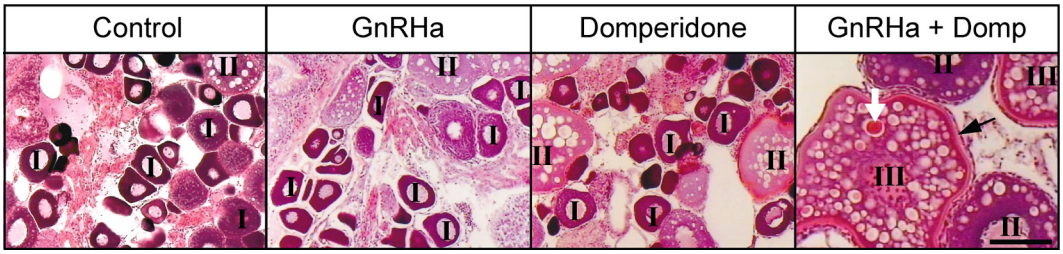

Figure 2. State of oocyte maturation in sexually regressed females after in vivo treatment with diluent (control), a GnRHa, a DA D2-R antagonist Domp, or a combination of both (GnRHa + Domp). Representative ovary sections of control sexually regressed female zebrafish and ones treated with GnRHa, Domp, and GnRHa + Domp, observed with an optic microscope. The zebrafish oocyte development was divided into three stages according to Selman criteria (47) and numbered from I to III. In control sexually regressed females, but also in females treated with GnRHa or Domp, stage I and II oocytes were observed. By contrast, in the females treated with GnRHa + Domp, enlarged oocytes were found, exhibiting a thickened zona radiata (black arrow) and containing in addition to large lipid vesicles, deeply stained yolk granules (white arrow) resulting from the incorporation of vitellogenin, two features characteristic of exogenous vitellogenesis (stage III oocytes). Scale bar: 100 μm.

(0.52 ± 0.03 g for controls and 0.497 ± 0.01 g for females treated with GnRHa + Domp), inducing a 2.57-fold increase in the GSI with respect to controls ($P < .05$).

1.3. Ovarian histology

The effects of the various treatments were further assessed by histological observation of the ovaries (Figure 2). In ovary sections of control sexually regressed females, we observed primary oocytes (stage I: small oocytes with eosinophilic ooplasm and relatively large nucleus) and oocytes at the cortical alveolus stage (stage II: with basophilic ooplasm containing lipid droplets). Similarly, in ovary sections of GnRHa- or Domp-treated females, only stage I or II oocytes were observed (Figure 2). By contrast, in females treated by Domp and a GnRHa, many vitellogenic (stage III) oocytes were found, exhibiting a distinct zona radiata and containing dense yolk globules, two features characteristic of exogenous vitellogenesis (Figure 2).

2. Innervation of LH-producing cells by TH-immunopositive fibers

In teleosts there is no hypophyseal portal system and neurohormones are directly carried to the adenohypophysis by nerve fibers from the preoptico-hypothalamic region (43). To assess the anatomical support of DA actions on *lh* expression in zebrafish, we used a double immunofluorescence technique to label TH and LHβ on sections of pituitary attached to the brain. Both in sexually regressed (Figure 3A) and cycling females (Figure 3B), a bundle of TH-immunoreactive (ir) fibers directly entering the pituitary gland was clearly observed, with a subset of these fibers innervating the PPD, where LHβ-producing cells are located. At a higher magnification (Figure 3, bottom panels), the double-labeling highlighted a dense network of TH-ir fibers threading between or terminating near LHβ-producing gonadotrope cells. No stage-related difference in the dopaminergic innervation of the PPD could be observed between sexually regressed and cycling females.

3. Localization of the three DA D2-R subtypes

In zebrafish, D2 receptors are encoded by three separate genes, *D2a*, *D2b*, and *D2c* (19). To determine whether the three subtypes were expressed in the pituitary gland, their respective distribution was studied by means of FISH.

3.1. Test of the three probes' specificity in adult zebrafish brain sections

To ensure that each RNA probe does not cross-react with several subtypes of transcripts in hybridization studies, we first examined D2-R subtype expression in adult brain sections, using triple FISH. Confocal images of a transverse brain section at the hypothalamic level (Figure 4) showed that the three probes mostly labeled different cell populations. Moreover, some cells expressed only one or two subtypes of D2-R transcripts: in the dorsal zone of periventricular hypothalamus (Hd; marked by an asterisk) some cells express D2a-R (in green) and D2c-R (in red) transcripts and thus appear in yellow in the merge picture, showing that the D2b-R probe does not cross-react with D2a-R or D2c-R transcripts. Cells in the ventral hypothalamus (Hv; marked by an arrowhead) express D2a-R (in green) and D2b-R (in blue) mRNAs and appear in light blue when merged, showing that D2c-R probe does not cross-react with D2a-R or D2b-R transcripts. Finally, cells in the preglomerular nucleus (PG; marked with a round dot), express only mRNAs encoding D2a-R, appearing in green when merged. Therefore the differential expression of the three transcripts in the adult zebrafish brain showed that the three RNA probes are specific for each subtype of D2-R and suggested a functional specialization of the three receptors.

3.2. Localization of the three D2-R subtype transcripts in the zebrafish pituitary PPD

The three-color FISH method was similarly used to detect simultaneously the expression of D2a-R, D2b-R, and D2c-R in the pituitary from sexually regressed (Figure 5A) or cycling females (Figure 5B). In both cases, the tran-

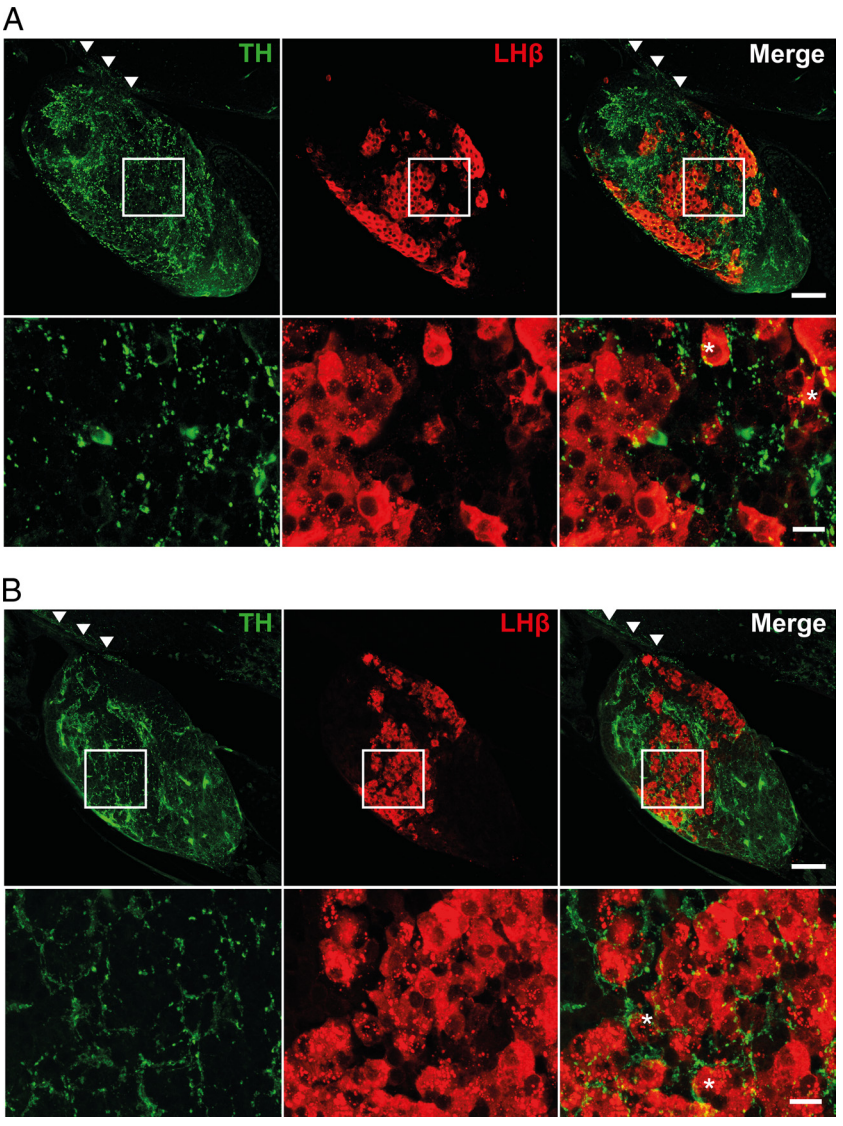

Figure 3. Chemoarchitectural evidence for the dopaminergic influence on LH-producing gonadotrope cells in the zebrafish pituitary from (A) sexually regressed and (B) adult cycling females. Top panels: Confocal z-stack (5 μm) of sagittal sections (anterior to the left) of pituitary attached to the brain, immunostained for TH (green) and LHβ (*red*). A bundle of TH-ir fibers (in green) is visible in the pituitary stalk (arrowheads), with a subset of these fibers innervating the PPD where LHβ-ir cells are located. Scale bar: 50 μm. Bottom panels: Confocal z-stacks (3 μm) of the PPD region framed in the top panels at higher magnification show a dense network of TH-ir fibers threading between LHβ-producing cells or terminating near some of these cells (marked by asterisks). Scale bar: 10 μm.

scripts for the three D2-Rs were all found in the pituitary and in largely overlapping areas. Hybridizing pituitaries with the three corresponding sense probes gave no signal (Supplemental Figure 2). While the three subtypes of D2-R exhibit similar distribution, the intensity of the labeling was different from one cell to another and from one region of the gland to another. Both in cycling and sexually regressed females, it is the median region (PPD), known to contain the gonadotrope cells, that exhibited the greatest labeling intensity for the three D2-R subtypes, which appear largely colocalized. Nevertheless, the anterior part of the gland or rostral pars distalis (RPD) in cycling females, as well as its caudal part or pars intermedia (PI) in all females, also expressed the three D2-R transcripts with a weaker intensity.

3.3. Localization of the D2-R subtype transcripts in LH-producing cells

To determine which D2-R subtype(s) was (were) expressed by LH cells and potentially involved in the inhibitory control of LH cells by DA, we coupled FISH of D2-R transcripts with LHβ immunofluorescence on adult pituitaries in toto. Comparative analysis of FISH and immunofluorescence results (Figure 6) showed that D2-R transcript expression domain of each subtype largely colocalizes with that of LHβ: the superimposition of the red and green images showed that most if not all of the cells expressing LHβ also express, more or less intensely, each type of D2-R transcripts (from light green to orange in the merge picture), indicating that each of the three subtypes of D2R is expressed by the vast majority of LH cells.

Discussion

DA of preoptico-hypothalamic origin directly acting at the pituitary level is a key and potent inhibitor of gonadotrope function in a number of teleost fish (for reviews, see Refs. 4, 44–46). However, the functional importance of this inhibitory pathway differs widely from one teleostean species to another. The zebrafish (*Danio rerio*) has emerged over the past decades to become a mainstream animal model, but the role of DA in the neuroendocrine control of its reproduction was not known.

Here we present evidence that DA plays a role in the control of zebrafish gonadotrope function.

Importance of the dopaminergic inhibitory regulation on the gonadotrope function in zebrafish

We examined the effect of removing a potential dopaminergic inhibition alone or together with a stimulation by

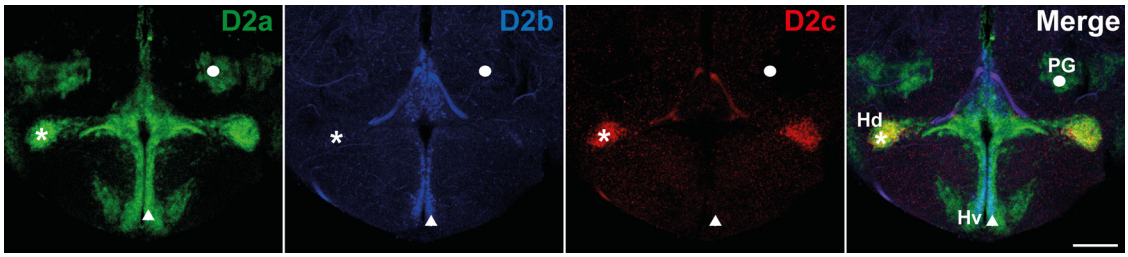

Figure 4. Differential expression of the three subtypes of D2-R transcripts in adult zebrafish brain demonstrates specificity of the probes: Transverse brain section (dorsal to the top) at the hypothalamic level stained by three-color FISH for D2a (green) D2b (blue) and D2c (red) receptor transcripts. The three cRNA probes recognize distinct, although partially overlapping, cell populations of the ventral diencephalon and hypothalamus. Note that only D2a probe labels the preglomerular nucleus (PG; marked with a round spot). D2a and D2c probes (but not D2b probe) label the dorsal zone of periventricular hypothalamus (Hd; marked by an asterisk). Finally, D2a and D2b probes (but not D2c probe) label the ventral hypothalamus (Hv; marked by an arrowhead). Scale bars: 100 μm.

a GnRHa on the zebrafish gonadotrope axis in sexually regressed females. We showed that although treatment of these females with either GnRH or Domp alone was unable to stimulate the gonadotrope axis, the combined treatment (Domp + GnRHa) was able to trigger an elevation in *lhβ* expression, leaving unchanged *fshβ* expression. We also showed that the same combined treatment induced a marked increase in the ovary weight and therefore in the GSI. Moreover, in the ovaries of control regressed females, but also of GnRHa- or Domp-treated females, all oocytes were arrested at stage II. By contrast, in the ovaries of females who received the combined treatment, an induction of vitellogenesis was observed, as shown by the numerous oocytes containing yolk, thus undergoing exogenous vitellogenesis (47). Exogenous vitellogenesis is a gonadotropin-regulated process, as demonstrated previously in the trout (48) and other teleosts. Thus, the combined treatment specifically activated the pituitary-gonadal axis, stimulating both LH synthesis and release and, consequently, vitellogenin production and incorporation.

All together, these results show that, in the old female zebrafish, the removal of the inhibition exerted by DA on LH cells is required for triggering GnRHa-stimulated LH synthesis and release, and for reactivating ovarian function. This is, to our knowledge, the first evidence of the existence of a dopaminergic inhibitory control on LH cells in this teleostean species. Indeed, the blockade of dopaminergic inhibition alone was not sufficient to increase *lhβ* expression in a significant manner, showing that in old females, the dopaminergic inhibition is accompanied with a deficient production of GnRH. The central involvement

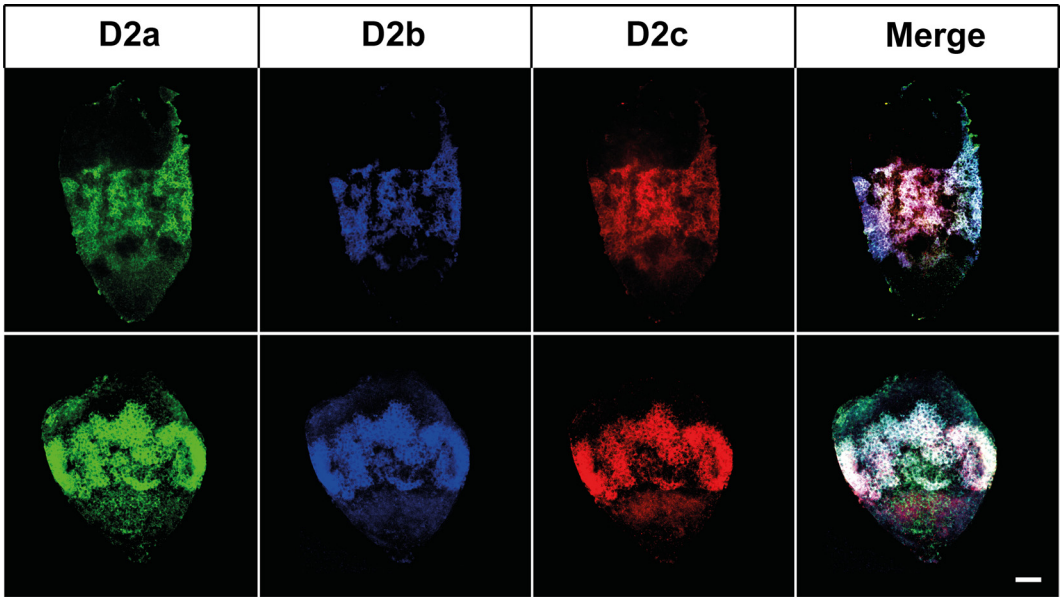

Figure 5. The three D2-R subtype of transcripts largely colocalize in sexually regressed (top panels) and in adult cycling (bottom panels) female pituitary: Projection of confocal z stacks of pituitary gland (anterior to the top) stained by three-color FISH for D2a (green), D2b (blue), and D2c (red) D2-R transcripts. The three transcripts are expressed in the gland and the central part or PPD exhibits the greatest labeling intensity. (a, anterior; p, posterior.) In the PPD, no difference could be observed for D2-Rs expression between adult cycling and sexually regressed females. Scale bars: 50 μm.

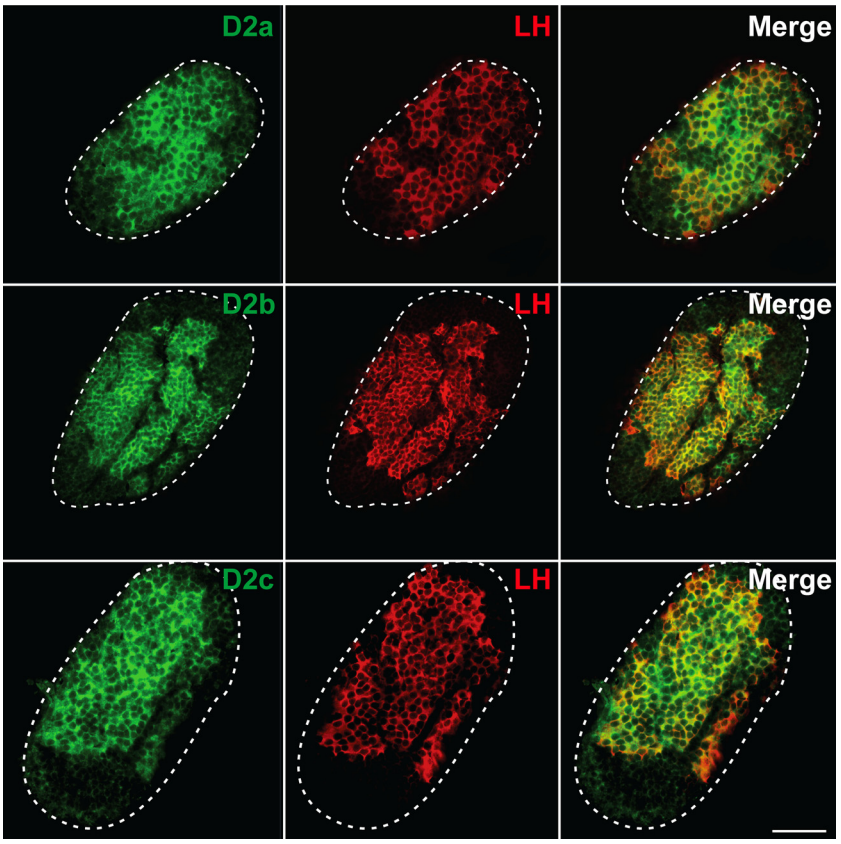

Figure 6. Coexpression of D2a, D2b, and D2c receptor transcripts and LHβ: Projections of confocal z stacks of adult cycling female pituitary. Single FISH for D2-Rs (green) combined with immunofluorescent staining for LHβ (red) showing an overall colocalization between each of the three D2-R subtypes and LH-producing cells. The dotted lines indicate the shape of the whole pituitary. Scale bar: 50 μm.

of GnRH in the regulation of LH secretion has been established functionally in all orders of teleosts (for review, see Ref. 49). Yet, in zebrafish the information on GnRH regulation of gonadotropin subunit genes is limited. Recent in vitro studies showed that treatment of zebrafish pituitary cells with salmon GnRH for 72 h had no effect on *fsh*β or *gh* expression, while significantly stimulating *lh*β expression in a dose-dependent manner (39). We similarly show herein that in vivo, in old zebrafish females, GnRHa is able to trigger a significant increase in *lh*β expression associated with an ovarian activation, with no change in fshβ expression, but only when the dopaminergic blockade was removed.

DA effects on LH synthesis have been investigated in a few species. An inhibitory action of DA on *lh* mRNAs was shown only in vitro in cultured pituitary cells from prepubertal grass carp (50). In contrast, in cultured pituitary cells from adult tilapia (51) or when administered in vivo in adult male striped bass (52), DA had no effect on *lh*β transcript levels. We report here an in vivo inhibitory effect of DA on *lh*β mRNA synthesis in sexually regressed female zebrafish. Moreover, an inhibitory effect of DA on *lh*β mRNA synthesis was shown herein in 4-month-old cycling females who received the same combined treatment, although no apparent change was detected at the ovary level. Actually, in control regressed females the GSI values were low (6.9 $\pm$ .8%), and the ovaries contained only stage I and II oocytes; the combined treatment conspicuously reactivated the ovary. By contrast, control cycling females exhibited already elevated GSI values (13.5 $\pm$ .9%, in the same range as those found after treatment in regressed females) because asynchronous ovaries contain oocytes at all stages of maturation. This may explain why, in cycling females, treatments fail to induce an additional ovarian stimulation. Nevertheless, the increase in *lh*β expression observed in these females after the combined treatment together with the constant dopaminergic innervation of LH cells and expression of the three D2-Rs revealed that the role of DA in the control of LH secretion is not restricted to the ageing period but may also be of importance during the spawning cycles. Additional experiments are needed to clarify the role of DA in the regulation of the LH preovulatory surge and its potential modulation by sex steroids.

It thus appears that, in zebrafish, DA may exert a powerful inhibitory effect capable of opposing the stimulatory action of GnRH on LH production and that, in this species, there is indeed a dual neuroendocrine control of reproduction. On that matter, the zebrafish is similar to other cyprinids, and our results are in keeping with what was discovered initially in goldfish (for reviews, see Refs. 1, 46), then in carp and loach (37), in which this inhibitory control of reproduction is powerful. Based on these data, cyprinid fish aquaculture currently uses coadministration of antagonists of D2-Rs together with GnRHa to induce ovulation and spawning (53).

Innervation of the gonadotrope cells by DA neurons

Teleost fish have no hypothalamo-pituitary portal system, and the neurohormones originating from the preoptic/hypothalamic region and controlling the activity of the different pituitary cell types are released directly by nerve

endings located near their target cells. Moreover, in the anterior pituitary, the cells of a given type are generally grouped together in a defined region of the gland, which is not the case in mammals (43). To get a morphological evidence of the dopaminergic control upon gonadotrope function in zebrafish, we studied the dopaminergic innervation of the pituitary with a specific focus on LH-producing cells, in sexually regressed and in adult cycling females. In both cases, we could show that numerous TH-ir fibers terminate in the PPD of female zebrafish pituitary, near LHβ-producing gonadotrope cells, without obvious changes in the innervation density with ageing. Although TH is the rate-limiting enzyme in the biosynthesis of both DA and noradrenaline (54), noradrenergic cell bodies are not found in the zebrafish forebrain or midbrain, and thus all TH-ir cells in these regions are dopaminergic (55). Our results agree well with previous data obtained in other studied teleosts showing a direct dopaminergic innervation of gonadotrope cells, using immunohistochemical detection of TH or DA (in goldfish, see Ref. 11, 56; in trout, see Ref. 12; in European eel, see Ref. 9; in Senegalese sole, see Ref. 57), and further support an integral role for DA in the neuroendocrine regulation of gonadotrope function in zebrafish.

The precise origin of the TH-ir fibers innervating gonadotrope cells in zebrafish is unknown. Previous studies in other teleosts, combining neural tract tracing and TH immunocytochemistry, have demonstrated that the major dopaminergic innervation of the pituitary originates from a subset of neurons in the anteroventral part of the preoptic area (differently named by the authors, see Refs. 3, 11, 58, 59). Additional studies are needed in zebrafish to selectively trace the fibers terminating in the LH-producing cells to determine which hypophysiotrophic DA cell group(s) control their functions.

Our anatomical data support the classical idea that DA acts directly at the level of gonadotropes through D2-R to inhibit LH secretion. The molecular mechanisms by which DA may interact with GnRH in this regulation have been extensively studied in goldfish and tilapia (for reviews, see Refs. 46, 60, 61).

Three DA D2-R subtypes in the zebrafish

Pharmacological studies in various teleost species showed that dopaminergic actions on LH release are mediated by receptors of the D2 type (for review, see Ref. 4). In mammals and birds, only one D2-R gene encodes two proteic isoforms (D2s and D2l), generated by an alternative splicing (16, 62). On the other hand, in teleosts such as zebrafish (19), European eel (17), and goldfish (18) several genes encoding D2-R were found. Full sequencing of some teleost genomes gradually lengthens the list of the species expressing at least two subtypes of D2 receptors (stickleback, medaka, tetraodon), although the physiological role of these multiple receptors has not yet been studied. In zebrafish, the three genes D2a-R, D2b-R, and D2c-R (19) result probably from the event of genome duplication, which took place at the basis of the teleost lineage (whole genome duplication three) (63, 64) and of an additional gene duplication specific to the zebrafish. To study the role of these multiple D2-Rs related to gonadotrope cells function, we used probes whose specificity was demonstrated by the differences in the distribution of the three subtypes of D2-R transcripts on brain sections of adult zebrafish. For example, in a region such as the hypothalamus, the nuclei labeled by our D2-R probes (Hd, Hv, and PG) have been described previously as expressing D2-Rs in other teleosts (65, 66). Moreover, within the same region, the three subtypes of transcripts were mostly expressed by different cells. These results are in agreement with the observations of Boehmler et al (19), who showed that in the embryonic nervous system the transcripts of the three genes presented different, although partially "overlapping," expression patterns.

Pituitary expression of the three D2-R subtypes in the zebrafish

The study of the localization of the D2-R transcripts in the pituitary gland, using three-color FISH, showed that the three subtypes were all strongly expressed and largely colocalized in the PPD, either in sexually regressed or in cycling females. The PPD is known to mainly contain gonadotrope cells (LH and FSH cells), as well as thyrotrope (TSH), and somatotrope (GH) cells. This distribution pattern is in agreement with earlier studies on D2-R expression in the PPD of other teleosts (in goldfish, see Ref. 36; in trout, see Ref. 13, 66; in European eel, see Ref. 17). In cycling females, we found some expression of the three D2-R transcripts in the RPD, known to contain lactotrope (prolactin) and corticotrope (ACTH) cells. The presence of D2-R transcripts in the RPD was also observed in the trout (66). Finally, the PI, located in the caudal region of the gland and which contains mainly melanotropes (α-MSH-expressing) cells, also weakly expressed the three subtypes of D2-R. The presence of D2-R transcripts in the PI is in line with results of previous studies in fish (66, 67) and mammals (68, 69), in which the secretion of α-MSH is similarly regulated by DA.

Expression of the three D2-Rs by LH-producing cells

As we observed a large colocalization of the three D2-R transcripts in the PPD, we further examined which of the three subtypes of D2-R was expressed by LH-producing

cells and potentially involved in the inhibitory control of gonadotrope function by DA. The coupled detection of one subtype of D2-R transcript and of LHß on the same pituitary gland revealed that, in female zebrafish, most of the cells producing LH express the three subtypes of D2-R. Therefore, they may all be potentially involved in the inhibitory control by DA.

Sequence alignment of the three deduced proteins (19) shows that a major difference between the subtypes of D2-R lies within the third intracellular loop, that is involved in G-protein coupling, regulatory interactions with other signaling molecules, and subcellular localization of the receptors. We can thus hypothesize that the activation of the three subtypes of D2-R expressed by LH cells may lead to a differential activation of intracellular signaling pathways.

In conclusion, current data demonstrate the existence in zebrafish of a dual neuroendocrine control of the gonadotrope function: on old females who have ceased cycling, we showed that the blockade of an endogenous DA inhibitory system allowed GnRHa to stimulate the pituitary expression of $lh\beta$ and reactivate ovarian function. These results revealed that in zebrafish DA represents a powerful counterpart to the well-known stimulating role of GnRH in the neuroendocrine regulation of the reproductive function. We demonstrated the existence of a dense dopaminergic innervation of LH-producing cells, providing the morphological basis of this control. In addition, we showed that the three subtypes of zebrafish D2-Rs were expressed in LH-producing cells, suggesting that they all may be involved in mediating this inhibition. Collectively, these physiological and anatomical findings support the existence of a direct and potent DA inhibitory tone regulating female gonadotrope function and reproduction in zebrafish.

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References

1. **Peter RE, Crim LW, Goos HJ, Crim JW.** Lesioning studies on the gravid female goldfish: neuroendocrine regulation of ovulation. *Gen Comp Endocrinol*. 1978;35:391–401.
2. **Chang JP, Peter RE.** Effects of dopamine on gonadotropin release in female goldfish, *Carassius auratus*. *Neuroendocrinology*. 1983;36:351–357.
3. **Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE.** Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish: existence of a dopaminergic preoptico-hypophyseal pathway. *Neuroendocrinology*. 1987;45:451–458.
4. **Dufour S, Sebert ME, Weltzien FA, Rousseau K, Pasqualini C.** Neuroendocrine control by dopamine of teleost reproduction. *J Fish Biol*. 2010;76:129–160.
5. **Aizen J, Meiri I, Tzchori I, Levavi-Sivan B, Rosenfeld H.** Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. *Gen Comp Endocrinol*. 2005;142:212–221.
6. **Saligaut C, Linard B, Breton B, et al.** Brain aminergic systems in salmonids and other teleosts in relation to steroid feedback and gonadotropin release. *Aquaculture*. 1999;177:13–20.
7. **Copeland PA, Thomas P.** Control of gonadotropin release in the Atlantic croaker (*Micropogonias undulatus*): evidence for lack of dopaminergic inhibition. *Gen Comp Endocrinol*. 1989;74:474–483.
8. **Dufour S, Lopez E, Le Menn F, Le Belle N, Baloche S, Fontaine YA.** Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. *Gen Comp Endocrinol*. 1988;70:20–30.
9. **Vidal B, Pasqualini C, Le Belle N, et al.** Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: a neuroendocrine lock for the onset of puberty. *Biol Reprod*. 2004;71:1491–1500.
10. **Kah O, Chambolle P, Thibault J, Geffard M.** Existence of dopaminergic neurons in the preoptic region of the goldfish. *Neurosci Lett*. 1984;48:293–298.
11. **Kah O, Dubourg P, Onteniente B, Geffard M, Calas A.** The dopaminergic innervation of the goldfish pituitary. An immunocytochemical study at the electron-microscope level using antibodies against dopamine. *Cell Tissue Res*. 1986;244:577–582.
12. **Anglade I, Zandbergen T, Kah O.** Origin of the pituitary innervations in the goldfish. *Cell Tissue Res*. 1993;273:345–355.
13. **Linard B, Anglade I, Corio M, *et al.*** Estrogen receptors are expressed in a subset of tyrosine hydroxylase-positive neurons of the anterior preoptic region in the rainbow trout. *Neuroendocrinology*. 1996;63:156–165.
14. **Weltzien FA, Pasqualini C, Sebert ME, et al.** Androgen-dependent stimulation of brain dopaminergic systems in the female European eel (*Anguilla anguilla*). *Endocrinology*. 2006;147:2964–2973.
15. **Chang JP, Peter RE, Crim LW.** Effects of dopamine and apomorphine on gonadotropin release from the transplanted pars distalis in goldfish. *Gen Comp Endocrinol*. 1984;55:347–350.
16. **Giros B, Sokoloff P, Martres MP, Riou JF, Emorine LJ, Schwartz JC.** Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature*. 1989;342:923–926.
17. **Pasqualini C, Weltzien FA, Vidal B, et al.** Two distinct dopamine D2 receptor genes in the European eel: molecular characterization, tis-

sue-specific transcription, and regulation by sex steroids. *Endocrinology*. 2009;150:1377–1392.
18. **Popesku JT, Navarro-Martin L, Trudeau VL.** Evidence for alternative splicing of a dopamine D2 receptor in a teleost. *Physiol Biochem Zool*. 2011;84:135–146.
19. **Boehmler W, Obrecht-Pflumio S, Canfield V, Thisse C, Thisse B, Levenson R.** Evolution and expression of D2 and D3 dopamine receptor genes in zebrafish. *Dev Dyn*. 2004;230:481–493.
20. **Jagadeeswaran P.** Zebrafish: a tool to study hemostasis and thrombosis. *Curr Opin Hematol*. 2005;12:149–152.
21. **Berghmans S, Jette C, Langenau D, et al.** Making waves in cancer research: new models in the zebrafish. *Biotechniques*. 2005;39:227–237.
22. **Zon LI, Peterson RT.** In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov*. 2005;4:35–44.
23. **Hill AJ, Teraoka H, Heideman W, Peterson RE.** Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci*. 2005;86:6–19.
24. **Scholz S, Fischer S, Gundel U, Kuster E, Luckenbach T, Voelker D.** The zebrafish embryo model in environmental risk assessment–applications beyond acute toxicity testing. *Environ Sci Pollut Res Int*. 2008;15:394–404.
25. **Ankley GT, Johnson RD.** Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR J*. 2004; 45:469–483.
26. **Segner H.** Zebrafish (Danio rerio) as a model organism for investigating endocrine disruption. *Comp Biochem Physiol C Toxicol Pharmacol*. 2009;149:187–195.
27. **McGonnell IM, Fowkes RC.** Fishing for gene function–endocrine modelling in the zebrafish. *J Endocrinol*. 2006;189:425–439.
28. **Ninkovic J, Bally-Cuif L.** The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. *Methods*. 2006; 39:262–274.
29. **Lessman CA.** The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries 2011. *Birth Defects Res C Embryo Today*. 2011;93:268–280.
30. **Kah O, Pellegrini E, Mouriec K, et al.** Oestrogens and neurogenesis: new functions for an old hormone. Lessons from the zebrafish. *J Soc Biol*. 2009;203:29–38.
31. **Menuet A, Pellegrini E, Anglade I, et al.** Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biol Reprod*. 2002;66:1881–1892.
32. **Palevitch O, Kight K, Abraham E, Wray S, Zohar Y, Gothilf Y.** Ontogeny of the GnRH systems in zebrafish brain: in situ hybridization and promoter-reporter expression analyses in intact animals. *Cell Tissue Res*. 2007;327:313–322.
33. **Steven C, Lehnen N, Kight K, et al.** Molecular characterization of the GnRH system in zebrafish (Danio rerio): cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. *Gen Comp Endocrinol*. 2003;133: 27–37.
34. **Biran J, Ben-Dor S, Levavi-Sivan B.** Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. *Biol Reprod*. 2008;79:776–786.
35. **Abraham E, Palevitch O, Gothilf Y, Zohar Y.** Targeted gonadotropin-releasing hormone-3 neuron ablation in zebrafish: effects on neurogenesis, neuronal migration, and reproduction. *Endocrinology*. 2010;332–340.
36. **Sloley BD, Trudeau VL, Dulka JG, Peter RE.** Selective depletion of dopamine in the goldfish pituitary caused by domperidone. *Can J Physiol Pharmacol*. 1991;69:776–781.
37. **Lin HR, Van der Kraak G, Zhou XJ, et al.** Effects of [D-Arg6, *Trp7, Leu8, Pro9NEt*]-luteinizing hormone-releasing hormone (sGnRH-A) and [*D-Ala6, Pro9NEt*]-luteinizing hormone-releasing hormone (LHRH-A), in combination with pimozide or domperidone, on gonadotropin release and ovulation in the Chinese loach and common carp. *Gen Comp Endocrinol*. 1988;69:31–40.
38. **Weltzien FA, Pasqualini C, Vernier P, Dufour S.** A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. *Gen Comp Endocrinol*. 2005;142:134–142.
39. **Lin SW, Ge W.** Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish–an in vitro approach. *Gen Comp Endocrinol*. 2009;160:183–193.
40. **Yamamoto K, Ruuskanen JO, Wullimann MF, Vernier P.** Two tyrosine hydroxylase genes in vertebrates New dopaminergic territories revealed in the zebrafish brain. *Mol Cell Neurosci*. 2010;43: 394–402.
41. **Dufour S, Delerue-Le Belle N, Fontaine YA.** Development of a heterologous radioimmunoassay for eel (*Anguilla anguilla*) gonadotropin. *Gen Comp Endocrinol*. 1983;49:404–413.
42. **Thisse C, Thisse B.** High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc*. 2008;3:59–69.
43. **Ball JN.** Hypothalamic control of the pars distalis in fishes, amphibians, and reptiles. *Gen Comp Endocrinol*. 1981;44:135–170.
44. **Pasqualini C, Vidal B, Le Belle N, et al.** An antagonist to GnRH in the control of reproduction in teleost fish: dopaminergic inhibition. Ancestral origin and differential conservation within vertebrates? *J Soc Biol*. 2004;198:61–67.
45. **Dufour S, Weltzien F, Sebert M, et al.** Dopaminergic inhibition of reproduction in teleost fishes: ecophysiological and evolutionary implications. *Ann NY Acad Sci*. 2005;1040:9–21.
46. **Popesku JT, Martyniuk CJ, Mennigen J, et al.** The goldfish (Carassius auratus) as a model for neuroendocrine signaling. *Mol Cell Endocrinol*. 2008;293:43–56.
47. **Selman K, Wallace R, Sarka A, Qi X.** Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *J Morphol*. 1993;218:203–224.
48. **Tyler CR, Sumpter JP, Kawauchi H, Swanson P.** Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. *Gen Comp Endocrinol*. 1991;84:291–299.
49. **Zohar Y, Munoz-Cueto JA, Elizur A, Kah O.** Neuroendocrinology of reproduction in teleost fish. *Gen Comp Endocrinol*. 2010;165: 438–455.
50. **Wang X, Zhao T, Wei H, Zhou H.** Regulation of dopamine D2 receptor expression in grass carp pituitary cells: a possible mechanism for dopaminergic modification of luteinizing hormone synthesis. *Gen Comp Endocrinol*. 2011;173:48–55.
51. **Melamed P, Gur G, Elizur A, et al.** Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II beta subunit and growth hormone in the teleost fish, tilapia. *Neuroendocrinology*. 1996;64:320–328.
52. **Hassin S, Holland MC, Zohar Y.** Early maturity in the male striped bass, *Morone saxatilis*: follicle-stimulating hormone and luteinizing hormone gene expression and their regulation by gonadotropin-releasing hormone analogue and testosterone. *Biol Reprod*. 2000; 63:1691–1697.
53. **Yaron Z.** Endocrine control of gametogenesis and spawning in the carp. *Aquaculture*. 1995;129:49–73.
54. **Nagatsu T, Levitt M, Udenfriend S.** Tyrosine Hydroxylase. the Initial Step in Norepinephrine Biosynthesis. *J Biol Chem*. 1964;239: 2910–2917.
55. **Kaslin J, Panula P.** Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (Danio rerio). *J Comp Neurol*. 2001;440:342–377.
56. **Kah O, Dubourg P, Chambolle P, Calas A.** Ultrastructural identification of catecholaminergic fibers in the goldfish pituitary. A high-resolution radioautographic study after in vitro ^{3}H-dopamine administration. *Cell Tissue Res*. 1984;238:621–626.
57. **Rodriguez-Gömez F, Rendön-Unceta M, Sarasquete C, Muñoz-Cueto J.** Localization of tyrosine hydroxylase immunoreactivity in

the brain of the Senegalese sole, *Solea senegalensis*. *J Chem Neuroanat*. 2000;19:17–32.
58. **Holmqvist BI, Ekstrom P.** Hypophysiotrophic systems in the brain of the Atlantic salmon. Neuronal innervation of the pituitary and the origin of pituitary dopamine and nonapeptides identified by means of combined carbocyanine tract tracing and immunocytochemistry. *J Chem Neuroanat*. 1995;8:125–145.
59. **Johnston SA, Maler L.** Anatomical organization of the hypophysiotrophic systems in the electric fish, *Apteronotus leptorhynchus*. *J Comp Neurol*. 1992;317:421–437.
60. **Chang JP, Johnson JD, Sawisky GR, et al.** Signal transduction in multifactorial neuroendocrine control of gonadotropin secretion and synthesis in teleosts-studies on the goldfish model. *Gen Comp Endocrinol*. 2009;161:42–52.
61. **Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B.** Regulation of fish gonadotropins. *Int Rev Cytol*. 2003;225:131–185.
62. **Monsma FJ Jr, McVittie LD, Gerfen CR, Mahan LC, Sibley DR.** Multiple D2 dopamine receptors produced by alternative RNA splicing. *Nature*. 1989;342:926–929.
63. **Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S, Venkatesh B.** Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Mol Biol Evol*. 2004;21:1146–1151.
64. **Vandepoele K, De Vos W, Taylor JS, Meyer A, Van de Peer Y.** Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. *Proc Natl Acad Sci U S A*. 2004;101:1638–1643.
65. **O'Connell LA, Fontenot MR, Hofmann HA.** Characterization of the dopaminergic system in the brain of an African cichlid fish, *Astatotilapia burtoni*. *J Comp Neurol*. 2011;519:75–92.
66. **Vacher C, Pellegrini E, Anglade I, Ferriere F, Saligaut C, Kah O.** Distribution of dopamine D2 receptor mRNAs in the brain and the pituitary of female rainbow trout: an in situ hybridization study. *J Comp Neurol*. 2003;458:32–45.
67. **Omeljaniuk RJ, Tonon MC, Peter RE.** Dopamine inhibition of gonadotropin and alpha-melanocyte-stimulating hormone release in vitro from the pituitary of the goldfish (*Carassius auratus*). *Gen Comp Endocrinol*. 1989;74:451–467.
68. **Chen CL, Dionne FT, Roberts JL.** Regulation of the pro-opiomelanocortin mRNA levels in rat pituitary by dopaminergic compounds. *Proc Natl Acad Sci U S A*. 1983;80:2211–2215.
69. **Meador-Woodruff JH, Mansour A, Bunzow JR, Van Tol HH, Watson SJ Jr, Civelli O.** Distribution of D2 dopamine receptor mRNA in rat brain. *Proc Natl Acad Sci U S A*. 1989;86:7625–7628.