EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

*European Journal of Neuroscience, Vol. 38, pp. 3292–3301, 2013* doi:10.1111/ejn.12348

DEVELOPMENTAL NEUROSCIENCE

# Relationships between radial glial progenitors and 5-HT neurons in the paraventricular organ of adult zebrafish – potential effects of serotonin on adult neurogenesis

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*Keywords*: dopamine, fish, hypothalamus, radial glial cell, serotonin

## Abstract

In non-mammalian vertebrates, serotonin (5-HT)-producing neurons exist in the paraventricular organ (PVO), a diencephalic structure containing cerebrospinal fluid (CSF)-contacting neurons exhibiting 5-HT or dopamine (DA) immunoreactivity. Because the brain of the adult teleost is known for its neurogenic activity supported, for a large part, by radial glial progenitors, this study addresses the origin of newborn 5-HT neurons in the hypothalamus of adult zebrafish. In this species, the PVO exhibits numerous radial glial cells (RGCs) whose somata are located at a certain distance from the ventricle. To study relationships between RGCs and 5-HT CSF-contacting neurons, we performed 5-HT immunohistochemistry in transgenic tg(*cyp19a1b*-GFP) zebrafish in which RGCs are labelled with GFP under the control of the *cyp19a1b* promoter. We show that the somata of the 5-HT neurons are located closer to the ventricle than those of RGCs. RGCs extend towards the ventricle cytoplasmic processes that form a continuous barrier along the ventricular surface. In turn, 5-HT neurons contact the CSF via processes that cross this barrier through small pores. Further experiments using proliferating cell nuclear antigen or 5-bromo-2′-deoxyuridine indicate that RGCs proliferate and give birth to 5-HT neurons migrating centripetally instead of centrifugally as in other brain regions. Furthermore, treatment of adult zebrafish with tryptophan hydroxylase inhibitor causes a significant decrease in the number of proliferating cells in the PVO, but not in the mediobasal hypothalamus. These data point to the PVO as an intriguing region in which 5-HT appears to promote genesis of 5-HT neurons that accumulate along the brain ventricles and contact the CSF.

## Introduction

Serotonin (5-HT) is a neurotransmitter involved in a wide range of physiological and behavioural processes (Lucki, 1998; Monti & Jantos, 2011; Olivier *et al.*, 2011; Tejada *et al.*, 2011; Haleem, 2012; Halford & Harrold, 2012; Aubert *et al.*, 2013; Crawford *et al.*, 2013; Dennis *et al.*, 2013). In addition, data from invertebrates to mammals, including human beings, demonstrated that 5-HT is a key player in neurogenesis and brain plasticity (Lauder *et al.*, 1981, 1983; Jacobs, 2002; Gaspar *et al.*, 2003; Powrozek *et al.*, 2004; Rosenbrock *et al.*, 2005).

In mammals, the serotonergic system is composed of the raphe nuclei, which project widely into many brain regions. The 5-HT system is also considered one of the first neuronal networks established during development, therefore potentially exerting regulatory influences on brain development (Gaspar *et al.*, 2003; Vitalis & Parnavelas, 2003; Gaspar & Lillesaar, 2012).

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*Received 28 February 2013, revised 26 July 2013, accepted 29 July 2013*

In addition to the 5-HT raphe nuclei and their projections, the hypothalamus of all non-mammalian vertebrates also contains a region named the paraventricular organ (PVO), which exhibits numerous 5-HT (Kah *et al.*, 1978; Kah & Chambolle, 1983; Kaslin & Panula, 2001; Lillesaar, 2011) and dopamine cerebrospinal fluid (CSF)-contacting neurons (Fremberg *et al.*, 1977; Kah *et al.*, 1978; Geffard *et al.*, 1982, 1984; Batten *et al.*, 1993; Meek, 1999).

On the other hand, the brain of adult fish exhibits a much higher neurogenic activity than other vertebrates (Kranz & Richter, 1971; Ekstrom *et al.*, 2001; Zupanc, 2001; Adolf *et al.*, 2006; Pellegrini *et al.*, 2007; Kaslin *et al.*, 2009). This adult neurogenesis is observed in all brain regions, and is due in large part to persistence of radial glial cells (RGCs) acting as progenitor cells (Pellegrini *et al.*, 2007; Lam *et al.*, 2009; Chapouton *et al.*, 2010; Marz *et al.*, 2010).

The functions of the aminergic CSF-contacting neurons of the PVO in non-mammalian vertebrates are completely unknown, as are their origin and their relationships with the neighbouring RGCs. In order to answer those questions, we investigated the relationships between 5-HT neurons and RGCs in adult zebrafish, focusing

particularly on the caudal region of the PVO. Additionally, by inhibiting 5-HT synthesis, we also investigated the potential role of 5-HT as modulator of brain proliferation in the PVO of adult zebrafish.

## Material and methods

### *Animals and brain dissections*

Animals were kept, handled and killed in agreement with the European Union regulation concerning the use and protection of experimental animals (Directive 86/609/EEC). All protocols used in these experiments were approved by the local ethics committee CREEA (Comité Rennais d'Ethique en matière d'Expérimentation Animale). All experiments were performed on adult males (6 months old) wild-type zebrafish (*Danio rerio*) and transgenic zebrafish tg *(cyp19a1b*-GFP) that expresses green fluorescent protein (GFP) in RGCs (Tong *et al.*, 2009; Diotel *et al.*, 2010; Brion *et al.*, 2012). Adult animals were kept in the zebrafish facilities of the Structure Fédérative de Recherche Biosit (INRA LPGP, Rennes, France). One week prior to experiments, fish were acclimated in the laboratory under standard conditions of temperature (28.0 ± 0.5 °C) and photoperiod (14 h light, 10 h dark) in tanks containing 40 L of water. At the end of each experiment, the fish were anesthetized on ice and killed by spinal cord sectioning. After sex determination by direct examination the gonads, the brains were removed and fixed overnight at 4 °C in phosphate-buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde for immunohistochemistry processing.

### *Immunohistochemistry*

Immunohistochemistry was performed on frozen sections (12 μm thick) mounted onto poly-L-lysine-coated slides (Thermo Scientific, Germany). For immunohistochemical staining of 5-HT neurons, we used a rabbit anti-5-HT antibody (1 : 5000, kindly provided by Dr Yves Tillet, INRA, UMR6175, Nouzilly, France; Tillet *et al.*, 1986). For immunohistochemical detection of proliferative cells, mouse anti-proliferating cell nuclear antigen (PCNA; 1 : 100; clone PC10; DAKO, Glostrup, Denmark) and mouse anti-5-bromo-2′-deoxyuridine (BrdU; 1 : 100, clone Bu20a; DAKO) antibodies were used. RGCs were labelled with a rabbit antibody raised against aromatase B (1 : 800, anti-zebrafish aromatase; Menuet *et al.*, 2005). After a brief rinse in PBS (pH 7.4), sections were dipped in PBS containing 0.2% Triton and 1% BSA (Sigma-Aldrich Co., France) to saturate non-specific binding. Sections were then incubated overnight at room temperature with the antibody of interest diluted in PBS containing 0.5% milk powder. On the next day, sections were washed three times in PBS with 0.2% Triton, and incubated with the appropriate secondary antibody (goat anti-mouse Alexa fluor 488 or 594, goat anti-rabbit Alexa fluor 488 or 594, 1 : 200; Invitrogen Molecular probes, Eugene, OR, USA). Brain sections were washed several times in PBS with 0.2% Triton and mounted with Vectashield medium (Vector Laboratories, Inc., Burlingame, CA, USA) with 4,6-diamino-2-phenylindole (DAPI) to visualize cell nuclei.

For PCNA immunohistochemistry, sections were first subjected to antigen retrieval in sodium citrate buffer (pH 6) at 80 °C for 30 min and then blocked in the non-specific binding buffer described above. For BrdU immunohistochemistry, sections were previously heated for 3 h at 65 °C in a buffer containing 50% formamide and 50% 2 × SSC. Slides were rinsed in 2 × SSC, placed in 2 N HCl buffer at 37 °C for 30 min and then washed in 0.1 M sodium tetraborate decahydrate (pH 8.5) at room temperature. Finally, slides were rinsed in PBS with 0.2% Triton before incubation with the anti-BrdU antibody. For the incubation with the secondary antibody, sections were treated as previously described.

The specificity of all antibodies for use in zebrafish has been evaluated previously (Tillet *et al.*, 1986; Menuet *et al.*, 2005; Pellegrini *et al.*, 2007; Marz *et al.*, 2010). The 5-HT antibody (Tillet *et al.*, 1986) yielded results identical to those obtained previously in goldfish (Kah & Chambolle, 1983) and zebrafish (Lillesaar, 2011). The aromatase B (Cyp19a1b) antibody has been largely validated previously by *in situ* hybridization and co-staining with GFP in a transgenic tg(*cyp19a1b*-GFP) zebrafish (Menuet *et al.*, 2005; Pellegrini *et al.*, 2007; Tong *et al.*, 2009). The PCNA and BrdU antibodies have been largely validated for use in zebrafish and results show the expected stainings (Marz *et al.*, 2010; Chapouton *et al.*, 2011; Rothenaigner *et al.*, 2011; Kishimoto *et al.*, 2012; Diotel *et al.*, 2013).

### In vivo *BrdU incorporation*

To determine whether 5-HT neurons of the caudal hypothalamus originate from RGCs localized in this region, fish were treated with BrdU (Sigma-Aldrich Co.; Sigma, St Louis, MO, USA), a synthetic nucleoside analog of thymidine which is incorporated into cells replicating their DNA. The BrdU was injected intraperitoneally at 50 μL/g (1 mM) body weight diluted in 110 mM NaCl, pH 7.0) twice at an interval of 2 h. The fish were killed 1, 3, 14, 21, 30 or 60 days after BrdU injection. The identity of the cells that havd incorporated BrdU was determined with BrdU immunohistochemistry, combined with 5-HT or aromatase B immunodetection according to Pellegrini *et al.* (2007).

### *Effects of 5-HT as a modulator of proliferation*

To highlight the effect of 5-HT on the cell proliferation rate in the caudal hypothalamus, adult wild-type zebrafish were treated with the 4-p-chloro-L-phenylalanine (p-CPA; Sigma-Aldrich), which acts as a selective and irreversible inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of 5-HT. The experiment was performed three times. p-CPA was diluted in alkaline PBS (PBS 1 ×, containing 0. 2% NaOH, pH 7.4,), and intraperitoneally injected (dose 100 μg/g) in adult wild-type zebrafish. The injected volume was 20 μL/g and the same volume of alkaline PBS was injected into the control group ($n$ = 50 fish per condition). Both groups were maintained in 5-L glass aquaria, under controlled conditions as mentioned above. Fish were killed at 0, 24, 48 or 96 h after the injection. Male fish were selected at each sampling time, with a minimum of six fish per time and treatment. Brains of killed fish were removed and processed for PCNA and 5-HT immunohistochemistry.

The concentration of p-CPA used in these experiments results in a significant decrease in the content of 5-HT in whole brain as demonstrated in the bluegill sunfish 24 h after the injection of the inhibitor (*Lepomis macrochirus*; Duffy-Whritenour & Zelikoff, 2008) and corresponds to the pharmacological dose applied in rats (Ito & Yamanouchi, 2010).

### *Counting of PCNA-immunoreactive cells and data analysis*

PCNA-immunoreactive cells in the control and p-CPA-treated groups were quantified using photographs of sections corresponding to the nucleus of the lateral recess, caudal hypothalamus and nucleus of postural recess (NRP). The quantification was performed with the ImageJ program (National Institutes of Health; http://rsbweb.nih.gov/ij/) on the photographs after monochromatic black-and-white transformation. Two persons, ignoring what they were counting, per-

formed pictures and cell counts in blind conditions and the mean of these very similar cell counts was then processed for statistical analyses. The differences between groups and sampling times were compared with an ANOVA of two factors and a subsequent mean comparison was carried out with the Bonferroni test. Differences with $P < 0.05$ were considered significant.

### *Microscopy*

Slides were observed with an epifluorescence microscope (Olympus Provis, equipped with a DP71 digital camera) or with Leica SP2 or Olympus BX61WI confocal microscopes. Images were processed with either the Olympus Analysis, Zeiss Cell or Olympus FluoView 3.0 software. Photographs were then assembled using Photoshop CS5.1 with no modifications except for light and contrast adjustments. The nomenclature of the brain nuclei and regions is according to that of the zebrafish atlas (Wullimann & Rink, 2002) with minor modification.

## Results

### *Distribution of 5-HT neurons and relation to radial glial cells in the hypothalamus*

The expression of 5-HT was assessed by immunohistochemistry in the brain of wild-type and tg(*cyp19a1b*-GFP) adult zebrafish, resulting in an expression pattern identical to that reported in other studies in zebrafish (Kaslin & Panula, 2001; Lillesaar, 2011) and other teleosts (Kah & Chambolle, 1983). Numerous varicose fibres were observed in virtually all regions of the brain, in particular in the telencephalon, similar to previous reports (Lillesaar, 2011). A strong 5-HT signal was observed in the pineal gland, where 5-HT acts as precursor in melatonin synthesis. A small group of 5-HT neurons was consistently detected at the level of the pretectal area, lateral to the anterior thalamic nucleus. As expected, the most prominent groups of 5-HT-positive cells were observed in the hypothalamus and consisted of three main sub-groups – anterior portion of the paraventricular organ (PVOa; Fig. 1A), intermediate part of the PVO (PVOi; Fig. 1B) located dorsal to the anterior aspect of the lateral recess, and nucleus recessus posterioris (PVOp; Fig. 1C). In contrast with other 5-HT cell bodies, these tightly-packed cells were localized close to the diencephalic ventricles or their recessi, and were of the CSF-contacting type of neuron. Indeed, all these cells extended a short process toward the lumen of the ventricle (Fig. 2B and D). Opposite this short process, a very fine axon left the cell body and these axons form dense fibre tracts that cannot be traced over long distances. Finally, as expected, many 5-HT neurons were localized in the superior raphe (Fig. 1D) and inferior raphe nuclei.

The use of the tg(*cyp19a1b)*-GFP transgenic line in which RGCs are labelled by GFP in many regions of the forebrain allowed analysis for the first time of the relationship between RGCs and 5-HT neurons in the posterior tuberculum and caudal hypothalamus (Fig. 1).

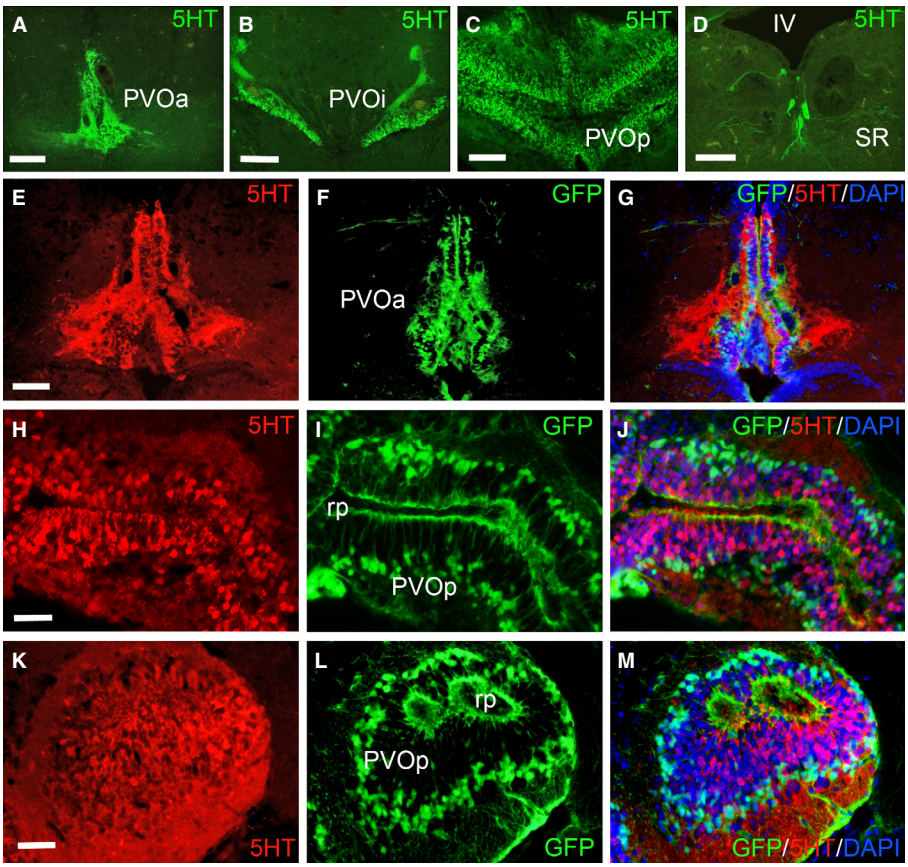


FIG. 1. (A–D) Transverse sections showing the distribution of 5-HT-immunoreactive cells in (A) the anterior (PVOa), (B) intermediate (PVOi), (C) posterior (PVOp) paraventricular organ- and (D) in the superior raphe nucleus (SR). (E–G) Transverse sections at the level of the PVOa of a tg(*cyp19a1*-GFP*)* adult zebrafish, showing the relationship between 5-HT neurons (red: E) and GFP-positive radial glial cells (green; F). (G) Merge with DAPI staining in blue. (H–M) Transverse sections at two levels of the PVOp of a tg(*cyp19a1*-GFP*)* adult zebrafish, showing that GFP-positive radial glial cells (green; I and L) surround the 5-HT neurons (red; H and K). (J and M) Merge, with DAPI staining in blue. Scale bars, 50 μm (A–D), 25 μm (E–M).

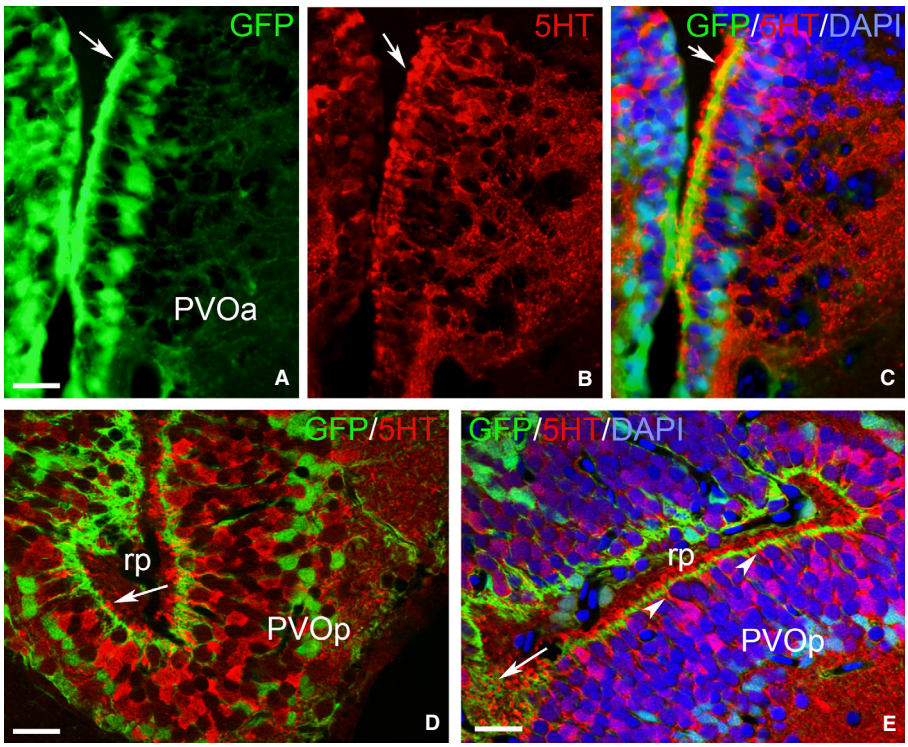


FIG. 2. (A–C) Confocal images (projection of a *Z*-stack) at the level of the PVOa showing the close relationships between GFP-positive radial glial cells (green; A) and 5-HT neurons (red; B). The merge (C) shows that radial processes establish a continuous barrier along the ventricle (V). This barrier is through small pores crossed by the CSF-contacting processes of the 5-HT cells (arrow). (D) Confocal image (projection of a *Z*-stack) at the level of the PVOp around the posterior recess (rp) showing the close relationships between GFP-positive radial glial cells (green) and 5-HT CSF-contacting neurons (red). Arrow points to CSF-contacting process ending into the posterior recess. (E) Confocal image at the level of the PVOp around the posterior recess (rp) showing the localization of GFP-positive radial glial cells (green) and 5-HT neurons (red). The arrows point to a part of the picture where the wall of the ventricle is cut tangentially, showing the small pores through which the extensions of 5-HT CSF-contacting neurons cross the barrier (arrowhead) formed by the radial glial cells. Scale bars, 12 μm.

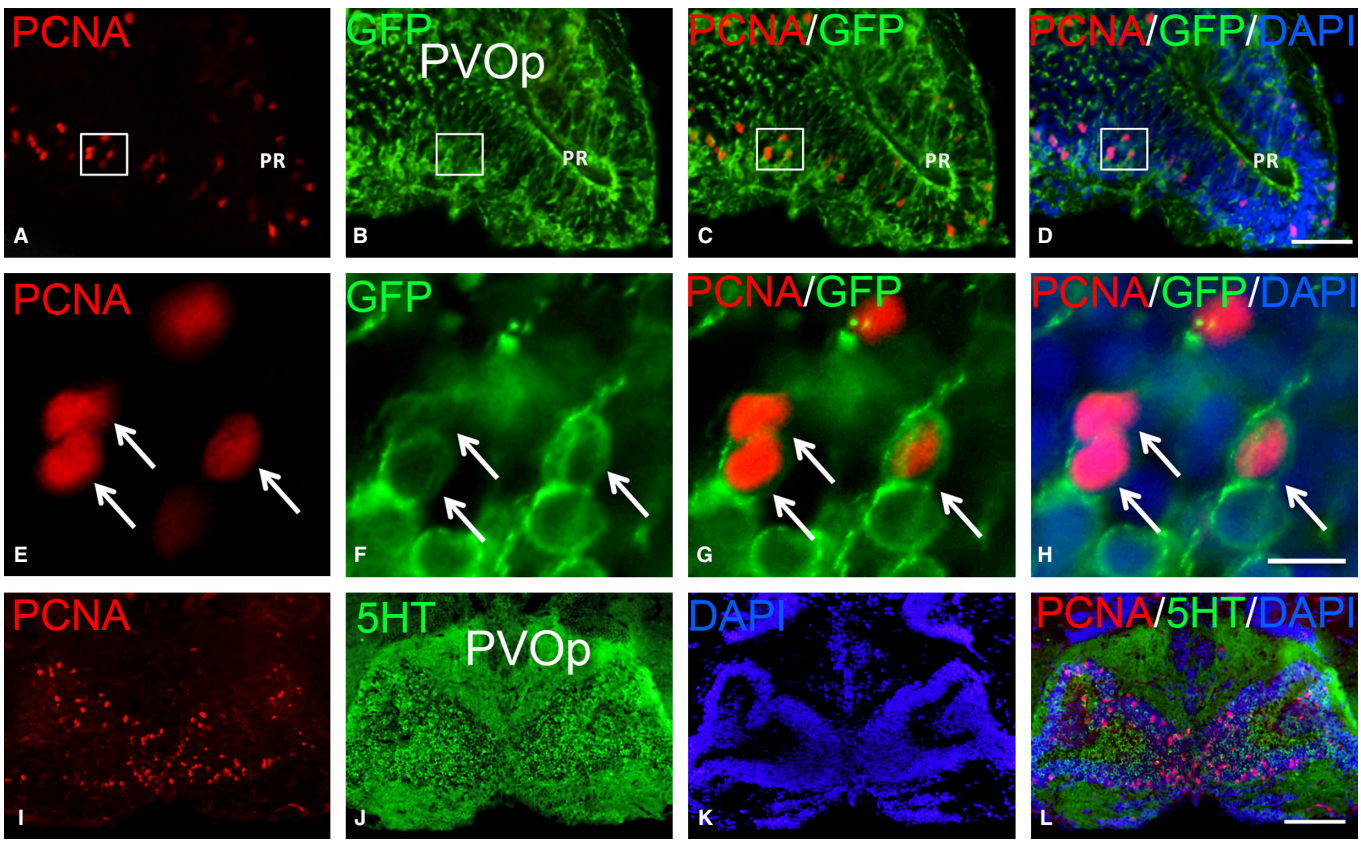


FIG. 3. (A–D) Transverse section at the levels of the PVOp of a tg(*cyp19a1*-GFP) adult zebrafish, showing that PCNA-positive cells (red; A) are located at the periphery of the nucleus recessus posterior, where the soma of GFP-positive cells are located. (E–H) Higher magnification of the areas boxed in (A–D) showing that PCNA-positive cells (red; E) correspond to GFP-positive radial glial cells (green; F, G and H). (I–L) Transverse section at the level of the PVOp of a tg (*cyp19a1*-GFP) adult zebrafish, showing that PCNA-positive cells (red; I) do not correspond to 5-HT-positive cells (green in J). (K) DAPI and L the merge. Scale bars, 50 μm (A–D), 8 μm (E–H), 75 μm (I–L).

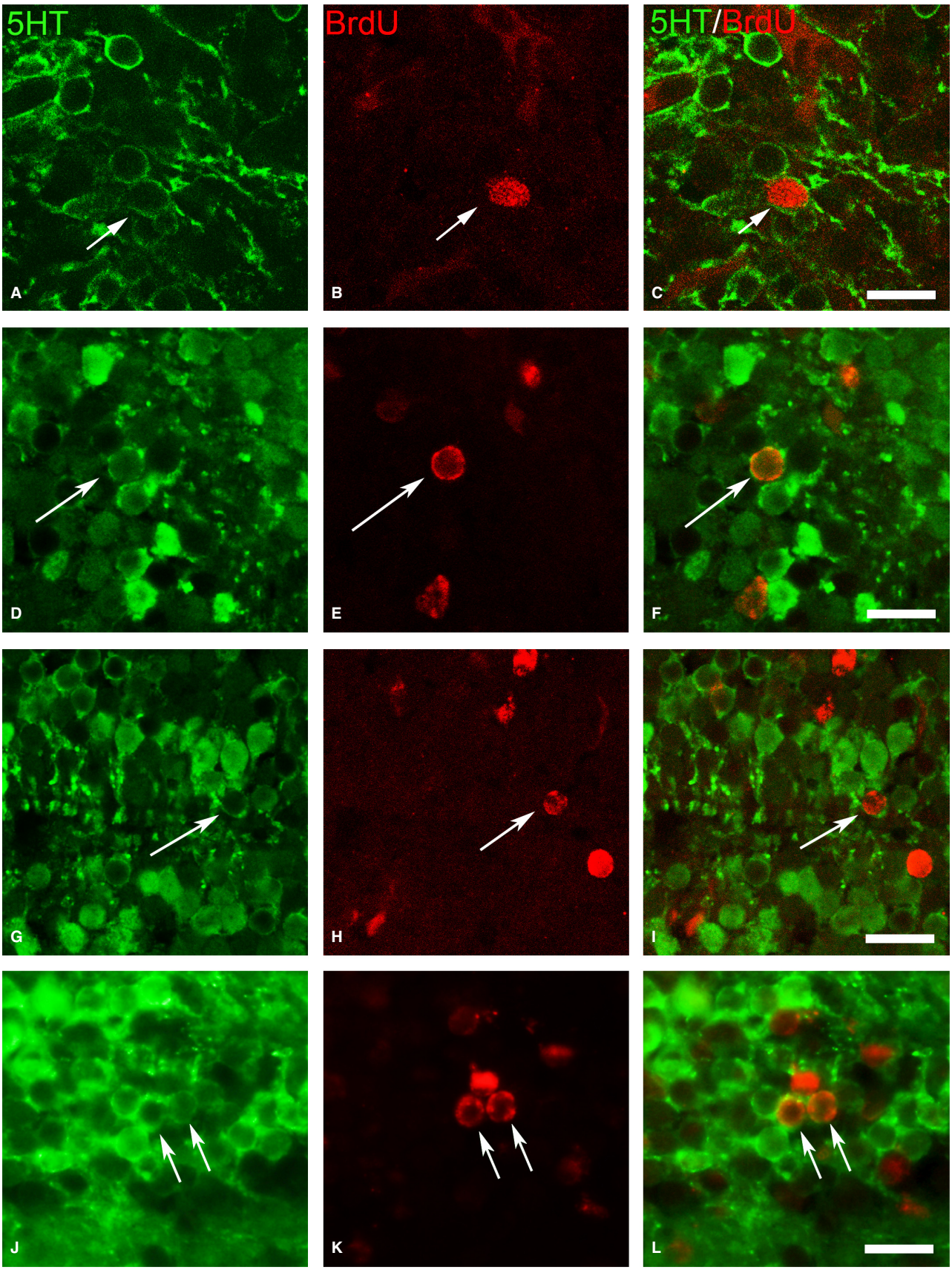


FIG. 4. (A–C) Transverse sections at the level of the PVOp of an adult zebrafish, showing that 14 days after BrdU injection some of the 5-HT neurons (green; A) exhibit a BrdU-positive nucleus (red; B). (C) Merge. (D–F) Examples of 5-HT-positive cells exhibiting a BrdU-positive nucleus 30 days after BrdU injection. (G–L) Examples of 5-HT-positive cells exhibiting a BrdU-positive nucleus 60 days after BrdU injection. Scale bars, 8 μm (A–C), 10 μm (D–L).

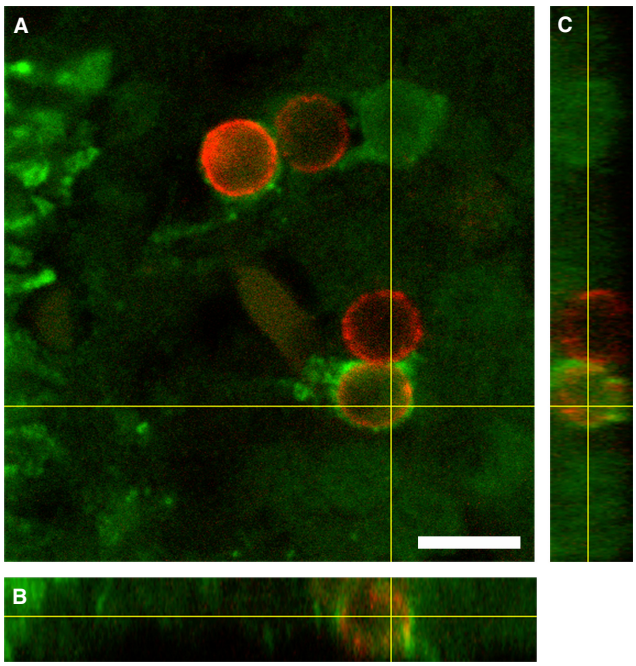


FIG. 5. (A–C) Transverse sections at the level of the PVOp of an adult zebrafish showing that 60 days after BrdU injection a 5-HT-positive neuron (green) exhibits a BrdU-positive nucleus (red). (B and C) Orthogonal projections of the *Z*-stack (23 optic sections of 0.23 μm; 60×/1.35 U PlanS Apo Oil). (A) 486 × 512 pixels; (B) 486 × 80 pixels; (C) 80 × 512 pixels. Original size, 23.08 × 33.79 μm. Scale bar, 6 μm.

This analysis showed a very close neuroanatomical distribution between these two cell populations in the PVOa (Fig. 1E–G), PVOi (not shown) and nucleus of the posterior recess in the caudal hypothalamus (Fig. 1H–J and K–M). Strikingly, in these regions, and particularly in the PVOp, the somas of the RGCs are located distant from the ventricles. This is in contrast with most forebrain regions where the nuclei of the RGCs line the ventricular surface (Menuet *et al.*, 2005; Pellegrini *et al.*, 2007; Marz *et al.*, 2010). In the PVO, RGCs exhibited a cytoplasmic process (~4–8 cell diameters long) to the ventricular layer where these processes connected with each other, forming a virtually continuous barrier along the ventricles (Figs 1I–L, 2, and 6D). Interestingly, the 5-HT cell bodies were closely associated with RGCs, in particular in the PVOp. In the nucleus recessus posterioris, RGCs perfectly surrounded the 5-HT-positive cells (Fig. 1H–M). Careful examination using confocal microscopy clearly shows that the proximal 5-HT-positive processes crossed this barrier through small pores and ended, under the form of a puff, within the ventricular lumen, in direct contact with the CSF (Figs 2 and 6D).

### *5-HT neuron derivation from proliferating radial glial cells*

To study the origin of 5-HT neurons in the caudal hypothalamus in more detail, PCNA stainings were performed in adult tg(*cyp19a1b*-GFP) zebrafish. As shown previously (Pellegrini *et al.*, 2007), RGCs exhibit proliferative activity. Figure 3A–D and I–L shows that most PCNA-positive cells were located at the periphery of the PVOp within the RGC layer. Double stainings further indicated, confirming previous reports (Pellegrini *et al.*, 2007; Marz *et al.*, 2010; Rothenaigner *et al.*, 2011), that a very large majority of PCNA-positive nuclei correspond to RGCs (Fig. 3E–H). In contrast, as would be expected, double stainings for PCNA and 5-HT did not show expression of PCNA in 5-HT-positive cells (Fig. 3I–L).

In order to investigate whether newborn cells generated by RGCs could differentiate into 5-HT neurons, adult zebrafish were injected with BrdU and killed periodically at 1, 3, 14, 21, 30 or 60 days after BrdU treatment. Double staining for BrdU–GFP and BrdU–5-HT were performed on consecutive brain sections of the PVOi and PVOp. At 24 h and 3 days after BrdU injections, BrdU-positive cells were observed at the periphery of the NRP and corresponded to GFP-positive cells, confirming the PCNA data (data not shown). At this stage, BrdU-positive nuclei were not detected in 5-HT neurons. However, at 14, 21, 30 and 60 days after injection, a small proportion (not exceeding 5%) of 5-HT-positive neurons in the PVOp and PVOi were also BrdU-positive cells (Fig. 4A–F), indicating that newborn cells derived from RGCs migrated centripetally and differentiated into 5-HT neurons. It was also common to find, between 5-HT-positive cells, cells labelled with BrdU but not with 5-HT (Figs 4D and F and 5).

### *Inhibition of 5-HT synthesis decreased RGC proliferation in the caudal hypothalamus*

The close relationships between radial progenitors and 5-HT neurons prompted us to investigate the potential effect of 5-HT on the cell proliferation rate in the caudal hypothalamus. This was performed using p-CPA, also known as Fenclonine, which is a synthetic amino acid acting as a selective irreversible inhibitor of tryptophan hydroxylase, causing a decrease in 5-HT content. Figure 6A, B, E and F show that, as expected, intraperitoneal injections of p-CPA caused a drastic reduction in 5-HT immunoreactivity in the brain of zebrafish.

PCNA staining was performed in control and treated animals to evaluate whether P-CPA treatment affected cell proliferation in the PVOi and PVOp, and also in the mediobasal hypothalamus taken as a control. Caution was taken to study only animals of the same age class (6 months), as preliminary studies have shown that after 6 months adult neurogenesis decreases significantly. Figure 6C, D, G and H illustrate the fact that the number of PCNA-positive cells shows a substantial reduction in the p-CPA-treated animals as compared to the controls. This reduction was quantified by double-blind analysis by two different persons. Statistical analyses of the data showed that 5-HT inhibition caused a decrease in cell proliferation in the caudal hypothalamus of adult zebrafish. In the PVOi, a significant reduction in proliferative PCNA-positive cells was detected after 24 h post-injection ($F_{3,32} = 6.12$, $P = 0.021$; $n = 6$) in the treated group relative to control (Fig. 6I). In the NRP, there was also a significant decrease in the PCNA cell number due to treatment ($F_{3,32} = 14.77$, $P = 0.0008$; $n = 5$) and time ($F_{1,32} = 22.86$, $P = 0.0001$; $n = 5$), and the interaction between these two factors ($F_{3,32} = 7.24$, $P = 0.0001$; $n = 5$), at 24 and 48 h post-injection in treated groups relative to controls (Fig. 6J). In contrast, in the neighbouring mediobasal hypothalamus, taken as a control, no significant differences between treated and control groups were detected at any sampling time ($F_{3,32} = 0.037$, $P = 0.84$; $n = 6$; Fig. 6K).

## Discussion

### *Distribution of 5-HT in the brain of zebrafish*

The present work confirms the distribution of 5-HT neurons in the brain of adult fish already documented in a number of studies (Kah & Chambolle, 1983; Ekstrom & Ebbesson, 1989; Johnston *et al.*, 1990; Corio *et al.*, 1991; Lillesaar, 2011). As expected, the most important

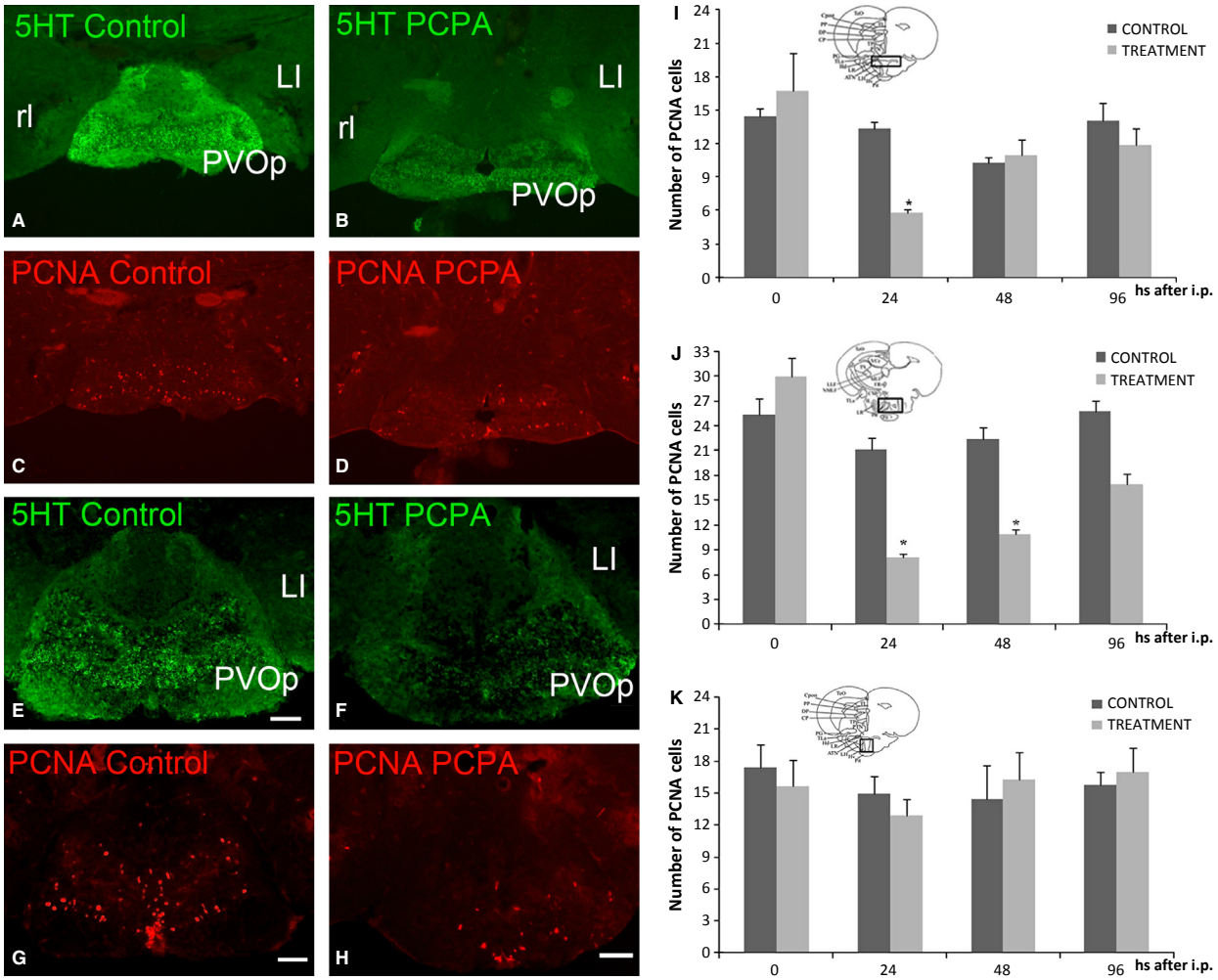


FIG. 6. (A–H) Examples of the effect of p-CPA 24 h after the injection on 5-HT immunoreactivity and cell proliferation in the PVOp. Compared to (A and E) controls, (B and F) p-CPA-treated animals exhibited much less 5-HT immunoreactivity and a smaller number of PCNA-positive cells. Compare C with D and G with H. Pictures A, B, E and F were taken at the same exposure time. (I–K) Effect of p-CPA on the proliferation as assessed by PCNA immunohistochemistry in the PVOi, the PVOp and the mediobasal hypothalamus 24, 48 and 96 h after p-CPA treatment. *$P < 0.005$ according to the Bonferroni test ($n = 5$ for condition and sampling time).

groups of 5-HT neurons were detected in the PVO, in addition to those in the raphe nuclei. This latter location of 5-HT CSF-contacting neurons in the diencephalon is a characteristic of non-mammalian species (Adrio *et al.*, 1999; Lillesaar, 2011; Bosco *et al.*, 2013). Many studies have documented the presence of 5-HT and dopamine neurons in the PVO of teleosts, amphibians, lizards and birds (Fremberg *et al.*, 1977; Yoshida *et al.*, 1982; Sano *et al.*, 1983; Ueda *et al.*, 1984; Meek & Joosten, 1989; Vigh-Teichmann & Vigh, 1989; Bennis *et al.*, 1990; Hirunagi *et al.*, 1992; Vigh & Vigh-Teichmann, 1992; Meek, 1999). Several authors have also pointed out that the number of serotonergic cells in the diencephalon is higher in the brains of fish than in those of frogs, lizards or birds (Smeets & Steinbusch, 1988; Lillesaar, 2011). Recent data from zebrafish have demonstrated that, in contrast to those of the raphe nuclei, the differentiation of 5-HT neurons in the diencephalon is independent of the ETS-domain transcription factor Pet1 and requires Fgf signalling acting through another ETS-domain transcription factor, Etv5b (Bosco *et al.*, 2013). Also, in adult zebrafish, 5-HT neurons of the PVO express a special variant of tryptophan hydroxylase, *tph1*, while *tph2* is expressed in the pineal, the pretectal region and the raphe (Bellipanni *et al.*, 2002). The functional signifi-

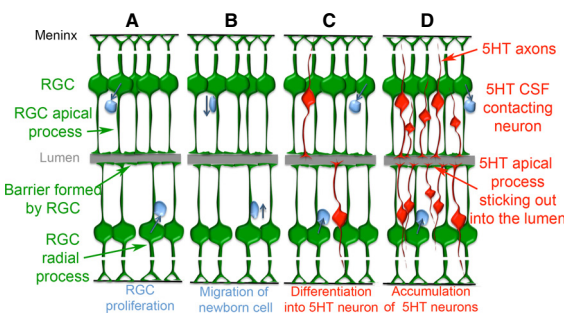


FIG. 7. Schematic representation of the relationships between RGCs (in green) and 5-HT CSF-contacting neurons in the PVOp, around the posterior recess. RGCs exhibit apical processes towards the lumen (in grey) and form a barrier along the ventricular surface. The long radial processes terminate with endfeet on the meninx. (A) RGCs can perform asymmetrical division generating newborn cells which (B), instead of migrating along the radial processes, migrate centripetally towards the ventricle. (C) Some of these newborn cells differentiate into 5-HT CSF-contacting neurons. (C and D) Such neurons have a short apical process that crosses the RGC barrier and protrudes into the ventricular lumen. (D) The repetition of this process leads to an accumulation of 5-HT CSF-contacting cells around the posterior recess. 5-HT axons leave in the opposite direction.

cance and adaptive relevance of the PVO are still subject to investigation.

### *Relationships between 5-HT and radial glial cells in the hypothalamus of zebrafish*

The present study provides for the first time information regarding the organization of the PVO and the relationships between RGCs and 5-HT CSF-contacting neurons. We show that the PVO is particularly rich in RGCs expressing brain aromatase, as documented previously (Diotel *et al.*, 2010). Such cells send long proximal processes to the brain ventricles and their lateral or posterior recessi. In the PVO, RGCs exhibit very tight association with 5-HT CSF-contacting neurons. We also demonstrate that, in adult fish, 5-HT neurons of the PVO derive from radial glia progenitors and, instead of migrating towards the parenchyma, they accumulate between the RGCs and the ventricle (Fig. 7) in contrast to what has been reported in the telencephalon of fish (Adolf *et al.*, 2006; Grandel *et al.*, 2006; Pellegrini *et al.*, 2007; Strobl-Mazzulla *et al.*, 2010; Marz *et al.*, 2011; Rothenaigner *et al.*, 2011; Grandel & Brand, 2013). Obviously, these PVO populations, in particular the PVOp, start increasing during development (Bosco *et al.*, 2013) and continue to increase throughout adulthood. Such a mechanism, which probably also applies to the dopaminergic neurons that exist in the same region, would explain why teleost fishes are regarded as exhibiting the highest 5-HT and dopamine contents in their constantly growing hypothalamus (Baumgarten, 1972).

Despite a large number of studies, the functional significance of these observations remains poorly understood, as the precise functions of the DA and 5-HT CSF-contacting neurons of the PVO are still unknown. These clusters of 5-HT neurons of the diencephalon project locally whereas there is strong evidence that ascending and descending 5-HT fibres originate in the raphe nuclei (Lillesaar *et al.* 2009). The role of the abundant local innervation from the PVO is not documented, but could be related to the modulation of diverse functions, such as the regulation of pituitary hormones, aggression or subordination behaviors and reproductive events (Somoza & Peter, 1991; Khan & Thomas, 1992; Saligaut *et al.*, 1992; Hernandez-Rauda *et al.*, 1996; Winberg *et al.*, 1997; Filby *et al.*, 2010). Although there have been claims that the PVO projects to the pituitary, it is probably not the case. Indeed, when DiI crystals were implanted in the pituitary gland in order to identify the hypophysiotropic neurons, CSF-contacting neurons of the PVO were not stained (Anglade *et al.*, 1993). In addition, there are no or very few 5-HT-positive fibres in the pituitary (Kah & Chambolle, 1983).

### *Effects of 5-HT synthesis inhibitors on adult neurogenesis*

Our study also documents for the first time that 5-HT itself modulates the proliferation of RGCs in the PVO, while this does not seem to be the case in a region devoid of 5-HT-CSF contacting cells such as the mediobasal hypothalamus. Fish treated with p-CPA exhibit, as expected, much lower 5-HT immunoreactivity. However, they also show a drastic reduction in the number of proliferative cells, as assessed by counting PCNA-positive cells in double-blind studies. Such an effect is reminiscent of that reported in mammals, where maternal p-CPA treatment-induced inhibition of 5-HT synthesis alters the brain development in the rat embryo (Lauder & Krebs, 1978; Lauder *et al.*, 1981, 1983). In the adult female rat, p-CPA treatment decreased the number of BrdU-immunolabelled cells in the dentate gyrus of hippocampus in intact (Brezun & Daszuta, 2000b) and ovariectomized animals (Banasr *et al.*, 2004). Furthermore, it is known that chemical lesions of the dorsal and medial raphe nuclei or inhibition of 5-HT synthesis induces a decrease in the number of proliferative cells in the subventricular zone and dentate gyrus. These inhibitory effects are completely reversed when serotonergic input is restored (Brezun & Daszuta, 1999, 2000a).

According to these results, 5-HT itself could promote adult neurogenesis in the PVO, possibly resulting in a further increase in the number of 5-HT neurons. However, the precise site of 5-HT action and the molecular and cellular mechanisms underlying such potential effects require further investigations. Observations from our laboratory suggest that the RGCs form a continuous barrier separating the CSF from the brain parenchyma. From the present study, it is clear that 5-HT-immunoreactive processes cross this barrier through small pores and thus are in direct contact with the CSF. It is still unclear whether 5-HT cells in the PVO take up 5-HT from the CSF or, conversey, release 5-HT into the ventricles as suggested by pioneer studies (Vigh-Teichmann & Vigh, 1989; Hirunagi *et al.*, 1992). According to more recent data, the PVO of fish is capable of synthesizing 5-HT (Lillesaar, 2011). In addition, the caudal hypothalamus is known to express several receptor subtypes, in particular the *htr1ab* receptor subtype, and the 5-HT transporter *slc6a4b* (Norton *et al.*, 2008). In mammals, the actions of 5-HT on neurogenesis are mediated throughout several serotonergic receptor subtypes. Activation of 5-HT1A receptors by a specific agonist increases the proliferation in SVZ and DG (Klempin *et al.*, 2010), and the fluoxetine-induced neurogenesis in the hippocampus is mediated by the 5-HT1A isoform (Santarelli *et al.*, 2003).

One of the most documented hypotheses regarding the role of 5-HT in the hypothalamus of fish concerns its potential involvement in sexual differentiation. According to studies in tilapia, p-CPA is capable of mimicking the effects of E2 on sexual differentiation (Tsai *et al.*, 2001). Furthermore, the expression of *tph2* in tilapia has been reported to be sexually dimorphic, also suggesting a role for 5-HT in sexual differentiation (Sudhakumari *et al.*, 2010; Raghuveer *et al.*, 2011). Because of the high expression of *cyp19a1b* in the PVO of fish, a potential link between 5-HT and aromatase activity is not impossible. In addition, the PVOp strongly expresses estrogen receptors in zebrafish (Diotel *et al.*, 2011). It is therefore possible that 5-HT neurons of the PVO are targets for E2 produced by the neighbouring aromatase-expressing RGCs.

In summary, this study demonstrates that the hypothalamus of adult zebrafish exhibits a strong expression of 5-HT in CSF-contacting neurons originating from RGCs located in their immediate proximity. Inhibition of 5-HT synthesis modifies the proliferation pattern in the periventricular hypothalamic regions, suggesting a link between 5-HT and neurogenesis. Finally, because radial progenitors express aromatase B in fish, one cannot exclude tight interactions between 5-HT and estrogens in the context of neurogenesis and sexual differentiation.

## Acknowledgements

This work was supported by the European INTERREG project TC2N. The authors express their gratitude to the staff of the zebrafish facility of the SFR Biosit (INRA LPGP, Rennes, France). We are grateful to Patrice Mascalchi from the BIOSIT microscopy platform for his invaluable help with confocal microscopy. We also thank Dr Yves Tillet (INRA, PRC, Nouzilly, France) for providing the 5-HT antibody.

## Abbreviations

5-HT, serotonin; BrdU, 5-bromo-2′-deoxyuridine; CSF, cerebrospinal fluid; DAPI, 4,6-diamino-2-phenylindole; GFP, green fluorescent protein; NRP, nucleus of the postural recess; PBS, phosphate-buffered saline, pH 7.4PCNA,

proliferating cell nuclear antigen; p-CPA, 4-p-chloro-L-phenylalanine; PVOa, anterior portion of the paraventricular organ; PVOi, intermediate part of the PVO; PVO, paraventricular organ; PVOp, nucleus recessus posterioris; RGC, radial glial cell.

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