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Multilineage Differentiation Potential of CNS Cell Progenitors in a Recent Developed Gilthead Seabream (*Sparus aurata* L.) Nervous Model

Maria Pia Santacroce[1]*, Antonella Tinelli[2], Anna Selene Pastore[1], Michele Colamonaco[3] and Giuseppe Crescenzo[3]

[1]*Unit of Aquaculture and Zooculture, Department of Veterinary Medicine, University of Bari "Aldo Moro", Italy*
[2]*Unit of Pathology, Deptartment of Veterinary Medicine, University of Bari "Aldo Moro", Italy*
[3]*Unit of Pharmacology and Toxicology, Department of Veterinary Medicine, University of Bari "Aldo Moro", Italy*

Abstract

Neural Progenitor Cells (NPCs) have gathered more and more attention in the field of Neural Stem Cells (NSCs). However, the multilineage differentiating behavior of these cells and their contribution to tissue regeneration, almost in lower vertebrate taxa, remain unknown. Since the early 1970s, many comparative studies have been performed using immunocytochemical screening on the brains of several vertebrate taxa, including teleosts, in order to identify these cells, even if the data are sometimes contrasting. This study aims: (1) to investigate *in vitro* the potential proliferative role of NPCs and Radial Glia Progenitors (RGP) in seabream neurogenesis; (2) to reveal the strict ability of fish NSCs to undertake the multilineage development and differentiation in neurons, astrocytes and oligodendrocytes.

By the use of double Immunofluorescence (IF) analysis and phase contrast microscopy, we identified the multilineage differentiation and the exact cell morphology. We demonstrated that NSC can self-renew and differentiate into different types of neurons or glial cells during extended culturing. Mature neurons expressed specific neuronal markers; they could differentiate during long term culturing, generating an extensive neurite growth. Glia was found highly mitotic and could developed mature astrocytes and oligodendrocytes. Glial cells were assessed by Glial Fibrillary Acidic Protein (GFAP) reactivity; neurons and myelinating oligodendrocytes were immunostained with cell-specific markers.

This work provide that the multilineage differentiation potential of seabream neural cell progenitors might be a useful tool for neurodegenerative diseases, being a promising approach for repairing the CNS injuries, also in other animals, as a new coming strategy for function recovery of damaged nerves.

Keywords: Teleost cell culture; *In vitro* fish neurogenesis; *Sparus aurata;* CNS cell progenitors

Introduction

The generation of new neurons in the adult Central Nervous System (CNS), so called adult neurogenesis, is a fundamental feature of the vertebrate brain during the post-embryonic neural growth [1]. It is well known that in mammals and birds, constitutive turnover of neurons occurs not only during embryogenesis but also during adult stages [2-5]. Adult neurogenesis has been observed in rodents and songbirds, where newborn neurons are added throughout life to highly restricted spatial regions in the telencephalon [5-9]. *In vivo* in mammals, endogenous Neural Progenitor Cells (NPCs), almost generated along the Sub Ventricular Zone (SVZ) in the adult brain, have been proposed as a potential source of newborn neurons for the neural tissue repair after various brain insults, such as ischemic stroke or traumatic brain injury [10,11].

Dissimilarly to birds and mammals, low non-mammalian vertebrates, as fish, amphibians and reptiles, display a considerable amount of adult neurogenesis in more numerous brain regions, even though such differences are poorly understood and investigated [11]. The adult CNS of teleost fish exhibits a high capacity for neuronal regeneration after injury [4]. Comparative studies in zebra fish and mammals show that the telencephalic Ventricular Zone (VZ) in the adult zebra fish brain generates NPCs that share characteristics with the NPCs in the mammalian SVZ: they migrate tangentially and differentiate into mature neurons into the Olfactory Bulb (OB) [5,11-15]. Recently, Kishimoto et al. [11] developed an *in vivo* fish model of telencephalic injury to demonstrate the strong ability of adult zebra fish to undergo neuronal regeneration. Prodigious regenerative processes were found in the injured zebra fish telencephalon, with the proliferation of endogenous NPCs, their lateral migration and neuronal differentiation at the injury site [11].

Studies on Neural Stem Cells (NSCs) defined these cells as self-renewing CNS cells that can differentiate into any of the three major neural cell lineages, specifically neurons, astrocytes and oligodendrocytes [16,17].

Even though the maintenance of vertebrate NSCs in the neural epithelium, as their controlled differentiation, is regulated by a conserved genetic network, many pro-differentiation factors have been explored for being involved in promoting the neural induction, as Fibroblast Growth Factor (FGF), Retinoic Acid (RA) and Epidermal Growth Factor (EGF) [18,19]. The mitogenic action of these growth factors can induce the *in vitro* proliferation and differentiation of NPCs that began to divide within the first week of culture *in vitro*. The first signal produced in the cluster of differentiating progenitors is the expression of nestin, an intermediate filament present in neuroepithelial stem cells, as revealed in cultures of albino mouse embryos [18].

***Corresponding author:** Maria Pia Santacroce, Unit of Aquaculture and Zooculture, Department of Veterinary Medicine, University of Bari "Aldo Moro", Italy, Tel: +39-080-544-3919 Fax: +39-080-544-392; E-mail: maria.santacroce@uniba.it

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In zebra fish, as in rodents, neuroblasts become quiescent after larval development; while in the adult brain, often after brain injury, a Notch signaling pathway prompted by growth factors regulate the transition of NSCs cells to a proliferative state, into neuroblasts toward neurogenesis. However, the extrapolation on the NSCs self-renewing capabilities arose mainly from *in vivo* studies of rodents and mammals, and we are still lacking evidences on the truly proliferative ability of multipotent NSCs and whether these cells can undergo multilineage differentiation to develop mature and self-renewing neurons or glial cells in brain.

In mammals, NPCs have been isolated and characterized *in vitro* from various regions of the fetal, adult and post-mortem brain tissues of various species [20-25]. Accumulating evidence suggests that in teleost fish brain, CNS has the capability to generate new neurons in the adult CNS continuously and in large numbers, probably more than in any other vertebrate taxon [4,26-34].

Nevertheless, none of these studies on fish has addressed in a definite manner the involvement of radial glia (RDG) in the generation of new neurons, by interplaying with the full development of the fish nervous tissue in an *in vitro* microenvironment. Due to several discrepancies, arose the need for improving differentiation procedures able to generate highly pure populations of mature neurons and glial cells in *in vitro* culture systems of lower vertebrates.

For this reason, we recently developed a seabream nervous model to study neuronal differentiation and regenerative processes *in vitro*. Using this model, we aimed: (1) to investigate *in vitro* the potential proliferative role of NPCs and Radial Glia Progenitors (RGP) in fish neurogenesis; (2) to reveal underlying the strict ability of fish NSCs to undertake the multilineage development and differentiation in neurons, astrocytes and oligodendrocytes.

This study provides a novel seabream nervous model for studying a series of regenerative processes involved in fish brain neuronal regeneration, giving new insights for the treatment of injury-impaired neurogenesis, even in mammals.

Materials and Methods

SaGliPs isolation and differentiation

Spaurus aurata juveniles (35 ± 5 g mean body weight, *n*=50) were used to obtain the primary neuroglial culture (SaGliPs), according to a recently developed method [35].

On arrival, fish were anaesthetized by immersion in seawater plus 3-aminobenzoic acid ethyl ester (MS222, 0.02%; Sigma-Aldrich) and rapidly sacrificed by spinal transection with a scalpel blade for decapitation. Whole brain tissue samples (weighing approximately 0.1-0.2 g/brain) were removed aseptically, pooled and collected in a sterile pre-cooled Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) (without Ca^{2+} and Mg^{2+}) supplemented with 10 mM HEPES, 0.5 mM EDTA, 25 mM $NaHCO_3$, 200 IU/ml penicillin, 200 µg/ml streptomycin, 200 µg/ml amphotericin B and 100 µg/ml gentamicin (Washing Solution, WS) [36,37]. Unless specified all chemicals were from Sigma-Aldrich-Aldrich Ltd., Milan, Italy.

Primary neuroglial cultures were rapidly isolated from freshly-enucleated brains [35]. After three washings with three volumes of HBSS (WS), brains were crushed by pressing a glass pestle into a stainless-steel sieve with 380 µm mesh for mechanical disruption. Cell suspension was filtered and collected with Digestion Medium (DM) lacking enzymes (7 mM $CaCl_2$, 200 IU/ml penicillin, 200 µg/ml streptomycin, 200 µg/ml amphotericin B, 100 µg/ml gentamicin, 10 mM HEPES, 25 mM $NaHCO_3$ in Leibovitz's L-15). The homogenate was then extracted by adding a cocktail of four enzymes including 0.05% Collagenase Type IV, 0.03% Hyaluronidase Type IV-S, 0.3% Dispase Type II, 0.03% DNase Type I for 20 min dissociations at 20°C to the homogenate in DM. The enzyme digestion was blocked adding Leibovitz's L-15 medium supplemented with 10% Foetal Bovine Serum (FBS; Bio Whittaker, Lonza Walkersville Inc., Italy). The digestion mixture was filtered through 104 µm and 60 µm stainless-steel filters, supernatant was discarded, and the pellet was resuspended in cold 1X Phosphate-Buffered Saline (PBS) 5% Fetal Calf Serum (FCS) and centrifuged twice at 150 g for 10 min at 4°C. The white pellet was recovered, washed and dissociated by adding 0.45 µg ml^{-1} DNase I in cold PBS to dissociate cell clumps by Pasteur pipette, then centrifuged at 70 g for 5 min at 4°C. The final collected pellets were resuspended in 10% FBS/L-15 medium and cell number was counted. Viability of cells was estimated by trypan blue exclusion. Brain cell suspension yielded 5.2×10^7 viable cells/g per tissue weight with a viability of 92.8 ± 0.06%.

The suspension of purified neuroglia was adjusted to a density of 1×10^6 cells/ml in basal culture medium (BM) consisting of Leibovitz's L-15 with 2 mM L-glutamine, 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml amphotericine, 50 µg/ml gentamycin, 1 mM Na Pyruvate, 5 mM D-Glu, 10 mM HEPES, 12 mM $NaHCO_3$, supplemented with 0.05% ITS *plus* (insulin/transferrin/sodium selenite plus oleic acid/linoleic acid/BSA), 0.01 mM MEM non-essential amino acid (MEM-NEAA; Bio Whittaker, Lonza Walkersville Inc., Italy), 0.01 mM MEM-vitamin mix (Bio Whittaker, Lonza Walkersville Inc., Italy) and 0.1 mM ascorbic acid. The BM osmolality was adjusted to seabream serum osmolarity (369 mOsm/kg) by adding 20 mMNaCl as reported by other Authors [38,39].

Cells were cultured in complete Leibovitz's L-15 (BM) and seeded in 0.01% collagen type I-coated plates and flasks at a density of 3×10^4 cells/cm^2, respectively, into 25 cm^2 Falcon Primaria™ culture flasks for morphological characterization of primary cell types by phase-contrast, and on 12-well plates Falcon BD Biosciences (BD Biosciences, Sismed s.r.l., Italy) for immunophenotypization by immunofluorescence. Firstly cells were allowed to attach for 12 hours, then fresh BM was added, thereafter replaced every 48 hours. Cells were cultured in a refrigerate incubator in humidified atmosphere at 97% air/3% CO_2 at 18°C (CO_2-170 Innova, New Brunswick Scientific, PBI, Milan, Italy). Cultures were used after approximately 3-4 weeks when the cells had become confluent monolayers. To obtain a glial cell-enriched culture, after the first week SaGliPs were cultured in Enriched Differentiation Medium (EDM) consisting in L-15 BM supplemented with 0.01 µg/ml Epidermal Growth Factor (EGF), 0.2 µg/ml hydrocortisone, 10 mM glucose, 10 µg/ml insulin and 4 mM glutamine. For assessment of cytostructural features, phase-contrast microscopy pictures were taken daily on adherent cultured SaGliPs with a CCD camera on a Motic AE31 inverted Epi-Fluorescence microscope.

SaGliPs characterization by immunofluorescence

Microscopy analysis was performed, for bright field and fluorescence, by a Motic AE31 Epi-Fluorescent Inverted Microscope, equipped with DAPI/TRITC/FITC fluorescence filter cube set (DAPI/Hoechst set: Exciter D350/50x /Emitter D460/50m, FITC/RSGFP/Fluo 3/DioAcridine set: Ex. D480/30x/Em. D535/40m, TRITC/DiI/Cy3 set: Ex. D540/25x/Em. D605/55m). Digital image capture was performed by Moticam 3000C Cooled CCD digital color camera (3.3 Megapixel, 1/2" CCD), capture system in origin Live Cam 1.0 (32-32) and Motic Images Advanced (V. 3.2) acquisition software (Motic, Seneco, Milan,

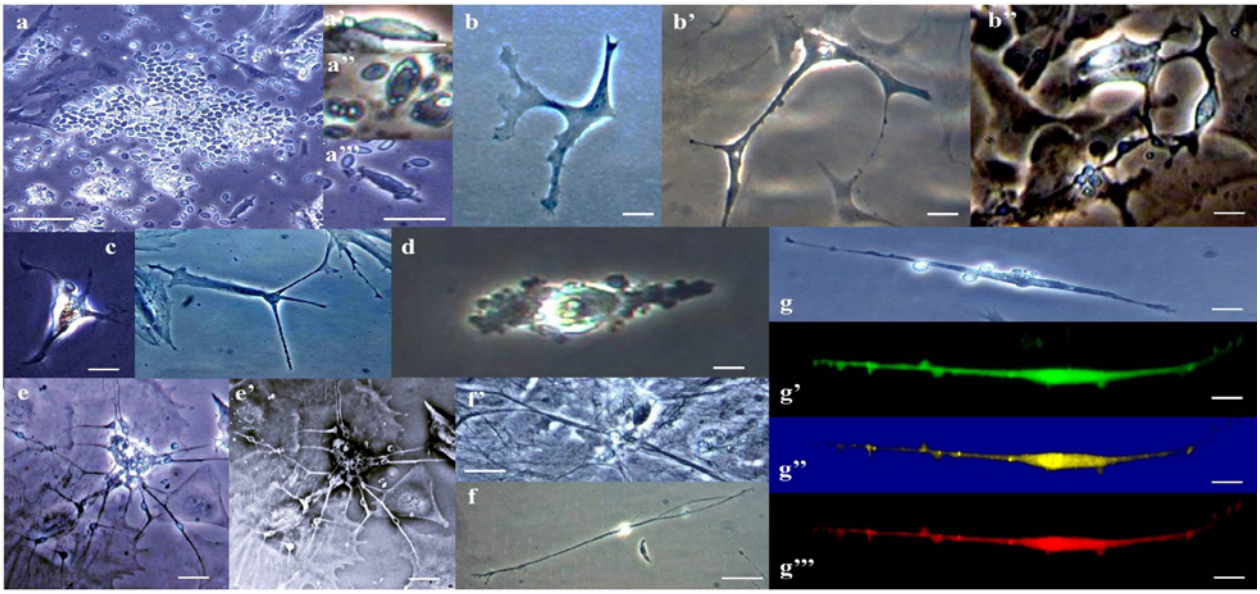

Figure 1: Morphological characterization of *S. aurata* primary neuronal cells. (a) Morphology of freshly isolated staminal neural cells (CNS) with the characteristic ovoid shape (a', a''), 1 day after seeding (×200). (b, b', b'') Early elongated attached glioblast progenitor after 2 days (×200). (b'') Negative inverted phase contrast image showing newly formed glioblasts from neuron precursor cells (NPC) in 6 day old culture (×200). (c) Glioblast (c') Neuroblast (d) Morphology of Neuron Precursor Cells (NPC) which generate neurons but not glia: here is visualized a colony showing new synthetized neurons (×200). (e, e') Morphology of neuronal Progenitor showing a cluster of newly synthetized neural cells, 4 day old culture (×200). (f) Morphology of a bipolar neuron after 2 day (f) and pseudo-unipolar neuron after 2 weeks of culture (f') showing the characteristic soma ovoid shape. (g) Bipolar shaped neuroblasts immunolabeled with GFAP-FITC (green, g'), with beta III tubulin-TRICT (red, g'''), merged image of g' and g''' (g''). Scale bar = 50 µm (a); 20 µm (f); 10 µm all.

Italy). Image analysis and assemblage was performed with Motic Images Advanced (V. 3.2) software (Motic, Seneco, Milan, Italy) and by using the Adobe Photoshop 8.0 (Adobe, Inc.) software for reunion from different filters.

For *indirect immunolabeling*, the following polyclonal (pAb) or monoclonal (mAb) primary antibodies were used at the dilutions indicated: mAb mouse IgG Anti-Glial Fibrillary Acidic Protein (GFAP) (G3893, clone No. G-A-5, Sigma-Aldrich, 1:500); pAb rabbit anti-UCHL1 (HPA005993, Sigma-Aldrich, 1:1000); mAb mouse IgM Anti-Vimentin, (V5255, clone VIM-13.2, Sigma-Aldrich, 1:200); pAb rabbit IgG Anti-Neurofilament 200 (NF), (N4142, Sigma-Aldrich, 1:200); mAb mouse Anti-Nestin [10C2] (ab22035, 1:250); pAb rabbit Anti-MBP (M3821, Sigma-Aldrich, 1:200); rabbit Anti-S100 beta antibody (S2644, Sigma); rabbit anti-b-III-tubulin antibody (Sigma). The secondary antibodies used were as follows: Goat Anti-Mouse IgG TRITC conjugate (T7028, Sigma); goat anti-rabbit IgG1 fluorescein isothiocyanate (FITC)-conjugate (F6005, Sigma); goat anti-rabbit conjugated with Alexa 594, Invitrogen (A11037); goat anti-rabbit conjugated with Alexa 594, Invitrogen (A11037); Goat Anti-Rabbit IgG-FITC (Product No. F0382); goat Anti-Mouse IgG FITC conjugate (F0257, Sigma); goat anti-rabbit conjugated with Alexa 594, Invitrogen; goat Anti-Rabbit IgG-FITC (F9887) at a 1:50 dilution for 45 minutes at RT. All primary and secondary antibodies were diluted in PBS/3% BSA. Cells were rinsed three times with PBS, fixed with 4% paraformaldehyde/PBS for 15 min at RT, washed twice with PBS and permeabilized with 0.2% Triton X-100/PBS for 10 min at RT. After three washes with PBS each of 5 min, cells were blocked in 3% BSA/PBS for 30 min, and then incubated in primary antibodies diluted in blocking buffer for 60 min at RT. For indirect labelling, after three PBS washes, cells were incubated with the appropriate secondary antibody for 60 min at RT. After other three PBS washes, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1.5 µg/ml) for 10 min at RT. To avoid photo bleaching of fluorescent dyes, stained cells were embed with antifade mounting medium PBS/glycerine/1,4-diazabicyclo[2.2.2]octane (90% Glycerol/100 mg/ml DABCO/PBS), adding the antifade directly onto the wells before microscope visualization. As a negative control, cells were stained with the same procedure without the primary antibody labelling.

For *direct immunolabeling*, the following monoclonal (mAb) antibody was used at the dilutions indicated: mAb mouse anti Proliferating Cell Nuclear Antigen (PCNA) FITC-conjugate (F0167, clone PC10, Sigma-Aldrich, 1:100).

Results

Neuronal and radial glial cells

Studies on NSCs defined these cells as self-renewing CNS cells that can differentiate into any of the three major neural cell lineages, specifically neurons, astrocytes and oligodendrocytes [16,17]. To study the potential role of NPCs and Radial Glia Progenitors (RGP) in fish neurogenesis, the neuroglial primary culture of the fish *S. aurata* has been used by our laboratory as a teleost CNS model system.

Multilineage development and differentiation of neurons, astrocytes and oligodendrocytes from *S. aurata* neuroglial cells in primary culture (SaGliPs), previously isolated in our laboratory, were morphologically characterized by inverted phase-contrast microscopy, and analyzed by Motic Images Advanced (V. 3.2) acquisition software (Motic, Seneco, Milan, Italy) [35]. In the panel of Figure 1 are shown microscopy images indicating the appearance, the morphologic features and the immunophenotyping of the neuronal lineage, with NSCs (Figures 1a-1d) and immature neurons (Figures 1e-1g''').

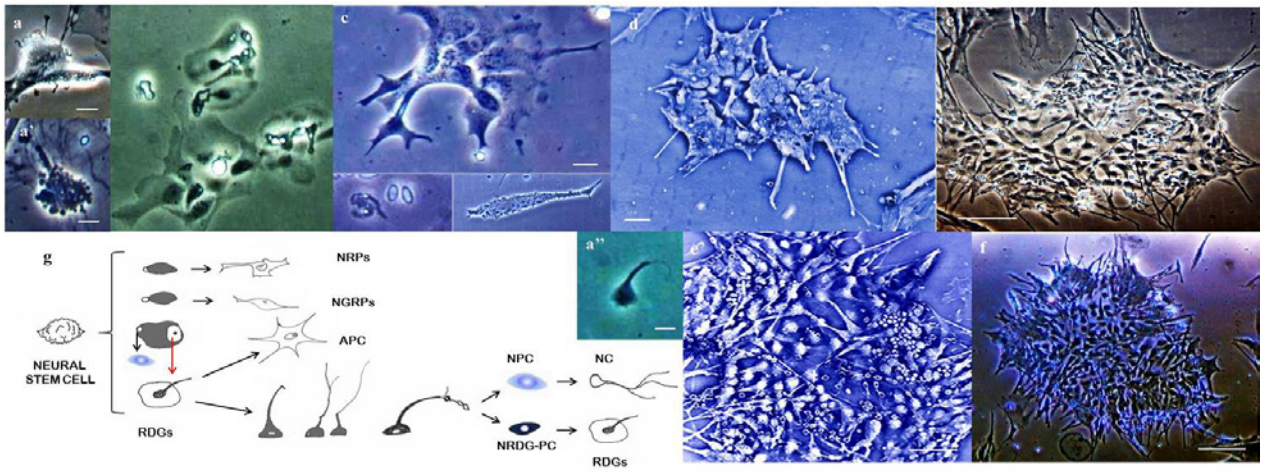

Figure 2: Morphological characterization of *S. aurata* primary neuroglial cultures. Phase-contrast microscopy pictures showing development and differentiation steps of Neuroglial Restricted Precursor cells (NGRPs). (a) NGRP cluster of stem cells at the early stage of neurosphere. (a') Highly branched neurosphere in adhesion morphology. (a'') Early stage of radial fiber generation: basal fiber cell of radial glia forming a short stub protrusive from the soma. (b, c, d) Early radial glia cluster producing basal glial cells; the NGRPs extend their basal process in a radial direction, thus gaining the typical form of radial glial rosette-like colony. (e) Phase-contrast image showing a rosette like colony of radial glia with early elongated protrusions after 2 weeks of culturing (×200). (e') An enlargement of cells in (e) showing a particular of the radial colony with areas of active neuronal proliferation and first dividing cells generating neurons (×400). (f) Phase-contrast image showing a rosette colony of radial glia with a blue filter enlightening newly formed neurons after 2 weeks of culturing. (g) Scheme represents development and differentiation of neuro-glial restricted precursor cells (NGRPs) astrocytes, oligodendrocytes, and radial glial cells. Scale bar = 10 µm (a-d); 50 µm (e-f).

In the panel of Figure 2 are presented microscopy images representative of the Neuro-Glial Restricted Precursors (NGRPs) in their appearance and morphologic features, with cluster of NSCs (Figure 2a) and glioblasts (Figures 2b-2f). Immunofluorescence images showed differentiated neuronal progenitor cells attached with pro-oligodendrocyte precursor cell expressing positive GFAP immunoreactivity (Figure 2b). In SaGliPs, after the first day of cell adhesion, the characteristic shape of neural stem cells appeared to the bottom of the flask as a round neurosphere rich of multi-extruding processes (Figure 2a); on the second day the shape of NGRPs changed both in morphology and in colour becoming flatten as in Figure 2a', while the first radial glial (RDG) rosette like colonies were observable (Figures 2e, 2f).

Within the first week after plating, the NGRPs extended their basal process in a radial direction, thus gaining the typical form of RDG rosette like colony (Figures 2b-2d). The contrast phase analysis has been shown that the new cell production occurred in specific mitotically active proliferation zones. Inside the RDG, a typical cell population of neuronal perikaryal appearance started to proliferate directly from the foot of cone-shaped radial cell, thus forming a cluster of highly proliferative cells (Figures 2e, 2e', 2f). From these proliferation zones, the newly formed young neuronal cells are guided, in their migration to the final target areas, by RDG fibers (Figures 3a,3b).

The neurogenic niches with astroglial morphology riches of highly replicating cells were observed during the 2nd week of differentiation (Figures 2 and 3). Fish neurogenic islands (generated by RGCs) showed a greater strong ability to regenerate neural cells, succeeding in a continuous generation of young neurons and RDGc renewal (Figures 2 and 3).

To determine whether RDGc were mitotically active, we tested their mitotic activity by fluorescence DNA markers (Figures 3e, 4d, 4e, 5e, 5f', 5h): (1) the UV-light excitable DAPI used as a DNA specific fluorochrome, as it appears to associate with AT clusters in the dsDNA; and (2) the Proliferating Cell Nuclear Antigen (PCNA), the most widely used expression marker of proliferation-associated proteins. PCNA is an antigen detected by antibody to cyclins D, E, A, and B which is specific for G1/2 and S, M phases of the mitotic cycle [40,41].

The morphology and distribution of the radial fiber indicates the type of dividing cells, as a consequence of different cleavage plane during cell cycle division of RDGc (Figures 3a-3f). During the early stage of fiber generation, the basal fiber of RDG remained elongated; it appeared like tired out forming a short radial stub projecting from the soma, that thins distally (Figure 2a'', 2b, 2g). Our results suggest that the vertical cleavage plane result only in neuronal production of daughter cells similar in shape (Figure 3a), while the horizontal cleavages produce two types of asymmetric divisions by generating (1) one new radial glial cell and one neuron progenitor (cell/cell horizontal cleavage) (Figure 3b); (2) one radial fiber and one neuron progenitor (fiber/cell horizontal cleavage) (Figure 3d). Furthermore, the contrast phase and immunofluorescence analysis revealed that young RDGc, during their cell division, generate two different types of daughter cells, one destined to become a long fiber process and the other destined to develop a neuron (Figures 3c, 3c', 3c''). In particular, the end of the fiber grew a thick process that would break, generating a neuron that started to create a line of newborn neurons by symmetric divisions (Figure 3). Such kind of neurogenesis was a peculiar characteristic of the seabream RDG model (Figure 3).

Two populations of glial fibers were apparent, one immunopositive for GFAP, the other for vimentin (Figure 4d). Immunocytochemistry analyses for double-or triple-immunofluorescent labelling were performed to define the status of differentiation of cultured cells. After 2 weeks of culturing, immunofluorescence revealed elongated DAPI-labelled nuclei, in close apposition to GFAP-labelled radial glial fibers (Figure 4d), thus signifying that NPCs grew closely opposed to the RDG fibers (Figure 4e). The GFAP-positive RDG fibers increased near the proliferating site of new neurons. It seems conceivable that these

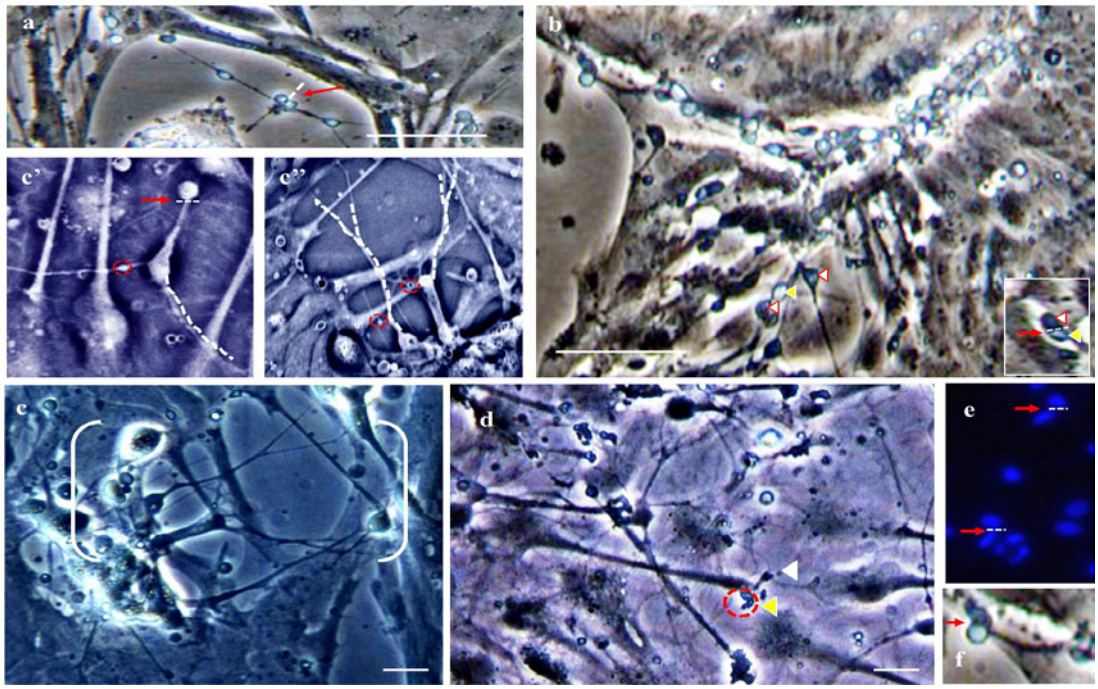

Figure 3: Symmetric and asymmetric division of *S. aurata* radial-glial cells (RDGs). (a) Phase contrast image showing symmetric divisions with vertical cleavages resulting only in neuronal production of daughter cells similar in shape. (b) Phase contrast image showing asymmetric divisions with horizontal cleavages by generating one new radial glial cell and one neuron progenitor (cell/cell). The bracketed area in (c) is shown in separate picture in (c') and (c"), as inverted phase contrast image displaying asymmetric division. Arrowheads in red indicate the horizontal cleavage plane generating the asymmetric RG cell division, white dotted lines indicate RG fibers, red circles indicate the neuron progenitor. (d) Phase contrast image showing asymmetric divisions on horizontal cleavage resulting in one radial fiber and one neuron progenitor (fiber/cell). Particularly (f) at the fiber extremity grows a thick process which collapses, generating a neuron that began to develop a line of newborn neurons by symmetric divisions. (e) DAPI counterstained nuclei show asymmetric divisions with horizontal cleavages

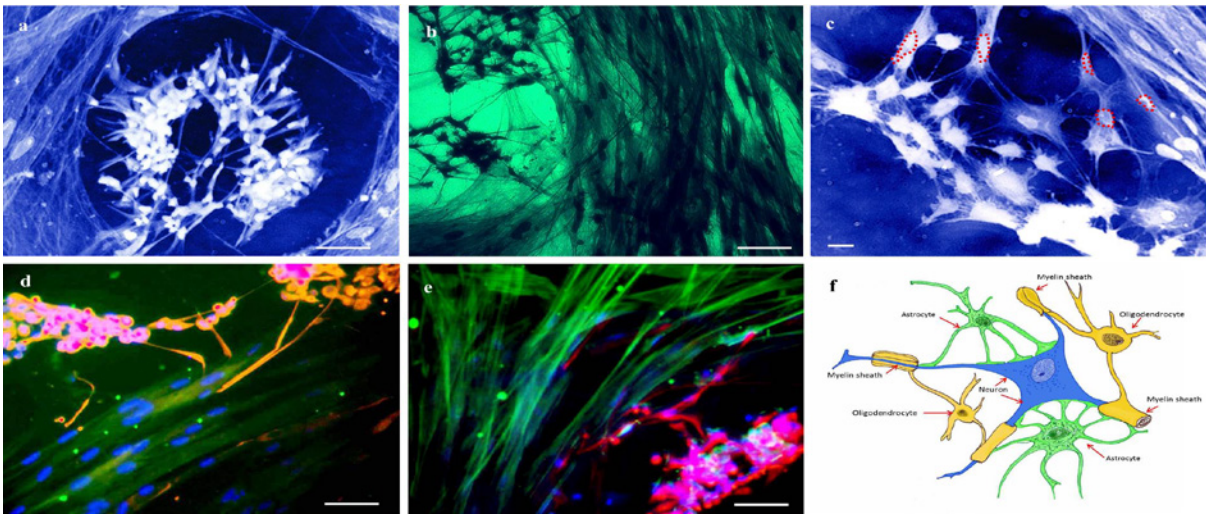

Figure 4: Mature radial glia from primary neuroglial cultures of *S. aurata* brain. Phase contrast microscopy images showing radial glia progeny from neural stem cells. (a, b) Rosette-like colonies of radial glia after 2 weeks of culture (200x). (c) Edge colony enlargement of (a); detail of the radial glial fibers, making an interlaced scaffold to which NPCs were closely apposed, showing the nuclei migration of new neurons (red dashed circles) within the radial migrating fiber towards the boundary (400x). (d) Immunofluorescence staining focusing GFAP positive astrocytes (red) differentiated into large cells with many filopodia while contacting multiple axons (green filament) in order to sustain oligodendrocytes during the myelination process (green spheres) (GFAP, red; vimentin, green filament; MBP, green spheres; DAPI/PCNA, blue/cyan). Merged picture (e) displaying colocalisation of MBP to label OPCs (green spheres) and GFAP to label astrocytes (red) near NF positive axons (green), with DAPI/PCNA (blue/cyan) to label cell nuclei. (d, e) RGCs can produce new generation of young neurons even in the old culture as revealed by PCNA within the inner cluster. (f) This diagram shows a schematic arrangement of nervous cells in culture. Astrocytes contact neurons being involved in metabolic exchange between neurons and blood; oligodendrocytes wrap themselves around axons forming the myelin sheath. Scale bar = 50 µm (a, b, d, e); 10 µm (c).

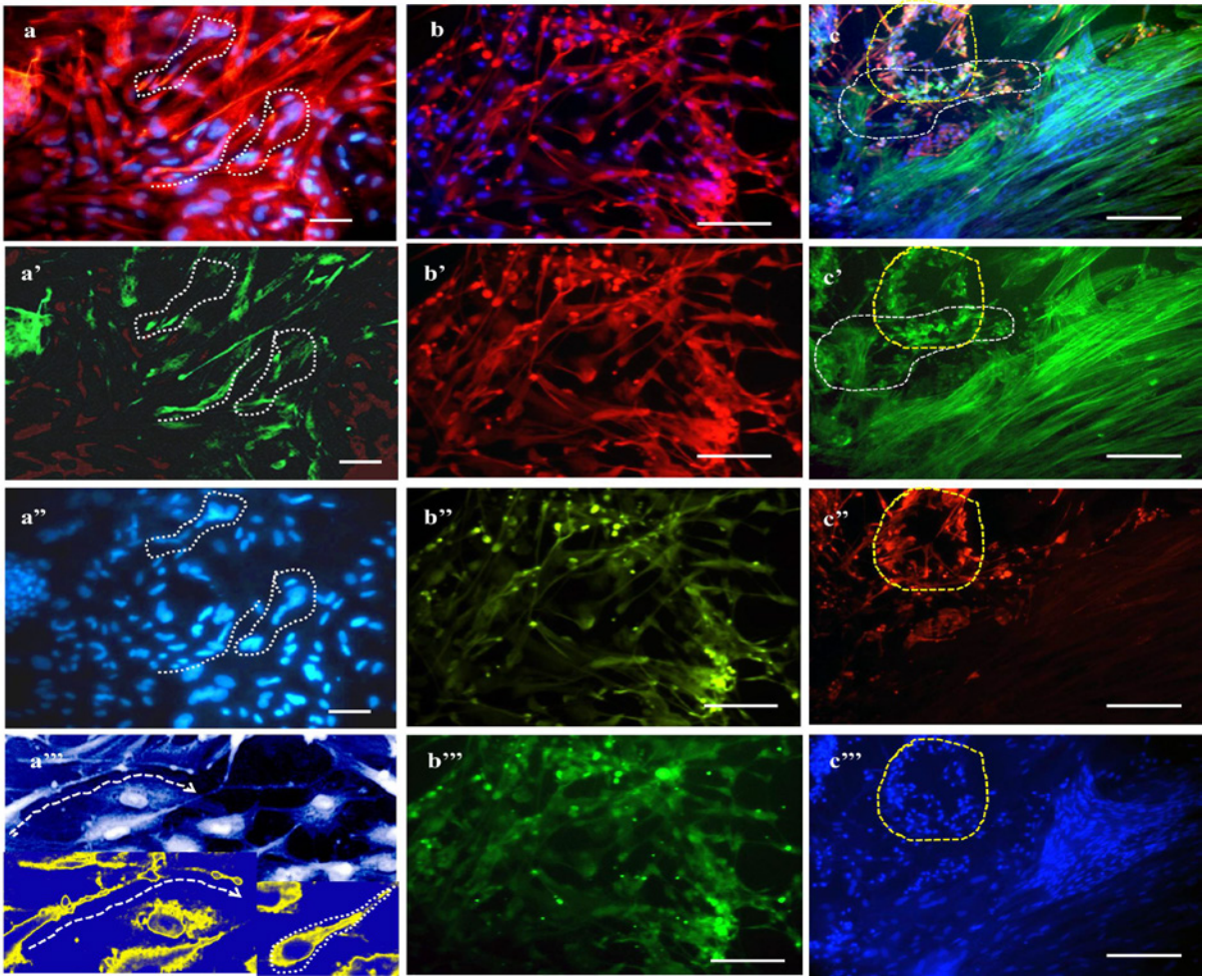

Figure 5: Indirect and direct immunofluorescence staining focusing development and differentiation in *S. aurata* brain cultured cells. (a) GFAP immunoreactivity at the tips of the astrocytes processes after 3 week of culture (×200); (a') Vimentin labeled fiber; Radial glia stained immunopositive for both glial fibrillary acidic protein (GFAP) and vimentin at 3 weeks old cultures; (a'') DAPI counterstained nuclei corresponding to the same picture of (a) and (a') (400x). The proliferative capacity of cells was shown by mitotic nuclei (white/cyan) double stained with Ab anti-PCNA-FITC and counterstained with DAPI (blue); the white dashed lines showed the actively dividing nuclei within the fiber extension. (a''') Phase contrast image showing basal RDG cells generating neurons (white dashed line showed the nuclear migration and fiber extension). (b-b') Intense fluorescence for Nestin here appeared to be highly expressed in the processes by most IPCs, 3 weeks old cultures. (b'') βIII-tubulin label immature neurons. (b''') NeuN was expressed nearly by mitotic figures. The mature post-mitotic neurons expressed in high density NeuN positive processes. (c-c''') Double immunofluorescence demonstrating colocalisation of neurons and astrocytes within the radial glial colony. (c) Several clusters of proliferative cells were observed (white and yellow circles); in particular, the central zone showed an intense granular production of neurons (UCHL1+/DAPI+/PCNA+) (white) inside the center of the colony of radial glia (GFAP+) (yellow circle). (c') 3 weeks old cultures, highly immunostained with UCHL1 (green), showed newborn neurons migrate along neuronal fiber. (c'') The outer boundary of the radial glia colony showed differentiated astrocytes with long GFAP positive filopodia (red) and neuronal fibers (c and c'), whereas in the inner zone there are clones of mitotic radial glia (c''', PCNA+/DAPI+). Scale bar = 50 μm.

neurons moved from end to the tip of the radial process by nuclear translocation, as visualized with the DNA marker (DAPI), thus reaching their final target site predominantly by the nucleus migration within the fiber (Figure 5).

Between the end of the second week and the beginning of the third of culturing, the seabream RDG colony assumed a specialized aspect, with RDG fibers arranged in an interlaced scaffold to which NPCs were closely apposed, possibly for their radial migration towards the final target site. The scaffold was organized with GFAP positive differentiated astrocytes with many filopodia contacting multiple axons (vimentin positive) in order to sustain oligodendrocytes during the myelination process (Figures 4d and 4e). Neurons were apparent when fish CNS cultures were at 2 weeks of culturing, emerging from the tip of RDG fibers (Figure 5). The morphology and the immunophenotyping of neural cells were displayed by the immunostaining with antineurofilament (NeuN), anti-βIII-tubulin, anti-nestin, anti-vimentin antibodies and the neuron-specific markers for ubiquitin carboxy-terminal hydrolase 1 (UCHL1) (Figure 5).

We tested neural differentiation of NPCs by examining the expression of maturation protein markers. To characterize the first early events of differentiation, we studied markers of NPCs during the immature status. After 1 week of culture, most immature precursors cells (IPCs) neural structures contained neuroepithelial cells immunoreactive for nestin. Nestin is an intermediate filament protein commonly expressed by many progenitor cells, and here appeared to be highly expressed in high density into neurospheres and IPCs processes (Figure 5). Most

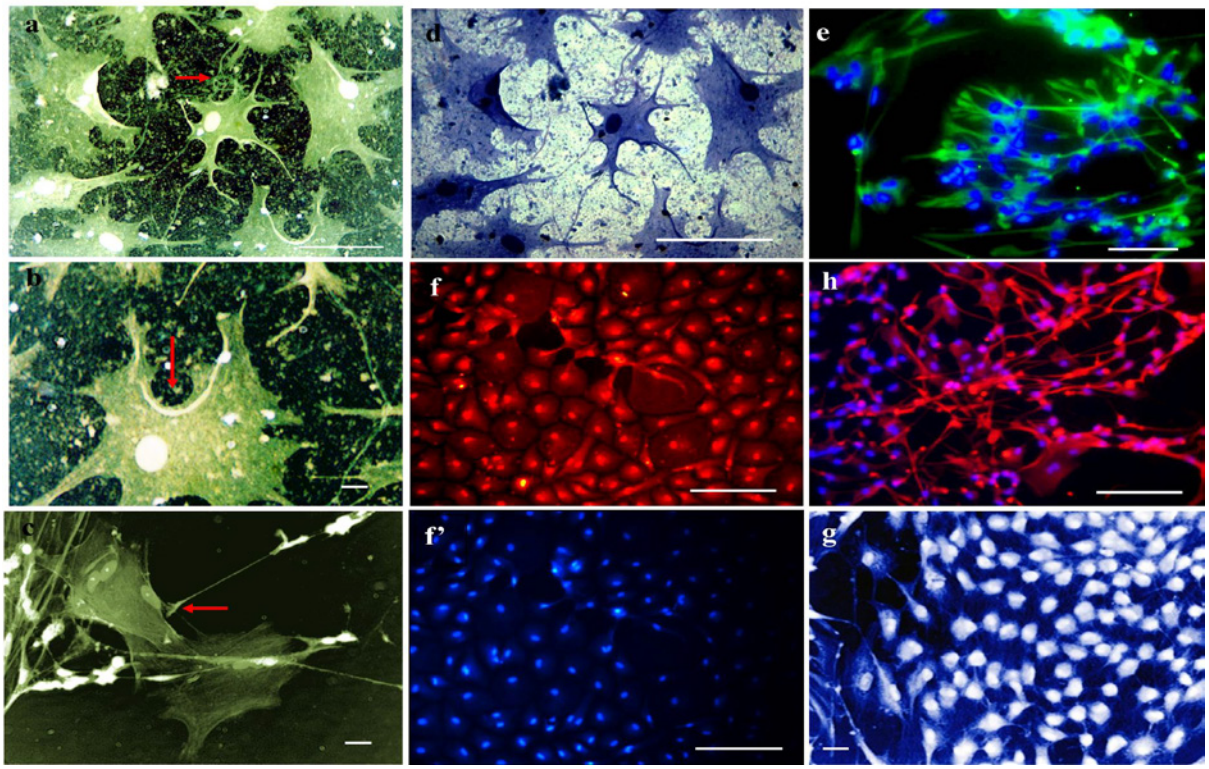

Figure 6: Characterization of *S. aurata* astrocyte monolayer. Phase contrast microscopy images showing the monolayer growth and differentiation in primary neuroglial cell cultures. (a) Astrocytes intercommunicated forming a monolayer after 2 weeks (×400), and (b) young neuron attached on the astrocyte border. (c, d) Phase contrast image showing fully differentiated communicating astrocytes connected with elongated neuritis overwhelming the cells after 1 month of culture (×400). (e) Mature astrocytes positive for UCHL1 labeling at the tips of the astrocytes processes (green) and DAPI (blue nuclei) after 1 week of culturing. (×400). (f, f') GFAP-immunoreactive mature astrocytes after 1 month of culture with the typical stellate shape and nuclei DAPI counterstained (×400). (g) Negative inverted phase contrast image showing 2 weeks old cultures of microglia (×400). (h) Mature astrocytes positive for GFAP labeling at the tips of the astrocytes processes (red) and DAPI (blue nuclei) after 1 week of culturing. (×400). Scale bar = 50 μm

nestin positive cells contained PCNA expression which colocalized with the DAPI counterstaining, thus counting for mitotic figures (Figure 5). RDGc can produce new generation of young neurons even in the old culture as revealed by PCNA within the inner cluster (Figures 4d and 4e). PCNA is a cell cycle dependent intranuclear protein, which assists the delta-DNA polymerase during DNA replication, whereby its expression is considered a marker of DNA synthesis, being detectable in G1/S phase [40]. By producing new neurons and guiding them to the growing edge of colony, radial glial cells confirmed their relatively strong ability as in the adult fish brain to regenerate neural tissues.

In a first early event, the basal fibers of RDGc supported the production and migration of new born neurons (Figure 5a-5a'''), around the edge of the RDG rosettes (Figure 5c and 5c'). Whereas during the phase of neuronal differentiation a new circuit formation of horizontal fibers occurred later, displaying an interlaced network, as a mesh woven of axonal fibres closely connected (Figure 4). Next, to evaluate the transition from IPCs to mature neurons, we tested two neuronal differentiation markers, βIII-tubulin to label immature neurons and NeuN the definite marker of mature neurons. After 14-20 days of differentiation, the cultures were highly enriched in neurons expressing the βIII-tubulin marker (Figure 5). The colocalization of βIII-tubulin/NeuN was expressed almost by differentiating neurons, while cells immunoreactive for NeuN were cells which completed the transition from immature to mature neurons. All differentiated neurons presented the multilineage neural phenotype, defined as coexpression of the following markers gradient oriented according the developmental status: GFAP, Nestin, Beta III-tubulin, NeuN, UCHL1 (Figure 5). During long term culturing the density of fibers markedly increased within 3 weeks, and differentiation up to 4 weeks achieved a complete coverage of the astrocyte bed with a massive arborisation of neurites (Figure 6). The density of vimentin-positive RDG fibers increased over the monolayer as long as the neuritis full-grown (Figure 6). By analyzing the sequence of events taken in the formation of astro-neuronal tissue *in vitro*, it appears apparent that the formation of the fiber network of RDG is a plastic prerequisite needed to enable the support of growth and migration of the newly generated neuronal cells (Figure 6), as confirmed by the presence of actively dividing nuclei, doublestained with Ab anti-PCNA-FITC and counterstained with DAPI (blue), within the fiber extension (Figure 5a"). Finally, even in the 3rd week old cultures were still observed mitosis both for newborn neurons migrating along neuronal fibers (UCHL1+/PCNA+/DAPI+) and within the inner rosette-like colony, suggesting the coexistence of differentiating astrocytes and neurons with newly formed cells.

Astroglial cells

Two specific progenitor cells, Glial-Restricted Precursor (GRP) cells and oligodendrocyte/type-2 astrocyte progenitor cells (O-2A/OPCs) have been studied as distinct glial precursor subtypes restricted to astrocyte development in mammals; they only differed in the differentiation potential and in the timing of their developmental

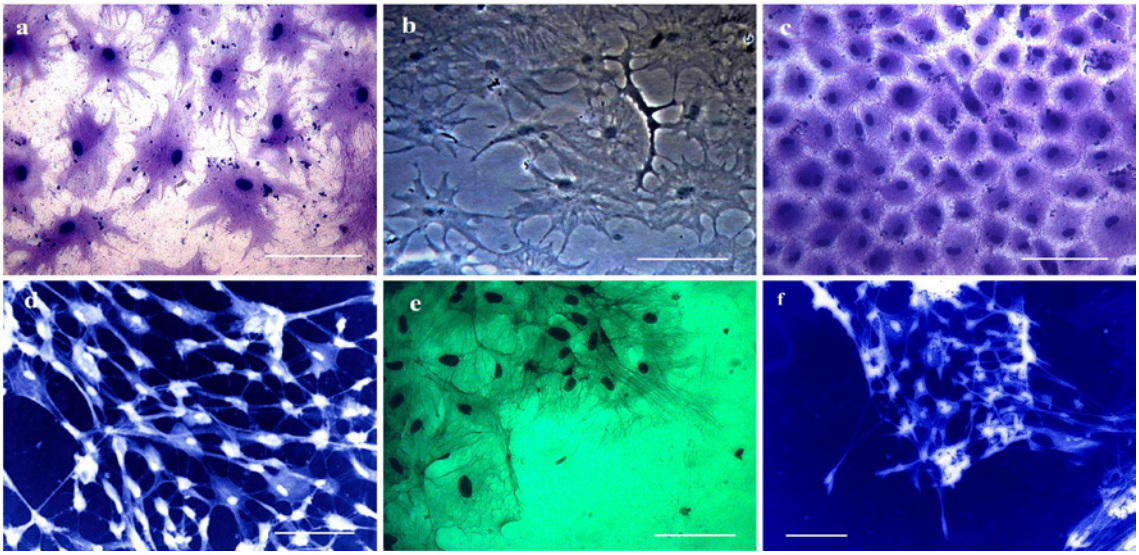

Figure 7: Characterization of *S. aurata* astrocyte monolayer. Phase-contrast microscopy pictures showing the type-I and type II mature astrocytes within the monolayer in SaGliPs. (a) Primary neuroglia cultures in enriched medium differentiated in type-II astrocytes during monolayer formation after 2 weeks of culturing. (b) Neural elongated stem cells (NS) while generating neurons and astrocytes (stellate cells) in 1 week old cultures. (c) Primary neuroglia cultures in enriched medium differentiated in type-I mature astrocytes forming a monolayer after 3 weeks of culturing. (d) Monolayer of astrocyte cells with dense dendrite and neurite branching, and neuron bodies after 4 weeks of culturing (×200). (e) Monolayer of mature type-II astrocytes. (f) Morphology of a radial glia growing islet after 5 day. Scale bar = 50 µm.

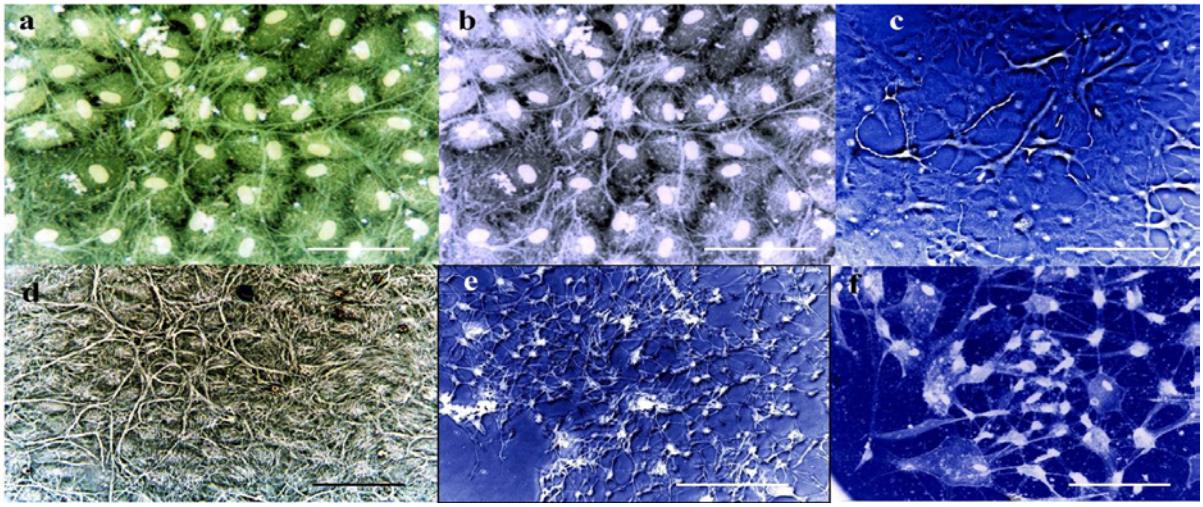

Figure 8: Morphological characterization of *S. aurata* primary neuronal cells. (a, b) Phase contrast image showing fully differentiated communicating neurons with elongated neuritis overwhelming the astrocytes bed after 1 month of culture (×400). (c) Inverted contrast phase image showing early elongated neuritis growing over the astrocytes bed after 1 week of culture (×200). (d) Phase contrast image showing fully differentiated neurons with elongated neuritis overwhelming the astrocytes bed, 1 month of culture (×200). (e) Negative inverted phase contrast image showing intercellular communications (vesicles) between neurons 2 weeks (×400). (f) Enlarged area of (e) picture. Scale bar= 50 µm.

appearance for the astrocyte subtypes produced, type 1 and type 2 astrocytes [42,43].

Neural seabream mixed cultures grew best in SaGliPs-enriched growth factors medium supplemented with serum, and could be maintained for at least 2 months. The astrocytes displayed a heterogeneous phenotype, including a highly branched fibrous astrocytes (type-II astrocytes) and polygonal stellate cells (type-II astrocytes) (Figures 7a, 7b, 7e and Figures 7c and 7d respectively). After 2 weeks, SaGliPs cultured in enriched medium started acquiring features of differentiation, such as the typical appearance of mature astrocyte forming a tight monolayer and generating neurons (Figure 7 and 8). According the lineage derivation the NGRPs-derived the type-I astrocytes, which firstly formed a confluent layer, and secondly the other cells grew on top; neurons grew in small clusters of RDGc with large bundles of axons. Next, after 3-4 weeks of culturing, neurons formed on the astrocyte bed a dense dendrite and neurite branching radiating outward (Figure 9).

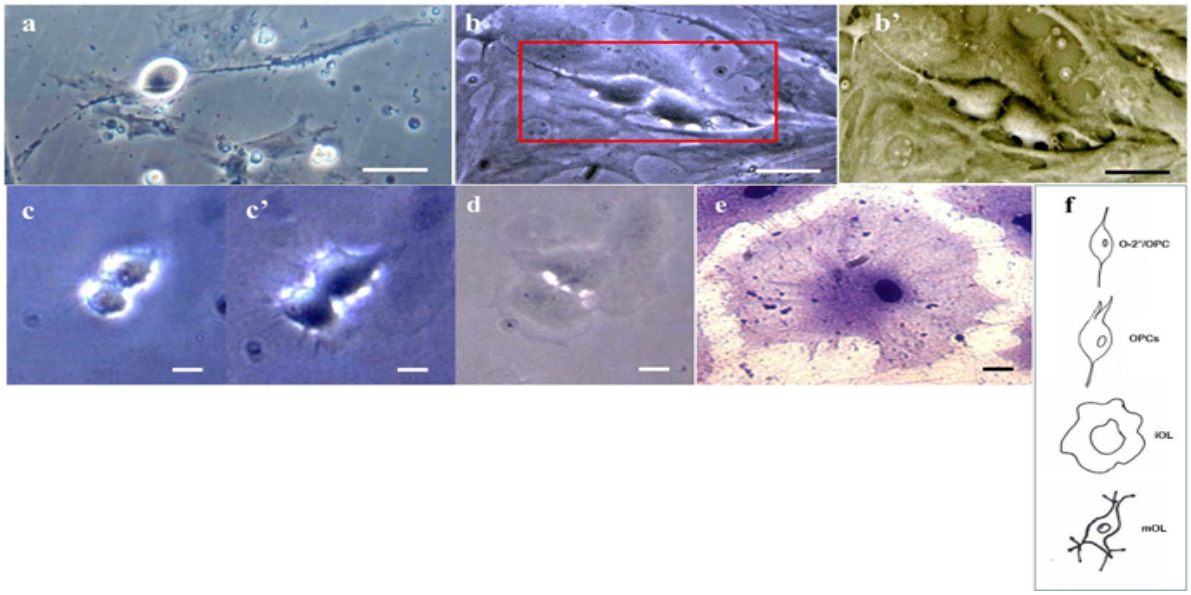

Figure 9: Oligodendrocyte lineage cell development and differentiation timing in neuroglial primary cultures from the brain of *S. aurata*. Phase-contrast microscopy images showing the lineage development of oligodendrocytes. (a) Glial-restricted precursor cells (GRPs); 2-day old cultures. (b, b') Oligodendrocyte precursors (OPCs); 1-week old cultures. (c, c') Enlargement of red area (d, e) Immature (iOL) and Mature Oligodendrocyte (mOL), 3 week old cultures.mOL showed a round cell body enriched of cytoplasmic granules and a dense filamentous meshwork in the peripheral areas often enwrapping other neuroglial processes. (f) Scheme of oligodendrocyte development. Scale bar = 50 µm (a, b, b'); 20 µm (c, c', d, e)e.

In seabream neural cell cultures, after 2 weeks of culturing, the growth velocity of fish astrocytes seemed to be higher, seabream cells developed quickly, reaching confluent culture within 2 weeks (Figure 7), fish cells were flat and stellate (Figure 7). The GFAP astroglial lineage marker was searched to highlight the presence of cultured astrocytes using anti-GFAP antibodies. In the fish model a typical astrocyte bed was depicted by the GFAP labelling after 3 weeks of culture.

Oligodendroglial cells

Oligodendrocytes, like other neural cells in the CNS, develop from the neuroepithelial precursors and are one of the last cell types to differentiate in the CNS.

Recently, some studies identified oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells only generate type 2 astrocytes and oligodendrocytes [42,43].

In 1 week old cultures we observed cells with a simple bipolar form identified as O-2A progenitor cells undergoing a morphological transition during the 2nd week of culturing (Figure 9) to pro-oligodendrocytes (pro OLIGO), with a more complex and flattened form enriched of multiple processes, accompanied by a change in cell surface antigens through the S100B expression acquisition which is essentially restricted to the oligodendroglial cell lineage (Figure 10b).

We observed, by CF and microscopic time lapse imaging, that proliferative oligodendrocyte progenitor cells converted into mature oligodendrocytes and after 2 weeks oligodendrocytes were able to recognize neurons and wrap their target axons (Figures 9 and 10).

After 3 weeks of culturing, fish oligodendrocytes stopped to divide, completed their differentiation transforming in mature Oligodendrocyte (mOL), and enveloped axons. In panel of Figure 10 we showed all stages of lineage development including proliferation, differentiation and long-term survival of oligodendrocytes up to the ultimate progression into fully-fledged oligodendrocytes expressing myelin products released in membrane sheats (Figures 9 and 10). In Figure 10, mOL showed a round cell body enriched of cytoplasmic granules, with a dense filamentous meshwork, initially enwrapping neuroglial processes in the peripheral areas.

Discussion and Conclusion

Since a long time, studies on NSCs defined as self-renewing CNS cells that can differentiate into any of the three major neural cell lineages, specifically neurons, astrocytes and oligodendrocytes, have been attempt almost in mammals [16,17]. Because of the lack of data on the culture of fish neural cells, with the exception on studies from the adult zebra fish injured brain, we carried out the characterization and lineage differentiation of NPCs from seabream CNS, primarily isolated by our research group and known as SaGliPs [35]. This is the first well characterized *in vitro* CNS teleost model, getting the chance of producing newly neural cells for long time in culture. The differentiation of neuronal lineages in seabream has never been described before. Previous studies supported the notion that, also in fish, radial glia may be a potential source of new neurons. For instance, while lower vertebrates display the capacity to generate new retinal neurons in response to injury, in teleost new neurons arise mainly from RDG following neural induction, and their subsequent development and differentiation [44].

We detailed in seabream the early stage of RDG generation; firstly the basal cell producing the fiber of Radial Glia (RG) remained elongated, appearing like tired out from the base and forming a short radial stub projecting from the soma. Studies from Miyata et al. [45] demonstrated that, during RDGc division, each neural connected fiber becomes thin but is neither lost nor divided, and it is inherited with an asymmetrical cleavage by one daughter cell. In these divisions were produced a neuron and a progenitor cell; the neuron inherits the pial

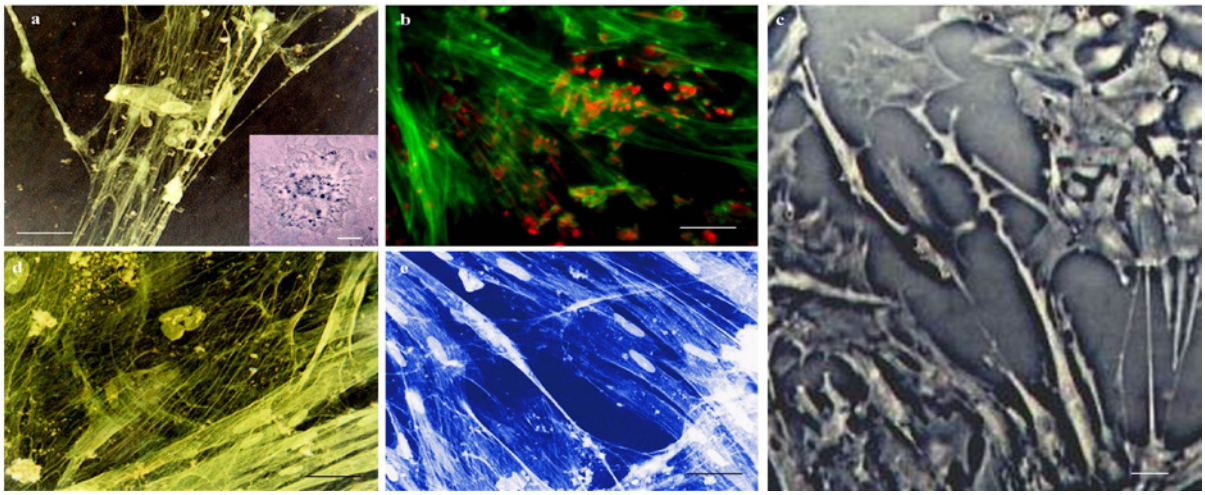

Figure 10: Differentiation of oligodendrocyte lineage from *S. aurata* brain cultured cells. (a) Early stages of fiber engulfment by oligodendrocytes by contrast phase inverted image. Insert within (a) showing the CF aspect of a mature Oligodendrocyte (mOL): mOL showed a flattened round cell body enriched with cytoplasmic granules while forming a dense filamentous meshwork in the peripheral areas; 3-week old cultures. (b) Double immunolabeling with S100B antibody (red) and β-III tubulin (green); mOLs (red) were closely apposed to neuron fiber while enwrapping the neuronal axons (green). The S100B expression is essentially restricted to oligodendroglial cell lineage after 5 days of differentiation. (c) mOL showed enwrapping the long axon of a neuronal cell. (d, e) particulars of wrapped layers and prominent inner/outer tongue processes of mOLs; CF inverted images. Scale bar= 20 μm (a, b, d, e); 10 μm (a', c).

fiber, and is therefore indistinguishable from the progenitor RDG. Our work confirmed results obtained from such recent *in vitro* and *in vivo* studies, examining the neurogenic potential of RDGc by characterizing the morphology and modes of neurogenic division planes as cited by Noctor et al. [33] Chenn and McConnel [47]. Moreover, our findings on the fate of the radial fibers, during RDGs development, revealed that in seabream the young RDGc, throughout their cell division, generate two different types of daughter cells, one destined to become a long fiber process and the other destined to develop a neuron. We found also that the cleavage plane will result only in neuronal production of two daughter cells similar in shape, while the horizontal cleavages will produce two types of asymmetric divisions by generating (1) one new RDGc and one NPC (horizontal cleavage versus cell/cell), and (2) one radial fiber and one NPC(horizontal cleavage versus fiber/cell). Recently, also in *in vivo* studies from the zebrafish, RDGc were found to be able to function as multipotent retinal stem cells that responded to the loss of photoreceptors by specifically regenerating the missing neurons [46]. The work of Chenn and McConnell [47] showed that as neurogenesis proceeded, vertical cleavages decreased, while horizontal cleavages increased. This appear very consistent with our finding, in that the vertical cleavages were symmetric divisions producing two similar precursor cells; whereas horizontal cleavages were asymmetric divisions, resulting in the production of one neuron and one RDG precursor cell [48].

In regards to the formation of fiber scaffold, after 2 weeks of culturing, we observed that NPCs grew closely apposed to the RDG fibers, thus providing strong evidence for their radial migration through the scaffold of RDG fibers, until cells reached the regenerating site and final confluence. We observed a characteristic fiber behavior: fibers initiate to grow from the tips of the protrusion of a basal cell, later propagate out of the tip origin, by splitting at the end into two lateral turns [49]. Afterward, they continue to run in two opposite lateral directions to the left and right side, generating a row of new neurons that resemble to a pearl necklace. This behavior is in accordance with the findings of Zupanc et al. [50,51] that observed, in the adult fish, RDGc actively divided to produce new neurons in the injured brain and guided them to the lesion site to regenerate the neural tissue [50,51]. Such evidences were supportive for the role exerted by RDGc in adult teleost brain to be responsible for the strong ability of neural tissue regeneration [52]. The possibility that RDGc might exist as radially oriented cells is absolutely dissimilar in mammals, where RDGc were transformed into multipolar astrocytes in the adult brain, no longer regenerating [52,53].

In seabream brain most dividing NPCs come from RDG progenitors. We demonstrated that RDGc maintained a vimentin-positive radial fiber throughout each stage of cell division, and an asymmetric inheritance of this fiber. Out of directing neuronal migration, radial fibers supported another important feature in the role of being neuronal progenitors; they were found highly mitotically active, and underwent nuclear migration. Radial glial divisions produced both additional radial glia and newly young neurons, while a separate population of neuronal precursors generated almost exclusively neurons.

Our findings overlap with the current understandings of rodent neurogenesis: two distinct types of NPCs were identified. The RG was the first type, being conceivably recognized as neuronal and glial progenitors also by other authors [30,33,45,54]. The island of RDGc behavior as a neuroepithelial niche, giving rise to radial clones of newborn neurons, where neuronal progenitor cells generated rounds of asymmetric division. The second type, known as (IPCs), was recently characterized as neuronal progenitors, generated from RG; it proliferate for one or two mitotic cycles, and produce neurons only [55-57]. In fish we found that RDGc generate IPCs, which divide to produce pairs of neurons. This is an intriguing result for the potential use of fish neural stem cells in regenerating nervous tissue [58]. In recent times, GRPs have been so well studied for the development of astrocyte transplantation therapies, highlighting new exciting potentials for regenerative therapies in the injured adult nervous tissue [48,49].

The self-renewing ability of NSCs which can differentiate neuroepithelial precursors into neural cell lineage of oligodendrocytes it

has been recently demonstrated [16,17]. In mammals, oligodendrocytes develop from the neuroepithelial precursors that line the so-called VZ. Soon after the migration, they can differentiate into either oligodendrocytes type or "type-2" astrocytes *in vitro*, depending on the composition of the culture medium. This is the reason for these cells are referred to as O-2A progenitor cells [59,60]. Dissimilarly to other neuronal progenitors, O-2A glial progenitors continue to divide after they leave the VZ, then they undergo a transition to pro-oligodendrocytes. Peculiar modifications characterized their morphology: they changed from a simple bipolar form to a more complex form with multiple processes, accompanied by a change in cell surface antigens [61]. Others Authors found that, in proximity of the site of terminal differentiation, the pro-oligodendrocytes stop to divide and enwrapped axons; here, they ultimate the lineage development by expressing myelin gene products and mature into fully-fledged oligodendrocytes [62]. All the previous discoveries overlapped with our findings; as a result we demonstrated all stages of lineage development including proliferation, differentiation and long-term survival. In the CNS, multiple precursor cells were found to develop both the neuronal precursor cells and glial precursors; among the latter, Glial-Restricted Precursor (GRP) cells and oligodendrocyte/type-2 astrocyte progenitor cells (termed also oligodendrocyte precursor cells, O-2A/OPCs) represent the two well-studied distinct glial precursor subtypes in mammals [42]. Although GRP cells and O-2A/OPCs were restricted to astrocyte development, they both differed in the differentiation potential, in the timing of developmental appearance and in the astrocyte subtypes produced (type 1 and type 2 astrocytes); they were also able to generate oligodendrocytes [42,43].

In seabream, multiple myelinating areas of single axons were easily identified by using fluorescent antibodies directed against components of myelin (i.e. S100B, MBP). Also, phase contrast analysis gave evidence of axonal myelin shapes, with some loosely wrapped layers and protruding processes. The excellent remyelinating fish ability of mOLs might be a promising approach for repairing the CNS injuries, also in other animals, as a new coming strategy for function recovery of damaged nerves.

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