The Dopaminergic Neurons Controlling Anterior Pituitary Functions: Anatomy and Ontogenesis in Zebrafish

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Dopaminergic (DA) neurons located in the preoptico-hypothalamic region of the brain exert a major neuroendocrine control on reproduction, growth and homeostasis by regulating the secretion of anterior pituitary (or adenohypophysis) hormones. Here, using a retrograde tract tracing experiment, we identified the neurons playing this role in the zebrafish. The DA cells projecting directly to the anterior pituitary are localized in the most anteroventral part of the preoptic area, and we named them preoptico-hypophyseal DA (POHDA) neurons. During development, these neurons do not appear before 72 hours post fertilization (hpf) and are the last dopaminergic cell group to differentiate. We found that the number of neurons in this cell population continues to increase life-long, proportionally to the growth of the fish. BrdU incorporation analysis suggested that this increase is due to continuous neurogenesis and not due to a phenotypic change in already existing neurons. Finally, expression profiles of several genes (*foxg1a*, *dlx2a* and *nr4a2a/b*) were different in the POHDA compared to the adjacent suprachiasmatic DA neurons, suggesting that POHDA neurons develop as a distinct DA cell population in the preoptic area. This study offers some insights into the regional identity of the preoptic area and provides the first bases for future functional genetic studies on the development of DA neurons controlling anterior pituitary functions.

Dopamine (DA), a neurotransmitter acting in the central nervous system (CNS) of vertebrates, is known to control a variety of physiological functions including motor programming, reward-related behaviors, visual and olfactory perceptions, thermoregulation, learning and memory, and emotions (for review, see Ref. 1). DA also represents the only nonpeptidergic agent exhibiting well-defined hypophysiotropic functions: it regulates the secretion of anterior pituitary hormones, such as prolactin (PRL), growth hormone (GH), thyroid-stimulating hormone (TSH) and melanocyte stimulating hormone (α-MSH) (for review, see Ref. 2). In addition, in some vertebrate species, DA exerts an inhibitory control on pituitary gonadotrope cells, counteracting the stimulatory effect of gonadotropin-releasing hormone (GnRH) on gonadotropin release and reproductive function. Such a dual neuroendocrine control of reproduction by GnRH and DA was first demonstrated in teleosts (3–5), but similar dopaminergic action on the control of reproduction has been shown in several species in various classes of vertebrates: amphibians (6), birds (7, 8) and mammals (9–12). However, the intensity of DA inhibition largely differs among vertebrate classes as well as within each class.

In amniotes, DA cell groups located in the preoptic/hypothalamic areas regulate anterior pituitary functions via the hypothalamo-pituitary portal blood system (2).

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Abbreviations:

Unlike amniotes, teleosts do not have a hypothalamo-pituitary portal system, and the pituitary cells are directly innervated by the neuroendocrine nerve terminals (13). Previous neuroanatomical investigations in various teleost species (goldfish: 5, 14; electric fish: 15; atlantic salmon: 16, trout: 17, and European eel: 18, 19), have demonstrated that the major dopaminergic innervation of the pituitary originates from a subset of neurons in the anteroventral part of the preoptic area. However the developmental characteristics of these dopaminergic neuroendocrine neurons have never been described. The zebrafish (*Danio rerio*) has emerged as a powerful model to study the development of DA systems and their physiology (20, 21). We recently demonstrated that DA exerts a potent inhibitory control upon female gonadotrope function in zebrafish (22). The presence of numerous fibers immunolabeled for tyrosine hydroxylase (THir), the rate-limiting enzyme of catecholamine synthesis, has been observed in the proximal *pars distalis* (PPD) of the female zebrafish pituitary, suggesting that a direct dopaminergic innervation may control the secretion of LHβ from gonadotrope cells (22).

In zebrafish, preoptic area (PO) has been subdivided into several subnuclei (23), the anterior (PPa) and posterior (PPp) parvocellular, the magnocellular (PM) preoptic nuclei, and the suprachiasmatic nucleus (SC). Although this terminology has been applied in studies of adult zebrafish dopaminergic systems (24–26), each DA subpopulation has not been thoroughly investigated. Moreover, all these cell groups have overlapping projection tracts, thus identification of the DA neurons that project onto the anterior pituitary gland has not been possible so far (27).

Here we identified the DA neurons projecting to the pituitary gland, providing a morphological support for the DA actions on the anterior pituitary hormone secretion in zebrafish. We showed that this neuroendocrine population possesses developmental peculiarities as compared to other DA populations, and displays neurogenesis even during adulthood. Our findings also offer some insights into the regional identity of the PO in zebrafish.

Materials and Methods

Animals. AB strain zebrafish (*Danio rerio*) and *otpa*$^{m866/m866}$ (28) and *otpb*$^{sa115/sa115}$ (obtained from Sanger Zebrafish Mutation Project) mutants were maintained at 28°C on a 14/10 hour light/dark cycle and fed once a day. From 3-month-old when the sex of the animals is distinguishable, only females were used. They were mature and able to lay eggs (GSI: 12 ± 0,8%). Embryos and larvae were raised at 28°C until 7 days post fertilization (dpf) in embryo medium (EM), and then in fish water. Developmental stages used are described by Kimmel et al (29). The genotype of *otp* alleles was determined by genomic PCR as described in Fernandes et al (30). Animal manipulations were performed according to the recommendations of the French Ethical Committee and under the supervision of authorized investigators (CP, SD).

TH Immunofluorescence. We used TH-immunoreactivity as a marker for DA neurons because all catecholaminergic neurons in the forebrain and midbrain are considered to be dopaminergic in all the species studied (31). We used a mouse antibody anti-TH (1:500; MAB318 Millipore) whose specificity was previously validated in zebrafish (25), and shown to mostly recognize TH1. Immunofluorescent staining (IF) was performed onto free-floating vibratome (VT1000S Leica, Germany)-sectioned brains (60 μm) embedded in 3% agarose, as described previously (22). After incubation with a secondary goat antimouse antibody coupled to AlexaFluor 488 (A11001 Invitrogen) or to Cy (Cy3: 115 165 044; Cy5: 115 175 003 Jackson laboratories) at a concentration of 1:1000, tissues were treated for nuclei staining with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride - 32 670 Sigma) at a concentration of 1/1000.

For ontogeny of TH cells, embryos of 48, 72, 84 and 96 hours post fertilization (hpf; at least 20 embryos in each stage) and brains of 1-month- and 3-month- old fish (at least 5 brains in each stage) were used. Numbers of animals for experiments using TH immunofluorescence combined with other techniques are described below.

DiI Retrograde tracing. 9-month-old cycling females (n = 40) were sacrificed with an overdose of MS-222. The skull was opened to facilitate the brain fixation in 4% paraformaldehyde (PFA; Cat No: 15 710 Electron Microscopy Sciences, Hatfield, Pennsylvania) in phosphate buffer saline (PBS) overnight at 4°C. Since the pituitary stalk of zebrafish is extremely fragile, the pituitary was exposed from the ventral side by removing the lower jaw and the surrounding tissues. A small hole was made using forceps in the bone over the middle part of the pituitary, where PPD is located. A microcrystal of DiI (1,1′-Dilinoleyl-3,3,3′,3′ tetramethylindocarbocyanine perchlorate, FAST DiI™ oil; D-3899 Invitrogen-Molecular Probes) was implanted. Tissues were then incubated in PBS in the dark at 37°C to allow dye migration. Different sizes of DiI crystals and different times of migration (from 2 to 50 days) were tested. Brains were then dissected and processed to make sagittal or frontal sections of 60 μm thickness using a vibratome.

BrdU incorporation experiments. 1-month-old juvenile fish (n = 30) and 3-month-old female fish (n = 16) were used, and each stage was divided into experimental and control groups. Fish were incubated continuously in 1 mM BrdU (5-Bromo-2′-deoxyuridine; B5002 SIGMA) diluted in fish water with DMSO (final concentration of 0.33%) for 3 days. BrdU solution was changed every day, one hour after feeding. After the treatment, the control groups were sacrificed with overdose of MS-222 and fixed in 4% PFA overnight. The experimental groups were raised for 3 weeks under standard conditions before being sacrificed and fixed in the same conditions. Brains were dissected, gradually dehydrated and stored in 100% methanol until used. After being rehydrated, brains were subsequently sectioned in sagittal or frontal planes (60 μm) using a vibratome. Immunofluorescent

staining of rat anti-BrdU (1:250; ab6326 Abcam), revealed with secondary antibody goat antirat coupled to AlexaFluor 546 (A11081 Invitrogen), was combined with mouse anti-TH (see above), and followed by DAPI staining.

Fluorescent in situ hybridization (FISH). Embryos of 48, 72, 84 and 96 hours post fertilization (hpf; at least 20 embryos in each stage) and brains of 1 month- and 3-month-old fish (at least 5 brains in each stage) were used. In situ hybridization were performed as described previously (22). Briefly, after fixation in 4% PFA, embryos and brains were gradually dehydrated and stored in 100% methanol until used. They were rehydrated, and treated with proteinase K for 30 minutes for the two latter stages. The following riboprobes were used: *th1* and *th2* (NM 131149, NM 001001829; 25), *otpa/b* (NM 001128703, NM 131100; 28), *nr4a2a/b* (NM 001113484, NM 001002406; 32), *foxg1a* (NM 131067; 33), *dlx2a* (NM 131311; 34), *prl* (NM 181437; 35), *shha* (NM 131063; 36), *nkx2.1a* (BC162296; 37), *lhx5* (NM 131218; 38) and *six3* (NM 131363; 39). Tissues were hybridized with the antisense riboprobes conjugated with either digoxygenin (DIG; 11277073910 Roche) or fluorescein (FITC; 11685619910 Roche), for 18 hours at 65°C. Tissues were then incubated with sheep anti-DIG or anti-FITC conjugated with peroxidase (POD; 1:500; respectively 11207733910 and 11426346910 Roche). The signal was revealed using FITC-conjugated tyramide (http://www.xenbase.org/other/static/methods/FISH.jsp). The in situ hybridizations were combined with TH immunofluorescence (visualized in red with Alexa 633) and nuclei staining with DAPI (see above).

Imaging. Stained tissues were mounted with the mounting medium for fluorescence Vectashield H-1000 (Vector). All images were acquired using a Zeiss LSM700 confocal microscope with 20× or 40× objectives. Channels were acquired sequentially to avoid signal crossover between the different filters. Images were processed using the ZEN software (Zeiss). Gene expression profiles of POHDA and SCDA populations were analyzed in each confocal plane, and Z-projections of images were obtained using Image J software (*http://rsbweb.nih.gov/ij/*). Composites were assembled using Adobe Photoshop and Indesign CS6 (Adobe Systems, San Francisco, California).

Cell counting and Statistics. All data were analyzed using GraphPAd Prism software. To correlate the number of the DA cells to body size of the fish, the latter was measured (without taking account of the tail fin) after PFA fixation. Parichy et al (40) indeed showed that zebrafish size is a robust indicator of developmental state. We manually counted the number of DA cells in the PO using cell counter plugin of the ImageJ software. We analyzed all Z-stacks of the entire PPa (sagittal sections of 50 μm) of 3dpf (n = 7), 7dpf (n = 8), 1 month- (n = 7), 3 month- (n = 4), 1 year- (n = 6), 2 year- (n = 6) and 3 year- (n = 3) old fish. Experimental data were expressed as means±S.E.M. The obtained data were compared using one-way ANOVA followed by Tukey's multiple comparison test. Significance was set at $P < .05$. Correlation between the number of the cells and body size was measured with Pearson product-moment correlation coefficient (Pearson's r). Comparison of the number of POHDA cells between *otp* mutants and wild type fish was also performed as described above. *otpa-/-* (n = 4), or *otpb-/-* (n = 4) and wild type (n = 5) adult female fish from 3 years-old were analyzed and one-way ANOVA test was performed. Double homozygous mutants (*otpa-/-* and *otpb-/-*; n = 3) and wild type (n = 3) of 6 dpf were analyzed and compared using Mann-Whitney test with a significance set at $P < .05$.

Results

Dopamine neurons projecting to the pituitary are located in the most antero-ventral part of the preoptic area

Since we previously showed that the pituitary gonadotrope cells in zebrafish are contacted by DA fibers (22), we searched for the origin of the DA fibers that directly innervate the pituitary in zebrafish. To this aim, we applied a DiI crystal in the middle part of the pituitary attached to the brain in adult female zebrafish. After one week of migration, numerous cell bodies were retrogradely labeled in the forebrain. Some labeled neurons were detected in the ventral telencephalon (data not shown), but the vast majority of labeled neurons were observed in the PO, (shown in red, Figure 1A-G). Subsequent immunofluorescent labeling against TH (THir; shown in green, Figure 1A-G) on frontal (Figure 1A-C) and sagittal (Figure 1D-G) sections showed that the colocalization of DiI and THir existed only within the anterior parvocellular preoptic nucleus (PPa), in the most anterior and ventral part of the PO (shown in yellow on Figure 1A-G Merge).

In the posterior part of the PO, we found DiI-labeled neurons without THir labeling (shown in red on Figure 1A-G Merge). Conversely, the THir-labeled cells in the posterior parvocellular preoptic nucleus (PPp), the magnocellular preoptic nucleus (PM), and the suprachiasmatic nucleus (SC), were not DiI-labeled (data not shown).

Since teleosts possess two TH genes (*th1* and *th2*) (41), we examined the expression of *th1* and *th2* mRNA by two-color FISH, combined with TH immunofluorescent labeling (TH-IF; Figure 1H). All *th2*-expressing cells co-express *th1*, and there were some cells expressing only *th1*. As already shown in our previous publications (25, 26), we also observed that all the *th1*-expressing cells were labeled with THir (Figure 1H). Because our TH antibody recognizes mostly TH1 (25), we can conclude that TH-IF labels all the *th* expressing neurons in the PPa.

Together, these observations show that the antero-ventral part of the PO would be the only source of the dopaminergic innervation to the zebrafish pituitary gland. We named these neurons the "preoptico-hypophyseal dopaminergic" neurons (POHDA), and in the rest of our study, we used THir to visualize these neurons.

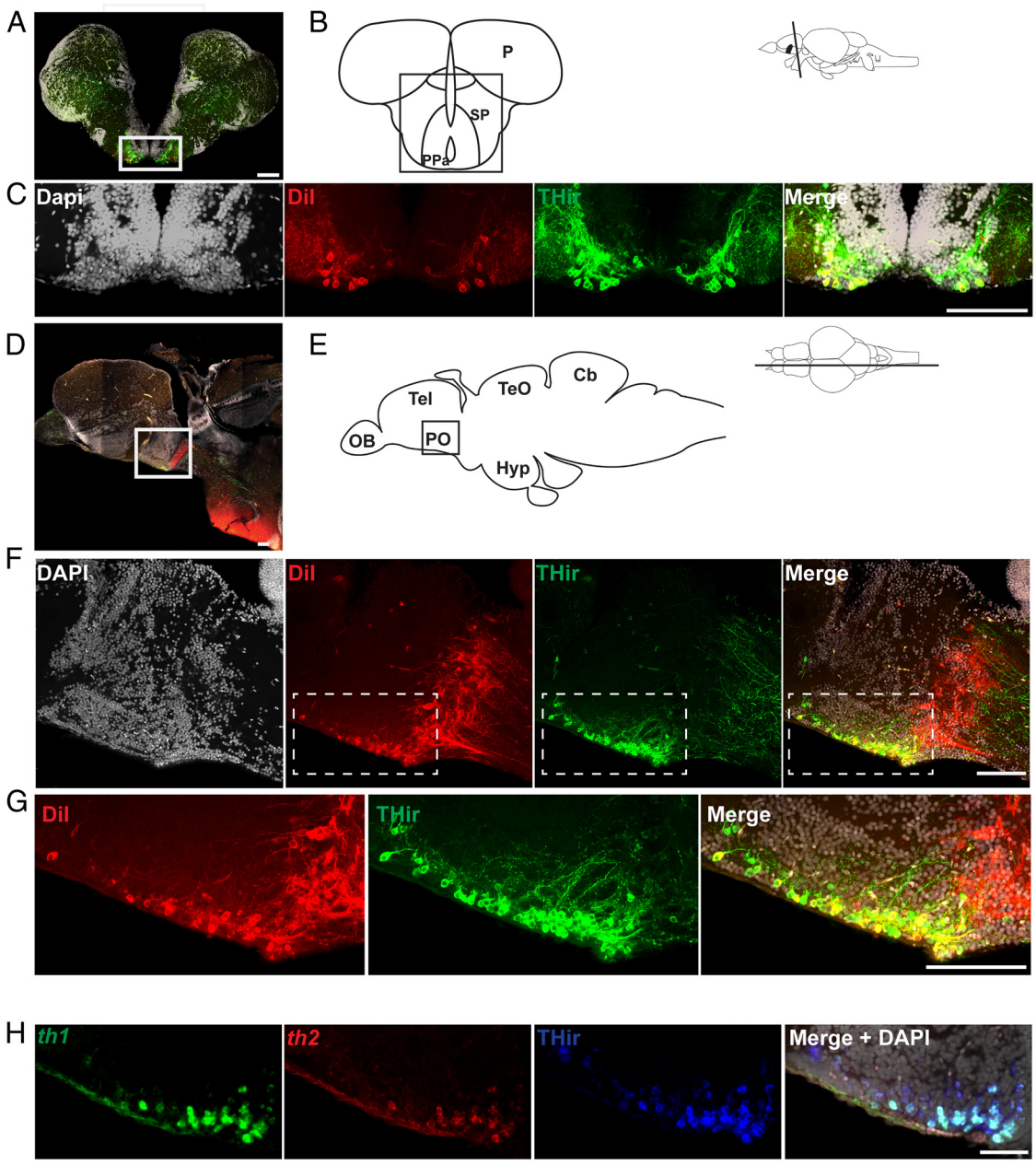

Figure 1. Frontal (*A-C*) and sagittal (*D-H*) views of POHDA neurons in the anterior preoptic area of the adult zebrafish. DAPI counterstaining (gray) shows the general anatomical structure of the brain sections. The rostral to the left in all the sagittal sections. ***A-G,*** Double labeling of DiI tracer (red) and TH immunofluorescence (THir; green). A small crystal of DiI was embedded into the pituitary and TH immunolabeling was performed after 8–10 days. *A*, Single confocal plane of a frontal section showing the anterior part of the preoptic area (PPa). ***B***, A schematic overview of the frontal section shown in *A*. ***C***, Single confocal plane presenting at a higher magnification of the area framed in *A*, showing the retrogradely labeled DiI tracer is found in TH immunolabeled cells (yellow in the merged image). ***D***, Single confocal plane of a sagittal section showing the anterior forebrain containing the preoptic area (PO). ***E***, A schematic overview of a sagittal section shown in *D*. ***F***, Single confocal plane presenting at a higher magnification of the area framed in *D*, showing that only the most antero-ventral subdivision of the PO is colabeled with DiI and THir (yellow in the merged image). The caudo-dorsal DiI labeling negative for THir (red only) corresponds to peptidergic neuroendocrine cells. ***G***, Further magnified views of the area framed in F (DiI in red and THir in green). ***H***, Single confocal plane of a sagittal brain section showing the expression of *th1* (green), *th2* (red), and THir (blue) in POHDA neurons. Two-color FISH for *th1* (green) and *th2* (red) with TH immunofluorescent labeling (THir; blue), reveal that all the THir cells express *th1* and that some *th1* cells coexpress *th2*. In contrast, cells expressing *th2*-only are not found in this region. All the *th1* and *th2* expressing cells colocalize with THir, thus THir is reliable to visualize all the POHDA neurons. Abbreviations: Cb, cerebellum; Hyp, hypothalamus; OB, olfactory bulb P, pallium; PO, preoptic area; PPa, anterior parvocellular preoptic nucleus; SP, subpallium; TeO, optic tectum; Tel, telencephalon. Scale bars: 100 μm for *A-G*, 50 μm for *H*.

Ontogeny of the POHDA neurons and development of their projections to the pituitary gland

The first step of our developmental analysis was to identify the precise location of the POHDA neurons within the zebrafish PO, using a constant anatomical landmark. We thus analyzed THir localization together with DAPI staining, in adult female zebrafish first, in order to visualize the general organization of the cells within the PO. PO is flanked by the anterior commissure (ac) and postoptic commissure (poc). From lateral views from a 3-month-old zebrafish brain, we could easily distinguish the POHDA neurons (Figure 2A-C), since they are located just anterior to the optic recess (OR). In contrast, the SC/PPp/PM DA populations are all located posterior to the OR. Using the OR as a landmark, we could easily follow back the ontogeny of the POHDA neurons at earlier developmental stages (from 3-month-old back to 48 hpf; Figure 2C-G). The first THir cell anterior to the OR (corresponding to POHDA) did not appear until 72 hpf (Figure 2F); in contrast several THir cells in the SC were already present at 48 hpf.

We then examined when POHDA axons reach the pituitary gland during embryonic development. To this aim, we attempted to observe the first THir fibers present in the pituitary, using prolactin transcripts (*prl*; green, Figure 3) as a marker of the hypophysis primordium at early stages. Indeed in zebrafish, the lactotroph lineage is the first one to differentiate and prolactin (*prl*) expression can be easily detected by ISH from 24 hpf onwards (35), a time when the expression of *fsh* and *lh* gene is extremely low (42). Up to 72 hpf, no THir fiber could be detected in the pituitary (data not shown), and the first THir fibers reached the gland between 72 hpf and 84 hpf (Figure 3), thus shortly after the appearance of the POHDA cells in the PPa. Although very few THir fibers are observed in the most anterior part of the gland (rostral *pars distalis;* RPD) containing *prl* expressing cells, DA is well known to control PRL secretion in zebrafish as early as 48 hpf (43). By contrast, numerous THir fibers were observed in the middle part of the gland (PPD) which mainly contains gonadotrope, thyrotrope and somatotrope cells in teleosts (35, 44), as well as in the *pars intermedia* (PI), suggesting that all these cells may be targets of the DA innervation, at least during development.

Continuous neurogenesis generates POHDA neurons throughout the fish growth

As the POHDA neurons differentiate late as compared to other preoptic DA neurons, we examined when this cell population reaches its full development. We thus counted the number of POHDA cells, just anterior to the OR, at different ages (Figure 4A). For sake of comparison, we also counted the number of DA cells in the SC (SCDA), just posterior to the OR. In contrast to the number of SCDA cells which reaches a maximum at about 72hpf and stay constant (12 $\pm$ 0.63 cells), the number of POHDA cells increased throughout the body growth, starting from one/two cells at 72 hpf, to 551 $\pm$ 29 cells at 3-years. Zebrafish, as other teleost fishes, is known to keep growing throughout life with an allometric growth of all the organs including the brain. We thus plotted the size of the fish, as well as the number of POHDA neurons, as a function of the age of the fish (Figure 4B, C). Both the body size and the number of POHDA neurons grow similarly. Finally, the body size and the number of the POHDA neurons appeared strongly correlated ($r^2 = 0.89$; Figure 4D), indicating that the number of POHDA neurons increased along the growth of the fish in an allometric fashion.

As the number of these neurons is increasing throughout growth, we aimed to determine whether such neurons are generated by recruiting new born cells, or by changing the phenotype of already existing cells. We performed BrdU incorporation experiments in 1-month-old juveniles (Figure 5A-C) and 3-month-old adults (Figure 5D-E). Fish were treated with BrdU for 3 days, and sacrificed 3 weeks after the BrdU treatments. In both 1-month-old juveniles (Figure 5B and 5B') and 3-month-old adults (Figure 5D and 5D'), BrdU and THir were colocalized in some cells of the PPa, showing that there were some progenitor cells in S phase at the time of the treatment that exited the cell cycle to give rise to new POHDA neurons soon afterwards. By contrast, no SCDA cell was colocalized with BrdU either in 1-month- (Figure 5C) or in 3-month-old fish (Figure 5E).

To eliminate the possibility that the BrdU labeling in THir is due to DNA repair, and not to DNA replication, we also analyzed the fish sacrificed just after the BrdU treatment (control groups). In this condition, there is no time for BrdU incorporating cells to differentiate, thus BrdU labeling in differentiated cells would be due to DNA repair. There was no POHDA or SCDA neurons labeled by BrdU in this condition (data not shown), thus we can interpret that all the double labeling of BrdU and THir is a sign of neurogenesis. Therefore, our results support the hypothesis that POHDA neurons, but not SCDA neurons, are generated de novo from dividing progenitors at these stages.

Regionalization and developmental characterization of POHDA neurons

We recently showed that the zebrafish PO comprises a morphogenetic entity organized around the OR of the brain ventricle, the optic recess region (ORR; 47). During

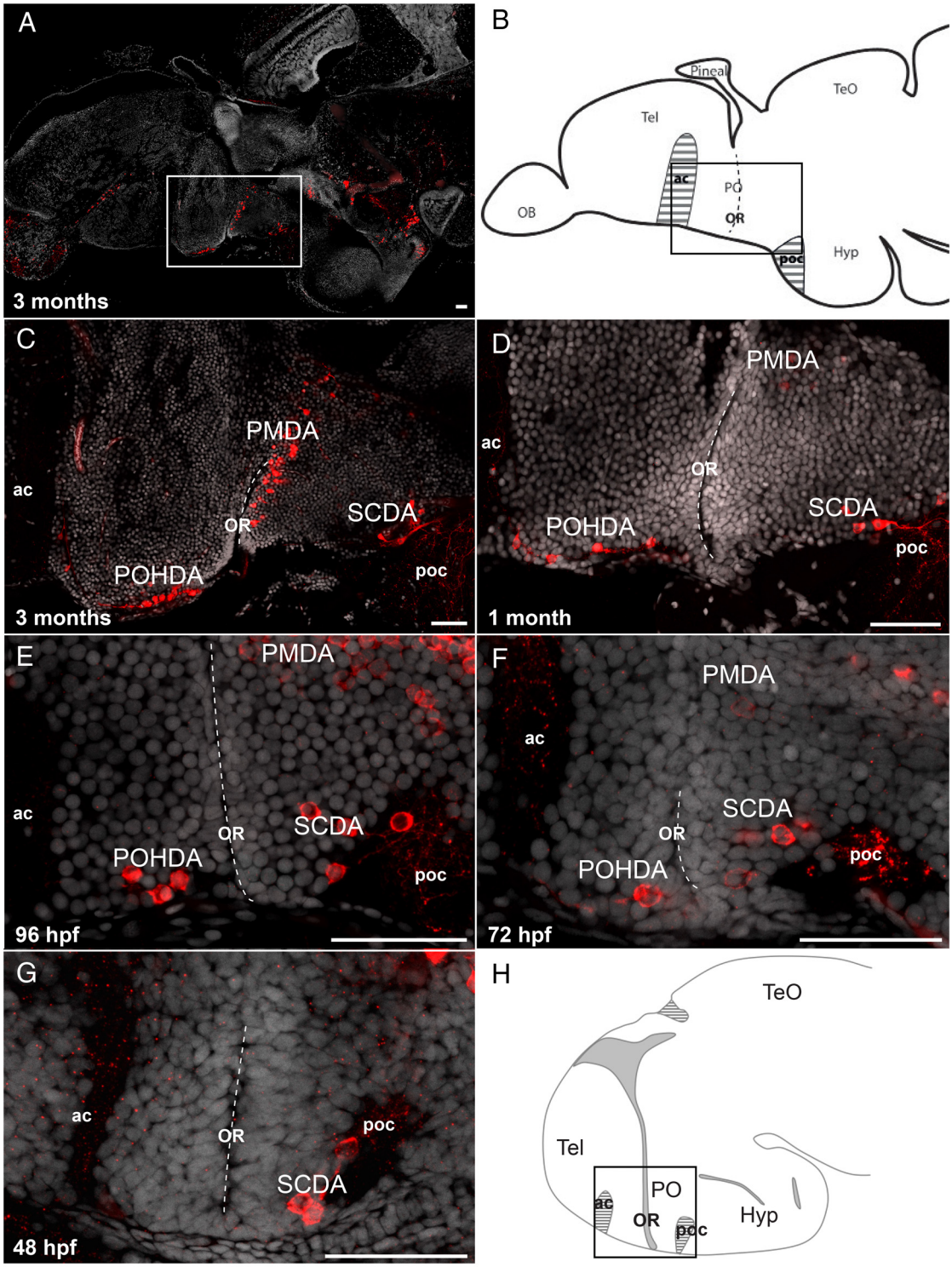

Figure 2. Ontogeny of the POHDA and SCDA neurons. TH immunofluorescence (THir; red) with DAPI counterstaining (gray), in sagittal sections of zebrafish brains (rostral to the left) at different developmental stages. A constant anatomical landmark, the optic recess (OR), allows to follow the ontogeny of preoptic neurons from adulthood (*A-C*) back to younger stages. ***A***, Z-projections (30 μm) of confocal images showing a global THir labeling (red) in a 3-month-old zebrafish brain, with a frame on our region of interest: the preoptic area (PO). ***B***, Schematic drawing representing the 3-month-old zebrafish brain showed in *A*. The dashed line represents the OR, and horizontal stripes represent fiber bundles, the anterior commissure (ac) and the postoptic commissure (poc) flanking the preoptic area (PO). ***C***, Higher magnification of the framed area in *A*. POHDA neurons are located anterior to the OR, in the most antero-ventral part of the parvocellular preoptic area. Posterior to the OR, DA neurons in the suprachiasmatic nucleus (SCDA) are located just above the poc, and those in the magnocellular preoptic nucleus (PMDA) are situated more dorsally. ***D***, Z-projections (30 μm) of confocal images showing POHDA and SCDA cells in a 1-month-old juvenile zebrafish. The magnification of this image is higher than *C* and PMDA neurons are found far more dorsally. ***E***, Z-projections (20 μm) of confocal images showing POHDA, SCDA

embryogenesis, borders of the telencephalon, ORR and hypothalamus can be delineated by abutting differentiated cells originated from different ventricular zones, and the ORR expresses a specific set of genes such as *foxg1a*, *dlx2a*, *sim1a* and *otpb* (Figure 6A; 47). The three DA cell populations, POHDA, SCDA, and PMDA, are located in the ORR (Figure 6B). Although POHDA neurons are near SCDA neurons at early stages of development, they are separated by the OR and we found that they are different in terms of the time course of development (see above). To check whether these two populations are also different in terms of gene expressions, we studied several genes which are implicated in the regionalization or the differentiation of DA cells.

High amount of the *foxg1a* transcripts were found in the most antero-dorsal region of the forebrain up to the OR (Figure 7A) including the PPa. At 72hpf (when the first POHDA neuron appears), it was colocalized with THir. Although the intensity of *foxg1a* expression reduced along the development, it went on being expressed in some POHDA neurons at least up to 1 month (Figure 6C). In contrast, *foxg1a* was never expressed in the SCDA neurons, which are located posterior to the OR (Figure 6C and Figure 7A).

Dlx2a transcripts are expressed in the ORR at the border areas with the telencephalon and the hypothalamus. We observed colocalization of *dlx2a* with THir in the SCDA neurons at 48 hpf, however, *dlx2a* expression was undetectable at later stages. By contrast, POHDA neurons start to express *dlx2a* at 72hpf (Figure 7B) when they are first detected, and some of them keep on expressing it even at 1 month (Figure 6C).

Orthopedia (*otp*), an important transcription factor for the specification of a subset of neuroendocrine cells in both mammals (48) and zebrafish (30), is also expressed in the lateral domain of the ORR. In zebrafish, *otpa/b* are also important transcription factors for the development of DA neurons in the posterior tuberculum (28, 30). Here, we observed that SCDA neurons never expressed *otp* genes (Figure 6C, Figure 8A, Supplemental Figure 1A). Only *otpb* was transiently expressed in POHDA neurons at 72 hpf (Figure 8A), and this expression was not found at later stages. Since new POHDA neurons are generated at later stages without *otpb* expression (Figure 6C), *otpb* may not be involved in the late stage of differentiation of POHDA neurons or in their maintenance. To confirm our hypothesis, we also compared the number of THir cells in the POHDA population between *otp* mutants and wild type fish. We analyzed *otpa-/-* and *otpb-/-* double mutant larvae (6dpf). Since the double mutants are lethal in juvenile, we could analyze only single mutant of either *otpa-/-* or *otpb-/-* in adult fish. We did not observe any difference in the number of POHDA neurons as compared to wild type fish (data not shown), further supporting this contention.

We also analyzed genes, which are considered to be important for the development of ventral forebrain regions including the eye field, such as *six3* and *lhx5* (49–51). The *six3b* transcripts were colocalized with THir in some POHDA and SCDA neurons (Figure 6C, Supplemental Figure 2A). In contrast, *lhx5* transcripts never appeared colocalized with THir (Figure 6C, Supplemental Figure 2B). *Nkx2.1* and *Shh* genes are key determinants for the development of hypothalamic regions (37, 52, 53) and they are not expressed in the zebrafish ORR. Accordingly, *nkx2.1a* and *shha* transcripts were never found located in POHDA or SCDA neurons (Figure 6C, Supplemental Figure 3).

Finally, the orphan nuclear receptors, *nr4a2a* and *nr4a2b*, have been shown to be critical for differentiation of some DA cell populations, including those of the posterior tuberculum and PO (32, 54). Here we found that SCDA neurons express both *nr4a2a* and *nr4a2b* at all developmental stages we have studied. By contrast, POHDA neuron never expressed either *nr4a2a* or *nr4a2b* (Figure 6C, Figure 8B, Supplemental Figure 1B). This suggests that the SCDA neurons are, as previously demonstrated, dependent on *nr4a2a/b* genes, whereas the POHDA neurons are not.

Together, these results suggest that POHDA and SCDA are distinct cell populations within the zebrafish PO. The differential gene expression patterns of POHDA neurons compared to posterior tubercular DA cells (eg, *otpa/b*, *nr4a2*) also suggest that the genetic network involved in the development of POHDA neurons is different from that of other dopaminergic neurons.

Legend to Figure 2 Continued. . .

and PMDA in 96 hpf zebrafish larva, at a higher magnification than *C* and *D*. ***F***, Z-projections (20 μm) of confocal images at 72 hpf. The first THir cell body anterior to the OR (POHDA) is observed at this stage, whereas numbers of SCDA and PMDA cells are already present. ***G***, Z-projections (20 μm) of confocal images at 48 hpf. No THir cell is observed anterior to the OR, and the DA cells in PO are represented only by those located near the poc, the SCDA. ***H***, Schematic drawing representing the 48 hpf zebrafish embryonic brain. The preoptic area (PO) shown in *G* is illustrated in the frame. The light gray represents the ventricles and horizontal stripes represent fiber bundles. As it is the case in the adult brain, PO is flanked by two commissures, ac and poc. Abbreviations: ac: anterior commissure; Hyp, hypothalamus; OB, olfactory bulb; OR, optic recess; poc: postoptic commissure; PO, preoptic area; Tel, telencephalon; TeO, optic tectum. Scale bars: 50 μm.

Discussion

Dopamine (DA) originating from the preoptico/hypothalamic region is a key regulator of the secretion of several hormones of the anterior pituitary gland in all vertebrates, and it is a strong inhibitor of gonadotrope function in some species (for reviews, see Refs 55, 56) including zebrafish (22). Zebrafish is an attractive model for developmental studies but also for physiological studies, with a growing number of tools which allow to reveal genetic networks or to follow the activity of cells in *vivo*. However, the anatomical organization underlying the actions of DA in the pituitary gland has never been studied in zebrafish, which prevents to take full benefit from the amenability of the model.

First, we identified DA neurons projecting to the pituitary *pars distalis* (PD) in the most ventral part of the PPa, just anterior to the OR. Our results agree with previous studies in goldfish (5, 57), trout (17), and European eel (18, 19), showing that the major dopaminergic innervation of the pituitary PD originates from a subset of neurons in the anteroventral part of the PO (differently named by different authors: NPOav in 5, 18, 19; NPOpc in 57; PPa in 15; SOC in 16). Nevertheless a difference is also found between our result in zebrafish and previous studies in goldfish. In our result, PPa was the only DA cell population found to project to the anterior pituitary. In contrast, goldfish studies showed that PPa dopaminergic neurons innervate the PD and suggested that other DA neurons, probably ones in the hypothalamus, innervate the PI (5, 58). In zebrafish all the dopaminergic sources to the pituitary seem to reside within the POHDA population. It is thus possible that POHDA neurons control the functions of both PD and PI. Functional studies need to be performed to confirm this hypothesis.

We found some hypophysiotropic nondopaminergic (DiI-only labeled) cells in the ventral telencephalon, as well as in the posterior part of the PO. Based on their localization, it is likely that the DiI-labeled cells in the ventral telencephalon correspond to the well-studied GnRH neurons (59, 60), and the ones in the posterior PO would represent peptidergic cell populations (eg, corticotropin-releasing hormone; CRH, thyrotropin-releasing hormone (TRH); TRH and somatostatin; SS, as well as oxytocin; OXT, arginine-vasopressin; AVP) which have been described in this region in zebrafish (61, 62).

Our result of ontogeny suggests that the POHDA neurons are probably the last dopaminergic cell group to be differentiated in the zebrafish brain during development. A previous zebrafish study has reported that DA neurons of the PO start to express TH between 48 and 60hpf, and that all the DA cell clusters are present in the 60 hpf embryonic brain (63). However, we showed that the first POHDA neuron appears only around 72 hpf, much later than the appearance of SCDA neurons located posterior to the OR. Therefore, POHDA neurons have not been taken into account in this previous study. In fact the DA cluster referred to as "preoptic" in previous embryonic studies (20, 64, 65) corresponds to the SC population. Because of the proximity between SC and PPa, and of the late appearance of the POHDA neurons as compared to the other DA populations, POHDA population has never been described in previous developmental studies.

Our BrdU incorporation experiments together with TH immunofluorescent labeling showed that the number of POHDA neurons increased all along the zebrafish growth, even in the adult, due to maintenance of neurogenesis of this cell population, which is not the case for SCDA neurons. This sustained neurogenesis would be due to the maintenance of undifferentiated progenitors in specific niches at adult stage (66). It has already been reported that PPa ventricular zone in the adult zebrafish is a region of neurogenesis (67–69). However, our study shows for the first time that this neurogenesis concerns DA neurons in this region. A previous study in embryos did not detect any proliferation marker (PCNA) in THir neurons in the PPa (20), probably because of the low proliferation rate of these cells. Indeed, in our study, a long term (3-days) incubation with BrdU and a long term chase (3-weeks) were necessary to evidence BrdU incorporation in newly formed THir neurons.

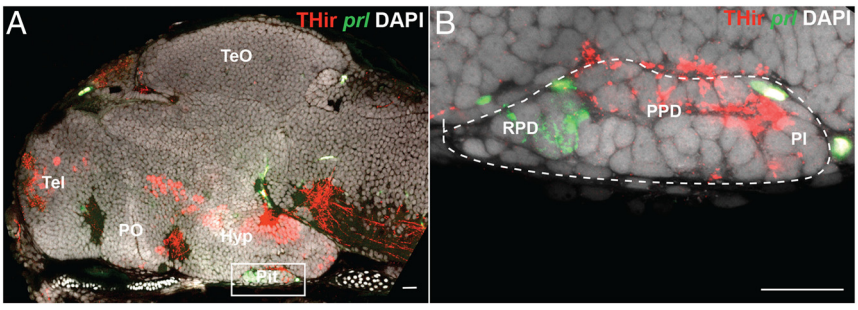

Figure 3. THir projections to the pituitary in 84 hpf larval brain. ***A***, Z-projections (3 μm) of confocal images showing TH immunofluorescence (THir; red) and prolactin FISH (*prl*; green) labeling, with DAPI counterstaining (gray), in a sagittal section (rostral to the left). The expression of *prl* was used as an early marker of the pituitary (framed). ***B***, The pituitary region framed in *A* at a higher magnification. The first THir fibers were observed in the pituitary at 84 hpf. Numerous THir fibers are found in the proximal *pars distalis* (PPD) and in the *pars intermedia* (PI), while very few THir fibers are observed in the rostral *pars distalis* (RPD), near *prl* expressing cells. Abbreviations: Hyp, hypothalamus; PI, *pars intermedia*; Pit, pituitary; PO, preoptic area; Tel, telencephalon. Scale bar: 20 μm.

The proliferation capability of POHDA progenitors could be estrogen-dependent. Estrogens are known to affect brain development, and to modulate embryonic and adult neurogenesis (70–75), and the embryonic PPa has been shown to express the three estrogen receptor subtypes (α, β1 and β2) from 24hpf onward (76). The role of estrogens in the proliferation capacity of POHDA progenitors thus awaits experimental evaluation.

A sexual dimorphism could be expected for such a DA population that is involved, at least in part, in the neuroendocrine control of reproduction. However, by comparing a group of male and female adult fish, we did not observe major differences in the number of POHDA cells. In addition, Pellegrini et al (69), comparing the actively proliferating brain regions (including PPa), did not observe difference between zebrafish male and females. We thus consider that the maintenance of neurogenesis all along the growth may be a general property of this cell population.

We showed that the number of POHDA neurons is proportional to the fish body size throughout life (allometry). The regulatory role exerted by these neurons may possibly depend on their number, that needs to remain proportional to the number of target cells in the pituitary, a gland which keeps on growing in zebrafish. For example, DA was shown to positively control GH cells, via the D1 class of DA receptors in goldfish (77, 78). It is possible that the number of GH cells increases throughout growth and that the increase of POHDA neurons may be correlated to it.

The differential gene expression profiles in POHDA and SCDA neurons during development (eg, *foxg1a* and *nr4a2a/b*) further highlighted the different characteristics of POHDA and SCDA, which are closely located during the embryonic stages. *Dlx2a* was expressed in both cell populations, but not in the same pe-

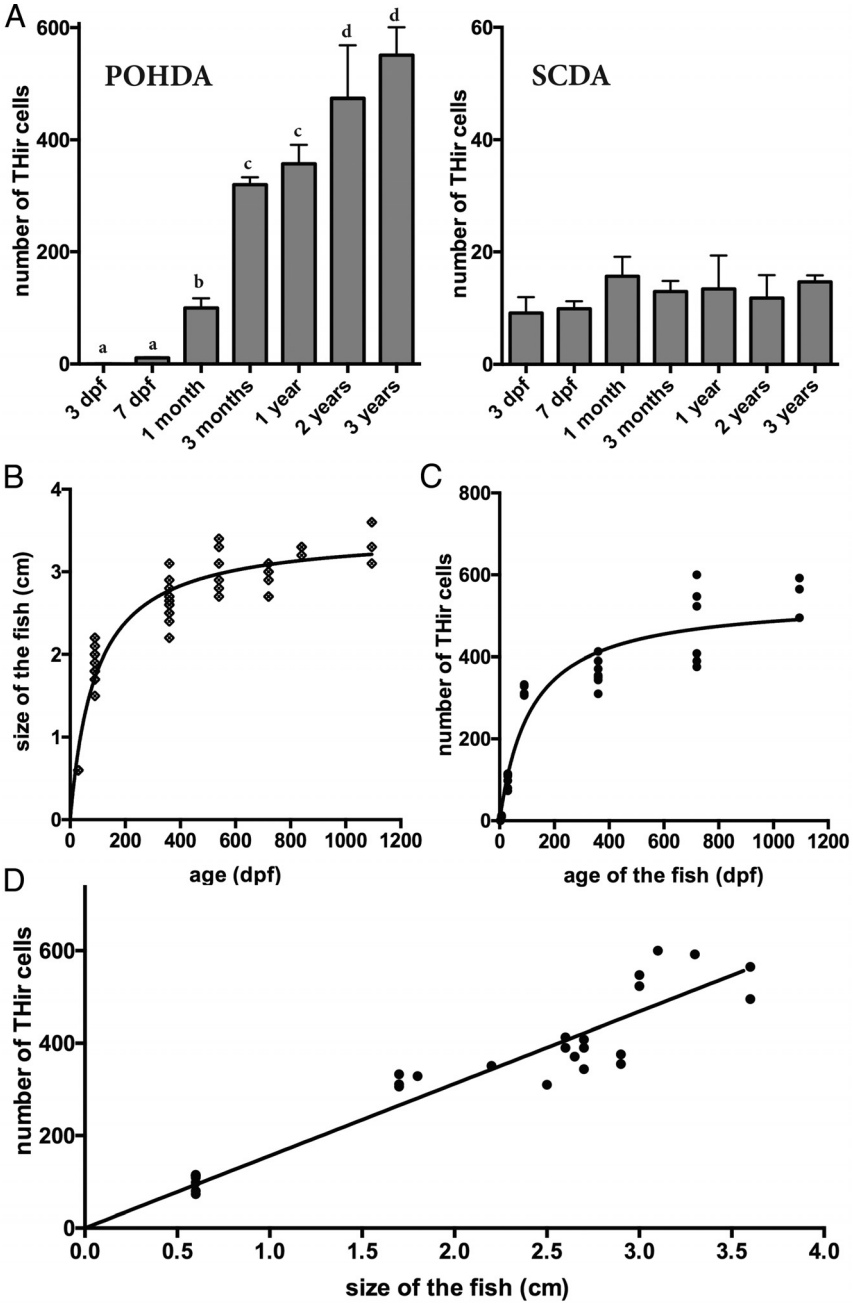

Figure 4. Consistent increase of the number of POHDA neurons all along the growth of the fish. ***A,*** The number of POHDA and SCDA neurons (detected as THir) in a brain was counted from confocal images of sagittal sections, and the means (±SEM) were plotted as a function of the age of the fish. The number of POHDA neurons among four age groups (a-d) were significantly different ($P < 0,05$) using ANOVA multiple comparison test, suggesting that POHDA neurons keep increasing all along the life. In contrast, no significant difference ($P > 0,05$) was observed in the number of SCDA neurons among the groups of different ages (with a mean of 12 ± 0.63 cells). (***B***) Body size (without taking account of the tail fin) of fish plotted as a function of the age (days post fertilization: dpf) of the fish. Each square represents individual fish (n = 57). (***C***) Number of POHDA neurons plotted as a function of the age (dpf) of the fish. Each circle represents individual fish (n = 43). The patterns of the curves in *B* and *C* both follow a similar exponential type. (***D***) Number of POHDA neurons plotted as a function of the size of the fish. There is a correlation (Pearson's r; r2 = 0.89), suggesting that larger fish have more POHDA neurons.

riod. Interestingly its expression accompanied the timing of differentiation both in POHDA and SCDA cells (Figure 6C). Although *Dlx2* gene is often used as a regional marker (79, 80), it appears to be present only at a specific differentiation cell state of the neural progenitors (81), colocalizing with a marker of differentiation (*elavl3*; 47). Our present results showing a continuous expression of *dlx2a* in some POHDA cells are consistent with this hypothesis. Together with the different anatomical location in relation to the OR, neurogenic properties and the gene expression profiles, our results support that POHDA and SCDA are two distinct DA cell populations in the developing embryo.

Although dopaminergic cell groups in zebrafish has been extensively studied (20, 24, 26, 45, 46), it has been difficult to study them in a comparative manner due to the different morphology of the brain and diversified DA systems among species. Our detailed ontogeny and gene expression studies also help for better comparison of the PO with other vertebrate species. We showed that zebrafish PO develops from a region flanked by the anterior commissure (ac) and the postoptic commissure (poc). Up to now the corresponding region has been considered as the "optic stalk" in zebrafish embryonic studies (82) as well as in mammalian studies (nicely demonstrated in Ref. 83). Because of the poor identification of this region during embryonic development, there was a discrepancy in the definition of the "preoptic area (PO)" in teleosts and tetrapods. While the tetrapod PO is considered to be a part of the telencephalon (84, 85) with a continuous expression

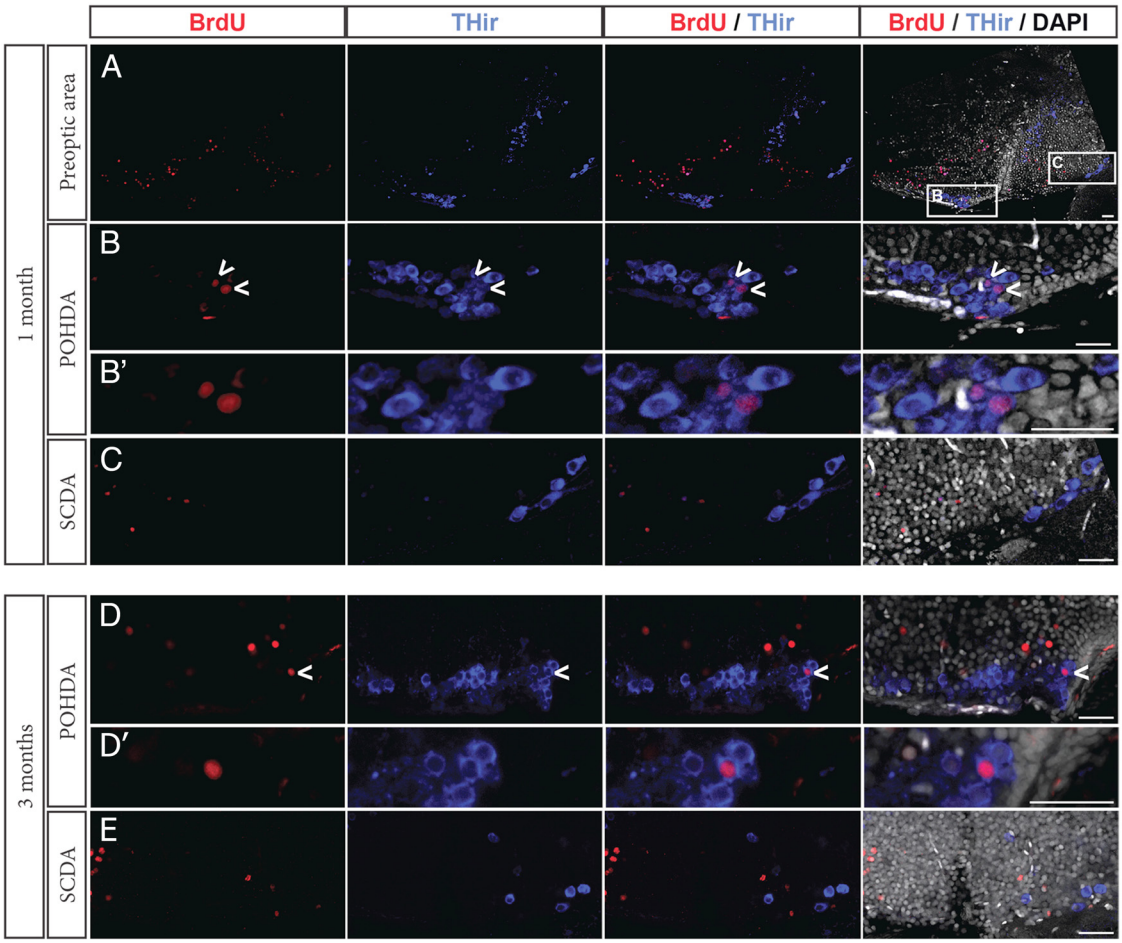

Figure 5. BrdU incorporation experiment reveals continuous neurogenesis in POHDA. 1 month (*A-C*) and 3 months old (*D*, *E*) zebrafish were treated with BrdU during 3 days, and analyzed 3 weeks later by BrdU (red) and TH (THir; blue) immunofluorescence. All images are sagittal sections of the brain (rostral to the left) showing the preoptic area (PO), corresponding to the framed region in Figure 2. ***A***, Z-projections (20 μm) of confocal images showing the PO in a 1-month-old fish. The framed areas contain POHDA (*B* and *B'*) and SCDA (*C*) populations. ***B***, Single confocal plane showing the framed area in *A* at a higher magnification. The arrow heads show the colocalization of BrdU and THir. ***B'*** Higher magnification of *B*. The clear BrdU/THir double-labeling suggests that these THir cells were born at 1-month-old. ***C***, Z-projections (10 μm) of confocal images showing the framed area in *A*, containing SCDA. There is no colocalization of BrdU and THir. ***D***, Single confocal plane showing the POHDA cells in a 3-months old fish. The arrow heads show the colocalization of BrdU and THir. ***D'***, Higher magnification of *D*. The clear BrdU/THir double-labeling suggests that these THir cells were born at 3-month-old. ***E***, Z-projections (10 μm) of confocal images showing SCDA cells in a 3-month-old fish. There is no colocalization of BrdU and THir. Scale bars: 20 μm.

of *foxg1a*, only the rostral half of the PO, containing the POHDA neurons, expressed *foxg1a* in zebrafish. Thus only the anterior part of the zebrafish PO would correspond to the tetrapod PO. The posterior part of the zebrafish PO would correspond to a part of the tetrapod hypothalamus. This agrees with the hypothesis that the *otp*-expressing peptidergic neuroendocrine cells in the zebrafish PO are homologous to those in the paraventricular nucleus of the hypothalamus (PVN) in mammals (61).

In mammals, seasonal dopaminergic inhibition of gonadotropin secretion is well demonstrated in ewe, and DA cells located in retrochiasmatic area (identified as A15) are critical for this function (86, 87). Topologically, the mammalian retrochiasmatic area is not likely to be homologous to the zebrafish PPa. Also taking into account the different manner of DA delivery to the anterior pituitary in mammals (via portal blood system) and teleosts (direct innervation), the similar role of DA in the control of pituitary between mammals and teleosts may be a consequence of convergent evolution.

As it is also illustrated by the presence of *th2* gene or the absence of mesencephalic DA cells, teleost DA systems are significantly different from the mammalian ones. Nevertheless the recruitment of nonhomologous DA cell populations for exerting similar functions in different species suggests that the nature of DA neurotransmission may be a common requirement for the neuroendocrine control of pituitary functions in vertebrates.

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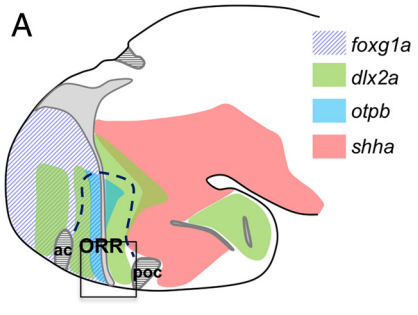

C

	48 hpf		72 hpf		84 hpf		96 hpf		1 month	
	POHDA	SC DA	POHDA	SC DA	POHDA	SC DA	POHDA	SC DA	POHDA	SC DA
six3b	*	+/-	+	+/-	+	+/-	+/-	+/-	+/-	+/-
nr4a2a	*	+	-	+	-	+	-	+/-		
nr4a2b	*	+	-	+	-	+	-	+/-		
foxg1a	*	-	+/-	-	+/-	-	+/-	-	+/-	-
dlx2a	*	+/-	+/-	-	+/-	-	+/-	-	+/-	-
otpa	*	-	-	-	-	-	-	-		
otpb	*	-	+/-	-	-	-	-	-		
lhx5	*	-	-	-	-	-	-	-		
shha	*	-	-	-	-	-				
nkx2.1a	*	-	-	-	-	-				

Figure 6. Gene expression profiles of the optic recess region (ORR) containing POHDA and SCDA neurons. *A,* A schematic representative of the zebrafish embryonic brains (around 48–72 hpf) showing the ORR and expression profiles of *foxg1a* (purple oblic lines), *dlx2a* (green), *otpb* (blue), *shha* (pink). The light gray represents the brain ventricles and horizontal stripes represent fiber bundles. The zebrafish preoptic area is flanked by the anterior commissure (ac) and the postoptic commissure (poc), thus corresponding to a morphogenetic entity organized around the optic recess, the ORR (47). ***B,*** A schematic representative of the zebrafish embryonic brains (around 48–72 hpf) showing the three DA cell populations within the ORR (= PO), POHDA, SCDA and PMDA. Confocal images of the framed region containing POHDA and SCDA are demonstrated in the Figures 7 and 8. ***C,*** A table summarizing gene expression profiles in POHDA and SCDA neurons. +: colocalized with all THir cells within the population; +/-: colocalized with some, but not all, of THir cells within the population; -: no colocalization with THir cells; *: no THir cells at this stage; Blank: not analyzed.

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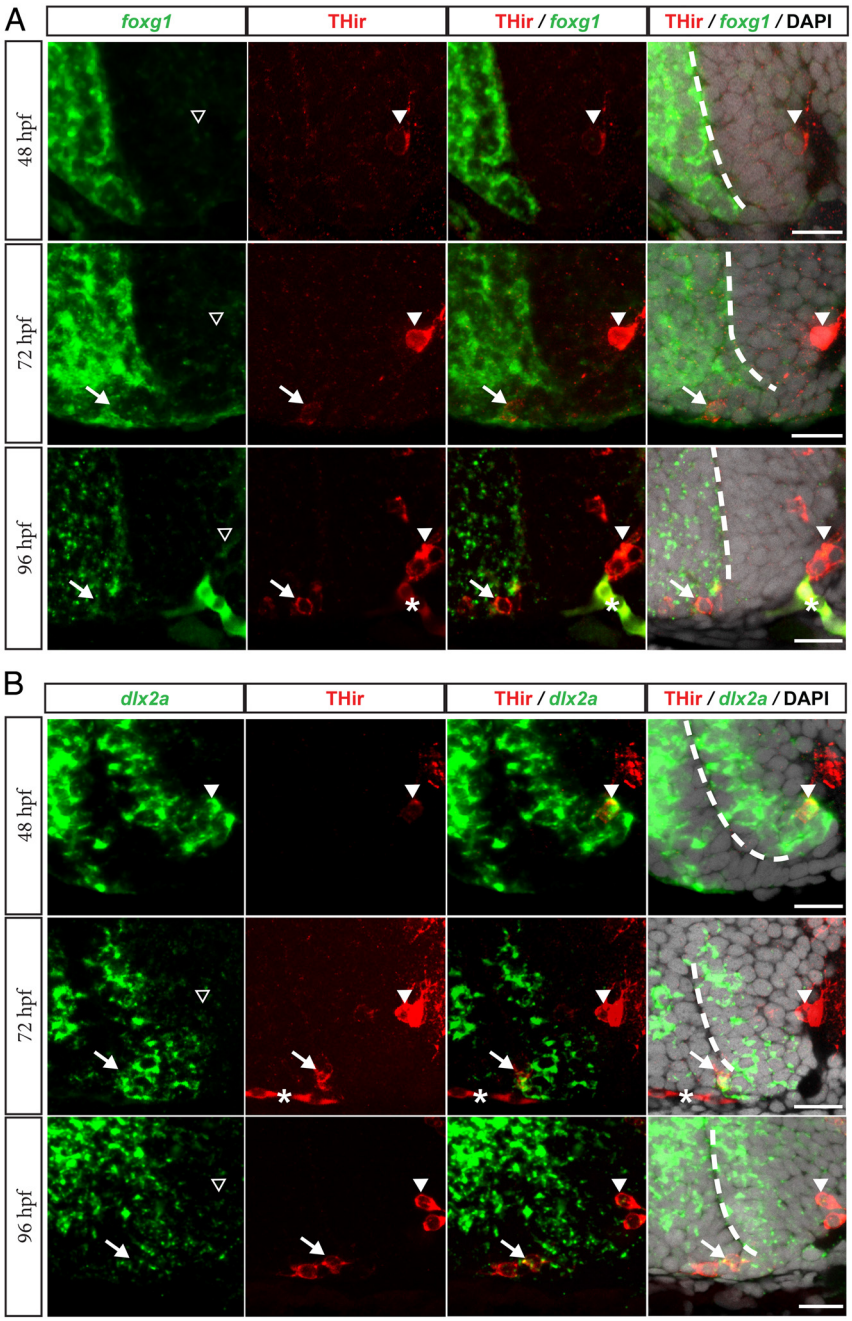

Figure 7. Gene expression of *foxg1a* and *dlx2a* in POHDA and SCDA neurons during the development. Z-projections (20 μm) of confocal images showing the preoptic area (corresponding to the framed area in Figure 6) in different embryonic stages: 48, 72, and 96 hpf. Dashed lines represent the optic recess (OR), and antero-dorsal part of the brain is shown to the left in all images. POHDA neurons (arrows) start to appear around 72 hpf, whereas SCDA neurons (arrow heads) start to appear earlier, around 48 hpf. Note that the arrows/arrow heads are filled when the POHDA/SCDA cell is labeled with the examined marker, while empty when the cell is not labeled. ***A,*** Double-labeling of *foxg1*a FISH (green) and TH immunofluorescence (THir, red), with a DAPI couterstaining (gray). *Foxg1* is expressed in a territory antero-dorsal to the OR. It is expressed in POHDA neurons, while not in SCDA. ***B,*** Double-labeling of *dlx2*a FISH (green) and TH immunofluorescence (THir, red), with a DAPI couterstaining (gray). *dlx2a* is first expressed in the SCDA at 48 hpf (arrow head). In 72 hpf, it is no more expressed in SCDA while starts to be expressed in POHDA. Abbreviations: ac: anterior commissure; poc: postoptic commissure. Scale bars: 25 μm.

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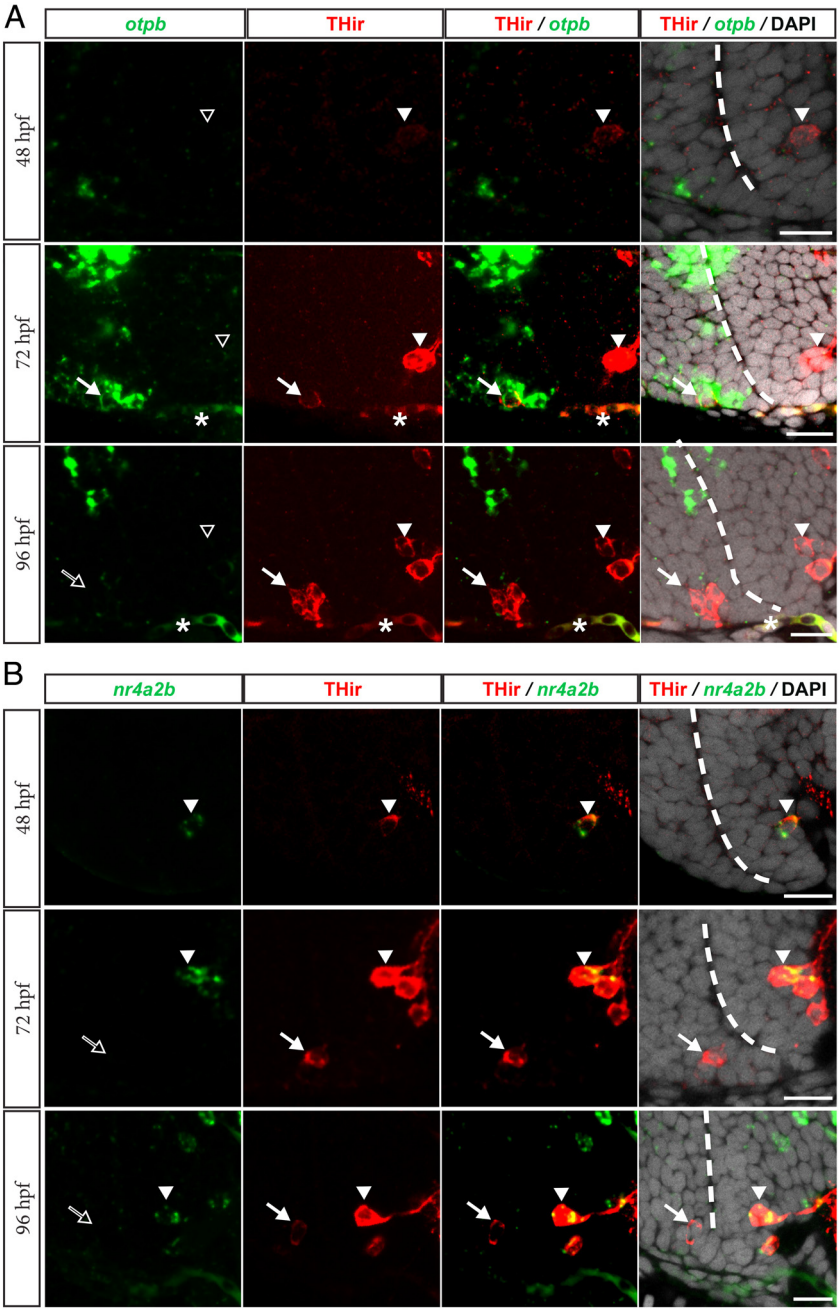

Figure 8. Gene expression of *otpb* and *nr4a2b* in POHDA and SCDA neurons during the development. Z-projections (20 μm) of confocal images showing the preoptic area (corresponding to the framed area in Figure 6) at different embryonic stages: 48, 72, and 96 hpf. Dashed lines represent the optic recess (OR), and antero-dorsal part of the brain is shown to the left in all images. POHDA neurons (arrows) start to appear around 72 hpf, whereas SCDA neurons (arrow heads) start to appear earlier, around 48 hpf. Note that the arrows/arrow heads are filled when the POHDA/SCDA cell is labeled with the examined marker, while empty when the cell is not labeled. ***A,*** Double-labeling of *otpb* FISH (green) and TH immunofluorescence (THir, red), with a DAPI couterstaining (gray). *otpb* is slightly expressed in POHDA neurons at 72 hpf, and not at all expressed in SCDA. ***B,*** Double-labeling of *nr4a2b* FISH (green) and TH immunofluorescence (THir, red), with a DAPI couterstaining (gray). *nr4a2b* is expressed in SCDA cells at all the three time points studied here. In contrast, no expression is found in POHDA. Note that the asterisks (*) indicate artifact labeling in blood vessels. Abbreviations: ac: anterior commissure; poc: postoptic commissure. Scale bars: 25 μm.

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Table 1.

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Bromodéoxyuridine		Anti-brdu	ABCAM (ab6326)	rat monoclonal	1/250
Tyrosine Hydroxylase		Anti-TH	MILLIPORE (MAB318)	mouse monoclonal	1/500
Digoxigenenin		Anti-Dig	ROCHE (11207733910)	sheep polyclonal	1/500
Fluorescein		Anti-FITC	ROCHE (11426346910)	sheep polyclonal	1/500

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