TESE DE DOUTORAMENTO

CHARACTERIZATION OF NEUROGENIC NICHES AND RADIAL GLIAL CELLS IN THE TELENCEPHALON OF SHARKS: COMPARATIVE ASPECTS AND EVOLUTIONARY IMPLICATIONS

ALBERTO DOCAMPO SEARA

ESCOLA DE DOUTORAMENTO INTERNACIONAL

PROGRAMA DE DOUTORAMENTO EN NEUROCIENCIA E PSICOLOXÍA CLÍNICA

SANTIAGO DE COMPOSTELA

2018

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DECLARACIÓN DO AUTOR/A DA TESE

CHARACTERIZATION OF NEUROGENIC NICHES AND RADIAL GLIAL CELLS IN THE TELENCEPHALON OF SHARKS: COMPARATIVE ASPECTS AND EVOLUTIONARY IMPLICATIONS

D. Alberto Docampo Seara

Presento a miña tese, seguindo o procedemento axeitado ao Regulamento, e declaro que:

1) A tese abarca os resultados da elaboración do meu traballo.
2) De selo caso, na tese faise referencia ás colaboracións que tivo este traballo.
3) A tese é a versión definitiva presentada para a súa defensa e coincide coa versión enviada en formato electrónico.
4) Confirmo que a tese non incorre en ningún tipo de plaxio doutros autores nin de traballos presentados por min para a obtención doutros títulos.

En Santiago de Compostela, 31 de Xullo de 2018

Asdo Alberto Docampo Seara

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DO DIRECTOR / TITOR DA TESE

CHARACTERIZATION OF NEUROGENIC NICHES AND RADIAL GLIAL CELLS IN THE TELENCEPHALON OF SHARKS: COMPARATIVE ASPECTS AND EVOLUTIONARY IMPLICATIONS

D. Miguel Ángel Rodríguez Díaz
Dna. Eva María Candal Suárez

INFORMA/N:

Que a presente tese, correspóndese co traballo realizado por D. ***Alberto Docampo Seara,*** *baixo a nosa dirección, e autorizo a súa presentación, considerando que reúne os requisitos esixidos no Regulamento de Estudos de Doutoramento da USC, e que como directores desta non incorre nas causas de abstención establecidas na Lei 40/2015.*

En Santiago de Compostela, 31 de Xullo de 2018

Asdo. Miguel Ángel Rodríguez

Asdo. Eva Candal

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

La investigación realizada fue financiada gracias a la concesión de los siguientes proyectos:

- TÍTULO DEL PROYECTO: Buscando la condición ancestral dela organización general de gnatóstomos: regionalización, migración, proyecciones y asimetrías en el cerebroen desarrollo de un tiburón.
  ENTIDAD FINANCIADORA: Ministerio de Economía y Competitividad-FEDER (BFU2010-15816-P)
  ENTIDADES PARTICIPANTES: Universidad de Santiago de Compostela
  DURACION: 2010-2014
  INVESTIGADOR PRINCIPAL: Isabel rodríguez-Moldes Rey

- TÍTULO DEL PROYECTO: Estudio de la neurogénesis en cerebro embrionario y adulto desde una perspectiva evolutiva
  ENTIDAD FINANCIADORA: Ministerio de Economía y Competitividad-FEDER (BFU2014-58631-P)
  ENTIDADES PARTICIPANTES: Universidad de Santiago de Compostela
  DURACION: 2015-2018
  INVESTIGADOR PRINCIPAL: Isabel rodríguez-Moldes Rey/Eva Candal Suárez

- TÍTULO DEL PROYECTO: Descodificación de las bases moleculares de la neurogénesis en el adulto mediante el análisis de la dinámica transcripcional de nichos neurogénicos de larga duración.
  ENTIDAD FINANCIADORA: Ministerio de Economía y Competitividad-FEDER (BFU2017-89861-P)
  ENTIDADES PARTICIPANTES: Universidad de Santiago de Compostela
  DURACION: 2018-2020
  INVESTIGADOR PRINCIPAL: Isabel rodríguez-Moldes Rey/Eva Candal Suárez

La realización de esta tesis fue posible gracias a la concesión de contratos de investigación asociados a proyectos:

- TÍTULO DEL PROYECTO: Estudio de la neurogénesis en cerebro embrionario y adulto desde una perspectiva evolutiva
  ENTIDAD FINANCIADORA: Ministerio de Economía y Competitividad-FEDER (BFU2014-58631-P)
  ENTIDADES PARTICIPANTES: Universidad de Santiago de Compostela
  DURACION: 2015-2018
  INVESTIGADOR PRINCIPAL: Isabel rodríguez-Moldes Rey/Eva Candal Suárez

La realización de la estancia predoctoral en el Observatorio Oceanográfico (CNRS, Banyuls-sur-mer, Francia), en el laboratorio de la Dra. Sylvie Mazan fue posible gracias a la financiación de “Rede Galega INBIOEST ED341D R2016/032” (Xunta de Galicia-FEDER),

Yo, Alberto Docampo Seara, con DNI 44831025T y con domiciliación a efectos de notificación Urb. Aldea Nova nº 177, **declaro** que esta tesis no presenta conflictos de interés. Y para que así conste firmo a día 31 de Julio de 2018.

Firmado:

Alberto Docampo Seara

AGRADECIMIENTOS

Y llegó…llegó el día…la luz al final del túnel…sé que parece dramático…pero es que yo soy dramático xD. No hay nada mejor que enfrentarse a una hoja en blanco al final de una etapa para darte cuenta de quién ha estado a tu lado. Yo me he dado cuenta que de que estoy rodeado de una familia generosa, de muy buenos amigos, de buenos compañeros y de grandes jefes. Así que, como es de recibo, quiero daros gracias a todos.

A Isa. Gracias por estar ese día en la fotocopiadora; por tu generosidad salió adelante mi TAD y gracias a ti me entró el gusanillo por este mundo.

A mis jefes Miguel y Eva. Gracias por ponérmelo tan fácil, por las sesiones en las que más que jefes erais psicólogos (si no psiquiatras) y por los buenos ratos. No habría imaginado unos jefes mejores. A partir de ahora los cafés lo pago yo xD.

A Fátima, alias polifish, alias WB boss, alias coordinadora de departamento. Porque hay que reírse y tú eres un claro ejemplo de cómo hacerlo.

A Ramón. Gracias por poner a mi disposición un cerebro tan privilegiado y por estar siempre al otro lado de la puerta dispuesto a ayudar.

A mis compañeros de laboratorio pasados y presentes. A Nuria. Gracias por apoyarme los primeros años de tesis. A Santi. Gracias por echarme siempre una mano y por interesarte por todo. A Isma. Es un lujo charlar y trabajar con una persona tan dispuesta y tan alegre; no pierdas eso. A Natalia y a Paula, las nuevas incorporaciones. Sois muy buenas niñas y ojalá nos sigamos cruzando en el futuro. A Idoia. Trabajar contigo y aprender de ti es un lujo; acuérdate de mi cuando ganes el Nobel. A Sheila, a Gabi, a Sol y a los que me faltan. Gracias.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

A los compañeros de Área. Al Dr. Romaus, Daniel y Rocío. Y a Blanca (no me olvido de ti). Gracias por estar a un café de distancia siempre que hizo falta. Y a los que no son tan del Área, en especial a Xoana. Gracias por ser tan buena persona y tan amable. Eres de esa gente con la que da gusto compartir cosas. No descarto que la señora Ana Viñas haya influenciado, que es otro cacho de pan.

A Merche. Una grande del confocal que hace que las horas se pasen volando. La magia que hemos hecho con algunas muestras queda entre tú y yo.

A los grandes de la cafetería de matemáticas. Vuestro café ha sido mi motor estos años. Gracias por tener siempre tan buena cara. No sé lo que cobráis, pero claramente no es suficiente.

A mis grandes amigas. Me alegro de estar rodeado de tan buena gente. A Andrea, Raquel y Sara. Aunque ahora no nos veamos tanto como quisiéramos, sois grandes pilares de mi vida. A Paula. Por compartirlo todo conmigo. Contigo lo que veo es lo que hay, y me gusta. Que no se pongan tontos que un día dominaremos el mundo. A Clara y Ana. Gracias por esos domingos (y no domingos) de arreglar el mundo. Mi mundo es un poquito mejor gracias a vosotras. A María y Marta. Por esos momentos donde el tiempo se detiene y podemos hablar hasta aburrirnos. No pasa con todo el mundo. A Sara y Miriam. Porque cuando hay cariño se nota y así lo siento de vosotras.

A mis amigas “ancestrales” Elena y Lorena. Porque las grandes mentes piensan igual. Con lo rápido que me canso yo de la gente lo vuestro tiene mucho mérito. Algo tendréis ;)

A Uxía. Me llevo buenos compañeros del master (Clara, Vanessa, Nacho, Samu, Esteban…), pero sobre todo a ti. Ojalá dure muchos años. Por lo pronto los paseos de viejitos por la alameda son un buen entrenamiento.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

I would also thank to Sylvie (Dr. Sylvie Mazan) for being so kind with me and the great opportunity that she gave to me by receiving me in her lab. Also to Ronan, Maxence, Leo and Charlotte, that where really nice with me, specially with my level of French (thank God we all speak English). Thank you all. I will surely come back for a visit.

Y por último a mi familia. Porque lo mejor siempre hay que dejarlo para el final. Gracias a mis padres y a mis abuelos. No sé qué haría sin vosotros. Gracias por entender este enrevesado mundo de la ciencia y no complicarlo más. A ti también Bro. Ahora que seré doctor pienso aumentar mi cupo de apertas :P.

Por todo esto, a todos, **GRACIAS.**

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CONTENTS

CONTENTS

Resume……………………...……………………………………………1

General Introduction………………………………………………………9

- 1. The telencephalon: anatomy and development in vertebrates
- 2. The relevance of studying cartilaginous fishes.
- 3. The telencephalon of *Scyliorhinus canicula.*
    - 3.1. Pallium and Subpallium.
    - 3.2. Olfactory system.
- 4. Developmental sources of evolutive variation in the telencephalon and olfactory bulbs.
    - 4.1. Neurogenesis in the telencephalic hemispheres and the relevance of progenitor radial glial cells
    - 4.2. Neurogenesis in the OB and the glial system

Rationale and Aims……………………………………………………….51

Chapter 1: Study of pallial neurogenesis in shark embryos and the evolutionary origin of the subventricular zone………...55

Introduction…………………………………………………………………. 57

Materials and Methods…………………………………………………… 61

- Experimental animals
- Tissue processing
- Immunohistochemistry
- Immunofluorescence
- BrdU labelling
- In situ hybridization
- Control and specificity of antibodies
- Imaging

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Results……………………………………………………………………….. 66

- Early stages of development: Characterization of pallial progenitor cells
- Pallial neurogenesis in *S. canicula*: exploring the existence of a SVZ in sharks
- Study of neurogenic transcription factors in S31 and S32

Discussion…………………………………………………………………….73

- The pallium of *S. canicula* contains radial-glia like apical progenitors and presents radial migration like other vertebrates.
- The shark pallium contains Tbr2 intermediate progenitors and a basic subventricular zone. Evolutionary implications.
- Sequence of transcription factor expression in pallial cells. Comparison with other vertebrates.

Conclusions………………………………………………………………….. 87

References…………………………………………………………………… 88

Tables and Figures………………………………………………………….. 104

Chapter 2: Expression of radial glia markers (GFAP, BLBP AND GS) during telencephalic development in the catshark (*Scyliorhinus canicula*)……..121

Introduction…………………………………………………………………. 123

Materials and Methods………………………………………………………126

- Experimental animals
- Tissue processing
- Immunohistochemistry
- Control and specificity of antibodies
- Image acquisition and analysis
- Cell counting

Results……………………………………………………………………… 130

- Expression pattern of GFAP in the telencephalic hemispheres of embryonic stages and juveniles.
- Expression pattern of BLBP in the telencephalic hemispheres of embryos and juveniles.
- Expression pattern of GS in the telencephalic hemispheres of embryonic stages and juveniles.
- Double immunofluorescence of GFAP/GS and BLBP/GS.
- Double immunofluorescence to PCNA and GFAP or BLBP.

Discussion……………………………………………………………………...141

- GFAP, BLBP and GS are expressed in cells with morphology of RG during embryonic development and in postnatal individuals.
- BLBP and GS adventricular positive cells may belong to the oligodendroglial lineage.
- Subsets of proliferating RG are present in the catshark embryonic telencephalic ventricular zone.

Conclusions…………………………………………………………………… 153

References…………………………………………………………………….. 154

Abbreviation List……………………………………………………………… 165

Tables and Figures……………………………………………………………..166

Chapter 3: Characterization of neurogenic niches in the telencephalon of juvenile and adult sharks………………..189

Introduction…………………………………………………………………… 191

Materials and Methods…………………………………………………………195

- Experimental animals

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

- Tissue processing
- In situ hybridization
- Immunohistochemistry
- Double in situ hybridization-immunohistochemistry
- Double-immunofluorescence
- Control and specificity of antibodies
- Imaging

Results……………………………………………………………………… 199

- PCNA expression pattern
- *ScSox2* expression pattern
- Double immunofluorescence against radial glial markers
- Expression of molecular markers of IPCs and neuroblasts: *ScDlx2, ScTbr2* and DCX

Discussion…………………………………………………………………...204

- Telencephalic proliferation across evolution: the origin of adult neurogenesis.
- aNSCs in the telencephalon on shark express radial glia markers and show proliferative activity
- Intermediate progenitor cells and neuronal commitment

Conclusions…………………………………………………………………. 211

References………………………………………………………………….. 212

Tables and Figures…………………………………………………………..224

Chapter 4: Expression of neurogenic markers and radial glial markers in the developing olfactory bulb of catshark (*Scyliorhinus canicula*)……...241

Introduction…………………………………………………………………. 243

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Materials and Methods……………………………………………………247

- Experimental animals
- Tissue processing
- In situ hybridization
- Immunohistochemistry
- Double in situ hybridization- immunohistochemistry
- Double-immunofluorescence
- Control and specificity of antibodies
- Imaging

Results…………………………………………………………………… 251

- Expression of *ScSox2*, Pax6, GFAP, BLBP and PCNA in the lateral pallium at stage 30.
- Expression pattern of transcription factors (*ScTbr2,* Tbr1 and *ScNeuroD*) during development of the OB
- Expression pattern of radial glia markers (GFAP, BLBP and GS) in the developing OB.
- Double immunofluorescence between GS-GFAP and GS-BLBP.
- Double immunofluorescence for BLBP and PCNA.

Discussion……………………………………………………………...256

- Pax6 positive cells present in the embryonic telencephalic VZ are proliferating stem cells with molecular hallmarks of radial glia.
- Neurogenic markers related with the differentiation of glutamatergic cell lineage are expressed in the developing OB of catshark.
- Expression of GFAP, BLBP and GS in later embryos: putative implications of glia in the development of the OB

Conclusions……………………………………………………………… 265

References…………………………………………………………….. 266

Tables and Figures……………………………………………………..276

General Discussion……………………………………………………291

General Conclusions………………………………………………….307

Resumen en castellano.. 311

RESUME

RESUME

The telencephalon is the part of the brain responsible for high cognitive processes, as well as the processing and association of sensory and motor information. One of the most important processes in which is involved is memory and learning, although it also essential for survival-related aspects such as the sense of smell. It is known as one of the most plastic brain structures and also as the most morphologically changing structure at the evolutionary level. Given the diversity of morphologies and subdivisions it presents, many studies have focused on understanding the basic morphogenetic mechanisms (early regionalization and specific patterns of neurogenesis, differentiation and cell migration) underlying this variation. The first genetic studies between species have shown that the telencephalon of vertebrates is divided into two regions: pallium (dorsal) and subpallium (ventral) separated by the pallium-subpallium boundary. Also, studies from fish to mammals have confirmed that the pallium is subdivided into 4 subdivisions: dorsal pallium, medial pallium, ventral pallium and lateral pallium, each of them with their respective derivatives, such as the cerebral cortex or the hippocampus in the case of mammals. The knowledge of how these structures arise as a result of variations in the rate and duration of the process of neurogenesis, in the specification/differentiation of different neuronal types or in the direction of cell migration, is essential to understand the great variability in the cytoarchitecture of the telencephalon.

Interestingly, the telencephalon (including the olfactory bulbs) of mammals is one of the few structures that presents active neurogenesis in adult stages. The number of studies that have focused on analyzing this process is extensive in mammals, but the mechanisms underlying it in other vertebrate groups are still poorly characterized. Up to date, the most studied organism outside the order of

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

mammals is the zebrafish, which, contrary to mammals, possesses a large number of neurogenic niches and high numbers of progenitor radial glial cells in the adult. However, the lack of studies in more basal vertebrates as sharks preclude any conclusions about the mandatory (conserved) versus specific (diverged) components of the germinal niches and the heterogeneity of the progenitor population, both during embryogenesis and in the adult life.

The aim of the present work is to study the basic morphogenetic mechanisms (specific patterns of neurogenesis and gliogenesis depending on different types of progenitor cells) that shape the telencephalon of the catshark, a basal vertebrate. With this aim, we analyze the expression pattern of several markers of different progenitor types and differentiated neuronal and glial cells in the developing and adult telencephalon. Our results are compared to those previously reported in other vertebrates, since changes in this basis morphogenetic mechanisms along evolution might contribute to the interspecific variations in the telencephalon morphology, size and function.

CHAPTER 1

The dorsal part of the developing telencephalon is one of the brain areas that has suffered most drastic changes throughout vertebrate evolution. During mammalian development, the dorsal pallium will locally generate pyramidal neurons that will build the main system of point-to-point connections of the cortex. These cells become specified and then differentiated through the expression of a well-known sequence of transcription factors (Pax6, Tbr2, NeuroD and Tbr1). The evolutionary increase in complexity of the dorsal pallium of mammals was thought to be partly achieved by the appearance of a new neurogenic niche in the embryonic subventricular zone (SVZ). Here, a new

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

kind of amplifying progenitors (basal progenitors) expressing Tbr2, undergo a second round of divisions, which is believed to have contributed to the expansion of the neocortex. Accordingly, the existence of a pallial SVZ has been classically considered exclusive of mammals. However, the lack of studies in ancient vertebrates precludes any clear conclusion about the evolutionary origin of the SVZ and the neurogenic mechanisms that rule pallial development. In this chapter we explore pallial neurogenesis in a basal vertebrate, the shark *Scyliorhinus canicula*, through the study of the expression patterns of several neurogenic markers. We found that apical progenitors and radial migration are present in sharks and therefore, their presence must be highly conserved throughout evolution. Surprisingly, we detected a subventricular band of *ScTbr2*-expressing cells, some of which also expressed mitotic markers, indicating that the existence of basal progenitors should be considered an ancestral condition rather than a novelty of mammals or amniotes. Finally, we report that the transcriptional program for the specification of glutamatergic pallial cells (Pax6, Tbr2, NeuroD, Tbr1) is also present in sharks. However, the segregation of these markers into different cell types is not clear yet, which may be linked to the lack of layering in anamniotes.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CHAPTER 2

Radial glial cells (RGCs) are the first population of glial nature to appear during brain ontogeny. They were initially thought to have a structural role during development by contributing to build scaffolds for neuronal migration. Nowadays, these cells are known to perform numerous functions in the brain of vertebrates, including an essential role in the neurogenic processes, both during development and in adult stages, as primary progenitor (stem) cells. The proliferative capacity of these cells, both in development and in adulthood, has

been subject of interest during past decades. In contrast with mammals where RGCs are restricted to specific ventricular areas in the adult brain, RGCs are the predominant glial element in adult fishes. RGCs have also been characterized in the developing brain of teleost fishes. However, developmental studies on the RGCs of cartilaginous fishes are scant. We have studied the expression patterns of RGCs markers including glial fibrillary acidic protein (GFAP), brain lipid binding protein (BLBP), and glutamine synthase (GS) in the telencephalic hemispheres of the catshark (*Scyliorhinus canicula*) from early embryos to post-hatch juveniles. GFAP, BLBP and GS are first detected, respectively, in early, intermediate and late embryos. Expression of these glial markers was observed in cells with radial glia morphology lining the telencephalic ventricles, as well as in their radial processes and endfeet at the pial surface, and their expression continue in ependymal cells and tanycytes in early juveniles. In addition, BLBP- and GS-immunoreactive cells morphologically resembling oligodendrocytes were observed. In late embryos, most of the GFAP- and BLBP-positive RGCs also coexpress GS and show proliferative activity. Our results indicate the existence of different proliferating subpopulations of RGCs in the embryonic ventricular zone of catshark. Further investigations are needed to determine whether progenitor RGCs similar to that found in embryos are also present in the adult brain (see Chapter 3).

CHAPTER 3

Neurogenesis is a multistep process by which progenitor cells become terminally differentiated neurons under the control of intrinsic and extrinsic factors. The study of adult neurogenesis has gathered increasing attention with the aim of understanding the mechanisms of plasticity involved in processes of memory-formation and decision-making, adaptation to new environments, or

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA USC

with the aim of developing new cell-based treatments for neurodegenerative diseases. Active sites of adult neurogenesis exist from fishes to mammals, although in the adult mammalian brain the number and extension of neurogenic areas is considerably reduced in comparison to non-mammalian vertebrates, and they become mostly reduced to the telencephalon. Much of our understanding in this field is based in studies on mammals and zebrafish, a modern bony fish. The use of the cartilaginous fish Scyliorhinus canicula (a representative of basal vertebrates) as a model expands the comparative framework to a species that shows highly neurogenic activity and persistent radial glia in the adult telencephalon. In this work, we studied the proliferation pattern in the juvenil and adult telencephalon of *S. canicula* by using antibodies against the proliferation marker PCNA. We have characterized PCNA immunoreactive zones by using stem cell markers (*ScSox2*), glial markers (GFAP, BLBP and GS), intermediate progenitor cell markers (*ScDlx2* and *ScTbr2*) and markers for migratory neuroblasts (DCX). Based in the expression pattern of these markers, we demonstrate the existence of different cell subtypes within the PCNA immunoreactive zones including non-glial stem cells, glial progenitors, intermediate progenitor-like cells and migratory neuroblasts, which were widely distributed in the ventricular zone of the pallium, suggesting that the main types that constitute the neurogenic niche in mammals are already present in cartilaginous fishes. The absence of PCNA immunoreactive cells in the brain of adult lampreys suggest that adult neurogenesis emerged in the transition from agnathans to gnathostomes.

CHAPTER 4

In mammals, the development of the olfactory bulb (OB) is related with the expression of transcription factors involved in the specifications/differentiation

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

of glutamatergic cells. In addition, interactions neuron-glia are necessary for the proper development of the OB. In chapter 1, a high molecular similarity was reported between mammals and cartilaginous fishes regarding the neurogenic mechanisms underlying the development of the telencephalon. However, information about the molecular mechanisms operating in the development of the OB are lacking in the catshark *S. canicula*, a cartilaginous fish. Using immunocytochemical and *in situ* hybridization techniques we have found that, previously to the appearance of the olfactory primordium (OP) in the catshark, proliferating cells expressing Pax6 and showing molecular features of radial glia were located in the pallial ventricular zone. Later in development, when the OP appears, a stream of Pax6 positive cells were observed between the ventricular zone and the OP. At the same developmental period, transcription factors involved in mitral cell development (*ScTbr2*, *ScNeuroD*, Tbr1) were first expressed in the OP. Later in development, these transcription factors are expressed in a cell band close to the protoglomerular region, which match with the expression pattern of *ScVglut1*, a marker of mitral cells. In addition, using antibodies against radial glial markers (GFAP, BLBP and GS), we observe morphologically different glial cells types in different domains of the developing OB; some of them showed morphology of radial glia, but astrocyte-like cells were also observed. Curiously, the glomerular layer is the region of the OB where numerous cell bodies and processes of glial nature were observed forming a glomerular glial shell, suggesting a role for these cells in glomerular formation. During development, numerous BLBP proliferating cells were also detected in the OB, but they were especially abundant in the olfactory nerve layer. These cells may correspond to olfactory ensheathing glia cells, suggesting that glial cells with different origins reside in the developing OB of catshark.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

GENERAL INTRODUCTION

Vertebrate brains are anatomically diverse. Though conserved segmental divisions common to all vertebrates have been identified (Puelles et al. 2013), important changes in the relative size and complexity of such divisions appear at taxonomic boundaries (Yopak et al. 2010). The developmental mechanisms underlying this evolutionary variation have been object of profound research. Developmental sources of such outcome include changes in the relative number of cells, which in turn depend on variations in the rate and duration of the process of neurogenesis, in the specification/differentiation of different neuronal types or in the direction of cell migration, among others (reviewed in Anderson and Finlay, 2014).

We employed a comparative approach for gaining understanding of the evolution of the telencephalon, one of the brain areas that has suffered most drastic changes throughout vertebrate evolution. We will address several developmental sources of evolutive variation with special focus on neurogenesis in various telencephalic domains, including the characterization of different neural progenitors and their progeny and the characterization of neurogenic niches in the adult.

1. The telencephalon: anatomy and development in vertebrates

The telencephalon comprises an intricate set of structures that are required for some of the most complex and evolved functions like cognition or memory to most basic abilities like the sense of smell. It arises from a primary vesicle called prosencephalon at the most anterior part of the neural tube. Later in the development this vesicle would be subdivided in two transverse segments: the secondary prosencephalon (rostrally, which includes the telencephalon and the hypothalamus) and the diencephalon (caudally) (Fig. 1, Purves et al. 2000).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

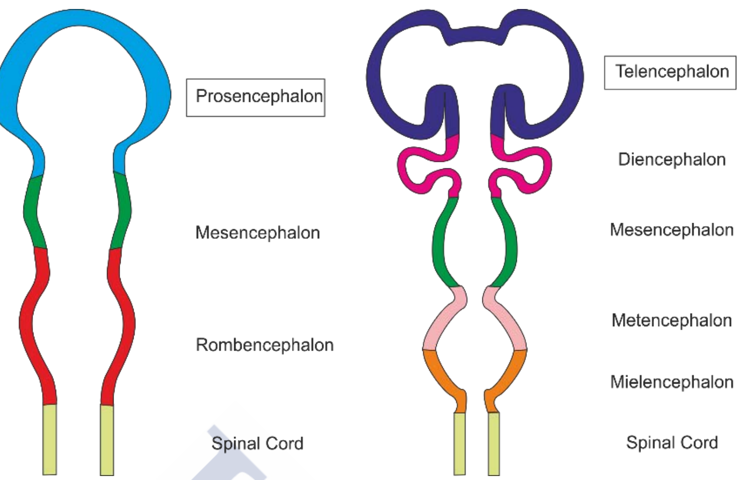


Figure 1. Development of the neural tube from a stage of 3 vesicles to a stage of 5 vesicles showing the main regions of the developing brain. **Adapted from Purves et al. 2000**.

In tetrapods, the telencephalon develops by a morphogenetic process known as evagination, which involves an enlargement of the central lumen of the neural tube, followed by an outward expansion of the telencephalic walls (Butler and Hodos, 2005). Lampreys follow the same morphogenetic process, but important differences regarding this process can be found in fishes. While the telencephalon of cartilaginous fishes develops as in tetrapods, the telencephalon of bony fishes follows a different process known as eversion, which means that the roof of the neural tube become thin and elongates meanwhile the pallium bends outward. Because of this process, the topography of all pallial areas gets reversed (Nieuwnhuys, 2009, Fig. 2).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

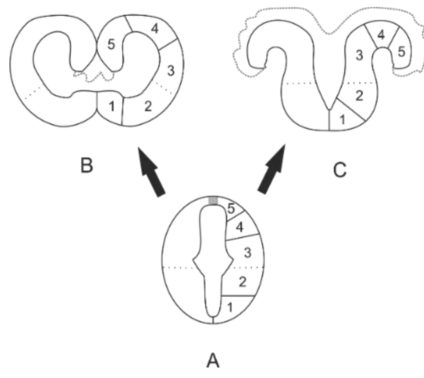


Figure 2. Schematic representation of transverse sections through the initial forebrain vesicle (A), the evaginated cerebral hemisphere of a tetrapod (B), and the everted cerebral hemispheres of a teleost (C). The topography of the main zones (1-5) and their derivatives are altered by the process. Both types of cerebral hemispheres can be divided (dashed line) into a ventral subpallium (1, 2) and a dorsal pallium (3-5). **Adapted from Northcutt (1995), with permission of Elsevier.**

Independently of the resulting topography, the telencephalon is broadly subdivided into a pallial (dorsal) domain and a subpallial (ventral) domain in all vertebrates, separated by a pallial-subapllial boundary (PSB). These domains are common to all vertebrates and have been molecularly defined by the combinatorial expression of several genes: Pax6 (paired box protein 6), Tbr1 (T-box 1) and Emx1 (empty spiracles homeobox 1) for the pallium; and Dlx2 (distal less 2) and Nkx2.1 (NK2 homeobox 1) for the subpallium (Puelles et al. 2000; Rodríguez-Moldes et al. 2017 and references therein)

During development, the pallial domain can also be subdivided in four areas: dorsal pallium (DP), medial pallium (MP), lateral pallium (LP) and ventral pallium (VP) (for review see Medina and Abellán, 2009). In mammals, the cerebral cortex derives from the dorsal pallium. This structure is the main responsible of decision making and of processing somatosensory information (Purves et al. 2000). The hippocampus is formed from the medial pallium and plays important roles in decision making, information encoding, short-term and long-term memory and spatial navigation (reviewed by Khalaf-Nazzal and Francis, 2013). Other structures derived from the pallium are the amygdala, related to fear conditioning (Medina et al. 2011), and the olfactory bulbs, two

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

bilateral evaginations derived from the rostral portion of the ventral pallium, which are involved in encoding, transfer, processing, and decoding of odorant-evoked sensory information (Marín and Rubenstein, 2002; Uteshev, 2014).

Regarding the subpallium, three histogenetic domains could be differentiated: lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), which give rise to striatal and pallidal subdivisions, respectively, and the preoptic area (POA). Both ganglionic eminences contribute to the basal ganglia system, which form part of a larger system involved with control of motor behaviour (reviewed by Medina et al. 2014). The POA plays a key role on thermoregulation and sexual behaviour (Paredes, 2003; Yaghmaie et al. 2005).

Two complex structures formed by a mixture of pallial and subpallial derivatives were described in the telencephalon of most tetrapods (reviewed by Moreno et al. 2009; Medina et al. 2011): the septum (at rostral levels in the medial telencephalic walls) and the amygdaloid complex (in the lateral telencephalic walls).

All of these subdivisions of the pallium and subpallium can be recognizable regardless of their topographical position by the expression of a battery of transcription factors. They have been documented in several groups of vertebrates including mammals, avians, reptiles, amphibians teleost fishes and sharks (Puelles et al. 2000; Bachy et al. 2002; Medina et al. 2005; Medina and Abellan, 2009; Moreno et al. 2009; Wulliman, 2009; Ganz et al. 2015; Rodríguez-Moldes et al. 2017). However, despite the existence of these common subdivisions, the telencephalon is one of the most variable brain structures between vertebrates and has also been considered as the most changing structure throughout evolution (Fig. 3).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

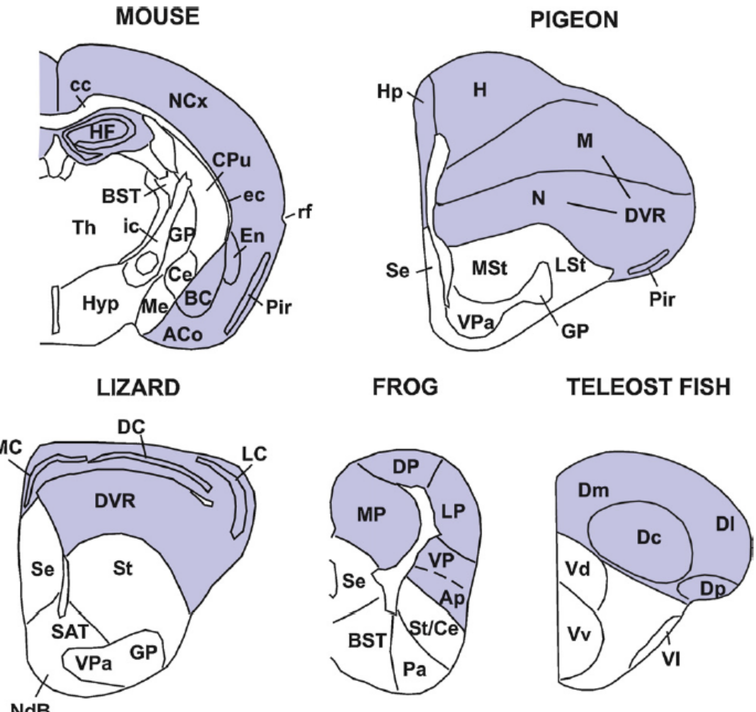


Figure 3. Schematic diagrams of frontal sections through the telencephalon of representative species of different vertebrates (mouse, pigeon, the lizard *Psammodromus*, the frog *Xenopus* and the teleost *Danio rerio*) showing the high variability of morphologies across vertebrates. The pallium is coloured in blue and the subpallium in white. Main abbreviations: Dc, dorsocentral; DC, dorsal cortex; Dl, dorsolateral; Dm, dorsomedial; DVR, dorsal ventricular ridge; H, hyperpallium; Hyp,hypothalamus; LC, lateral cortex; LP, lateral pallium; M, mesopallium, MC, medial cortex; MP, medial pallium; NCx, neocortex; Se, septum; St, striatum; Th, thalamus; Vd, ventrodorsal; Vl, ventrolateral; VP, ventral palliu; Vv ventroventral. **Taken from Medina and Abellán, 2009, with permission from Elsevier.**

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Indeed, the use of classic model organisms has helped to identify not only conserved developmental processes but also some derived traits that might be indicative of evolutionary trends (Rodríguez-Moldes et al. 2017). Studies in an emergent model organism, the catshark (or lesser-spotted dogfish) *Scyliorhinus canicula* are essential to deepen our understanding of vertebrate telencephalon evolution.

2. The relevance of studying cartilaginous fishes.

Cartilaginous fishes or chondrichtyans, represent an ancient radiation of vertebrates considered the sister group of gnathostomes with a bony skeleton that gave rise to land vertebrates. This group includes two major radiations: Holocephali or non-articulated jaw vertebrates, and Elasmobranchi or articulated jaw vertebrates. This last group includes sharks, skates and rays (Fig. 4).

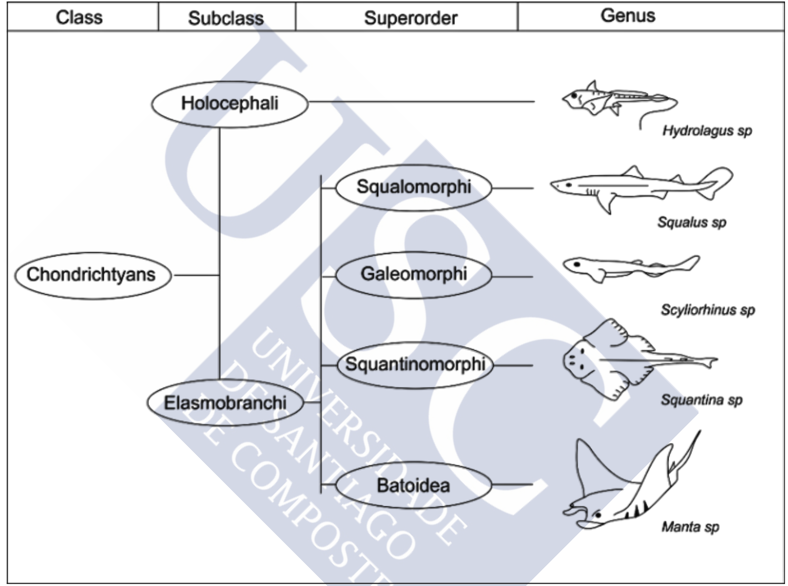


Figure 4. Schematic representation of the taxonomic classification of Chondrichthyans. **Elaborated from data included in Compagno, 1977**.

The phylogenetic position of this group of basal vertebrates as an out-group to all other living jawed vertebrates (gnathostomes) makes chondrichthyans essential in assessing the ancestral organization of the brain of jawed vertebrates (Rodríguez-Moldes et al. 2017).

The main animal model representing cartilaginous fishes is the lesser spotted dogfish or catshark *Scyliorhinus canicula*. Catsharks are easily reared in

captivity and abundant embryonic material can be obtained throughout a year. Eggs are easily maintained under laboratory conditions until hatching and their transparency allows staging of the embryos (according to Ballard et al. 1993) and their selection for particular experimental approaches. Despite their ancient origin, the brain of this small shark is less simple in organization that was previously thought. Besides, studies on brain size and encephalization have shown that cartilaginous fishes are a highly encephalized group that possesses brain to body mass ratios that were comparable to all major groups of gnathostomes including avians and mammals (Northcutt, 1989; Yopak, 2012). Indeed, cartilaginous fishes are said to have larger brains at similar body sizes than teleosts (reviewed in Rodríguez-Moldes et al. 2017).

Moreover, the telencephalon in chondrichthyans, as in most vertebrates, develops by evagination (Fig. 2). This process is similar to that observed in tetrapods, lobe-finned fishes, and agnathans, but different from the eversion process observed in ray-finned fishes (reviewed in Rodríguez-Moldes et al., 2017). Therefore, cartilaginous fishes display lateral ventricles and telencephalic structures more easily comparable with tetrapods, which make them an advantageous fish model for comparative and evolutionary studies of the telencephalon.

3. The telencephalon of *Scyliorhinus canicula*

The telencephalon of chondrichthyans, as in other vertebrates, consists of two main histogenetic subdivisions: the pallium and the subpallium (which includes the preoptic area). The olfactory bulbs, which emerge as bilateral evaginations from the pallium, are usually considered as a major, distinct, component of the brain (Yopak et al. 2010).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.1. Pallium and subpallium

Numerous studies have been made in the adult telencephalon of this species in the past century by using classic stain techniques in order to identify cells masses and fiber organization in the telencephalon (summarized by Smeets et al, 1983).

The main cell mases defined in the palliumare: *area periventricularis pallialis* (app), nucleo septalis (Ns), *pallium dorsalis* (Pd), *pallium dorsalis superficialis (*Pds*)*, *pallium dorsalis centralis* (Pdc), *Pallium medialis* (Pm) and *Pallium lateralis* (Pl); while those described in the subpallium are: *area superficialis basalis* (asb*), area periventricularis ventrolateralis* (apvl) and *striatum* (str) (Fig. 5).

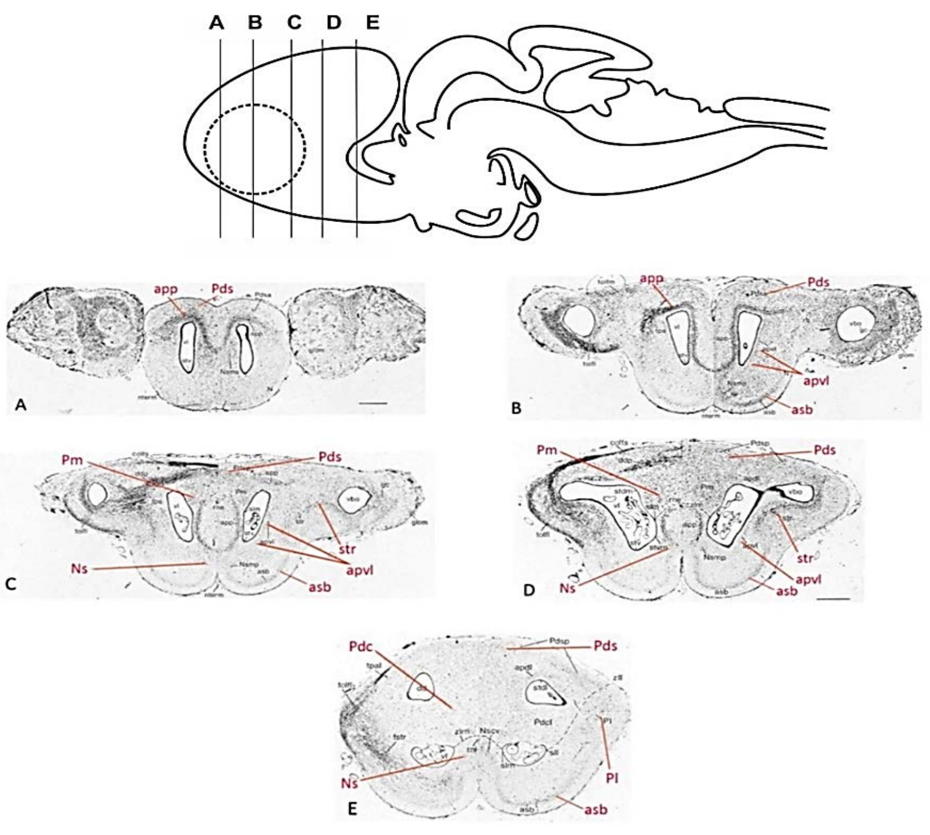


Figure 5. Principal cell masses of the telencephalon of *S.canicula* showed in transverse sections. **Modified from Smeets et al. 1983**.

Several studies have been performed in the last decades in order to characterize neurochemically the telencephalon of *S. canicula.* Using immunohistochemical techniques the distribution pattern of several neurochemical markers such as serotonin (Carrera et al. 2008b) glutamate acid descarboxilase (GAD; Carrera et al. 2008a) tyrosine hydroilase (TH; Carrera et al. 2012), glycine (Anadón et al. 2013), peptides like the neuropeptide Y and substance P (Vallarino et al. 1988; Rodríguez-Moldes et al. 1993) have been analysed in the mature/adult brain of this species.

Studies in the last years have provided a detailed description of the development of various neurochemical systems in the telencephalon of sharks (Carrera et al. 2008a, 2008b, 2012). More recently, studies have been mainly focused in ontogeny and homology of diverse brain structures, including various telencephalic domains (reviewed by Rodríguez-Moldes et al. 2017). Main subdivisions of the developing pallium and subpallium have been tentatively identified by analysing the distribution of various genes (or gene products) whose expression has been largely associated with specific pallial or subpallial subdomains, including *Pax6, Dlx2, GAD, Nkx2.1, Lhx9* or *Tbr1* (Carrera et al. 2008a; Ferreiro-Galve et al. 2008; Rodriguez-Moldes, 2009; Quintana-Urzainqui et al. 2012; Quintana-Urzainqui et al. 2015; reviewed by Rodríguez-Moldes et al. 2017). These studies allowed us to successfully identify the pallial-subpallial boundary, the homologue of lateral ganglionic eminences, the homologue of medial ganglionic eminence and also gave insights on the boundaries and topology of pallial subdivisions (Rodriguez-Moldes et al. 2017).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Recently, studies on neurogenesis during development and adulthood have been performed in the telencephalon of this species (Quintana-Urzainqui et al. 2015). These studies revealed a conserved pattern of development of GABAergic cells, unravelling migratory routes similar to that described during mammalian development and adulthood. Moreover, using antibodies against the proliferating cell nuclear antigen (PCNA) numerous proliferative cells have been evidenced in the telencephalon of adult specimens (Quintana-Urzazinqui et al. 2015). However, the there are no studies about development of (glutamatergic) projecting neurons and detailed studies about adult neurogenesis are lacked.

3.2. Olfactory system

The olfactory system consists of: an olfactory epithelium (OE), which originates from an olfactory placode and contains the olfactory receptor neurons (ORNs); the olfactory nerve composed of the axons from ORNs and supporting elements; and the olfactory bulb (OB), the region of the telencephalon where olfactory axons contact with central neurons. The primary olfactory pathway is formed by the olfactory nerve while the secondary olfactory pathway includes several extrabulbar areas, which are innervated by OB projection neurons.

The OBs in cartilaginous fishes are essential for their survival; they confer sharks a very developed sense of smell and they are considerably large with respect to the size of the brain (Yopak et al. 2015). Though the OBs are laminated structures, they do not present the six cell layers described in mammals. The citoarchitectonic organization of the OB in the catshark has been investigated by classic staining techniques and summarized by Smeets et al. (1983). Three main layers can be observed in this species: the olfactory nerve layer, the glomerular layer and the granular layer. Two main types of cells have been described in the OB: interneurons and projection neurons. Interneurons are

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

located in the granular and glomerular layer and have already been characterized in the mature brain of the catshark by using antibodies against neurochemical markers such as glutamic acid decarboxylase (GAD), tyrosine hydroxylase (TH), Met-Encephaline (ENK) or neuronal nitric oxid synthase (nNOs) (Sueiro, 2003; Ferrando et al. 2012). On the other hand, mitral cells, which are the main projection cells in this structure, are not located in a specific layer as in mammals; in contrast, they are diffusely distributed between the glomerular and granular layer in the catshark (Zielinsky and Hara, 2006). In some species of cartilaginous fishes, two subtypes of mitral cells can be observed regarding their morphology and branching (Dryer and Graziadei, 1993); however, different subtypes of mitral cells have not been reported so far in *S. canicula* and no molecular characterization of these cells has been reported. Curiously, in these species a mitral cell layer can be recognisable. The main extrabulbar connectivity of the OB has been studied in the adult by using carbocyanine dye (Yañez et al. 2011). For a comparison between the OB of mammals and catshark see Figure 6.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

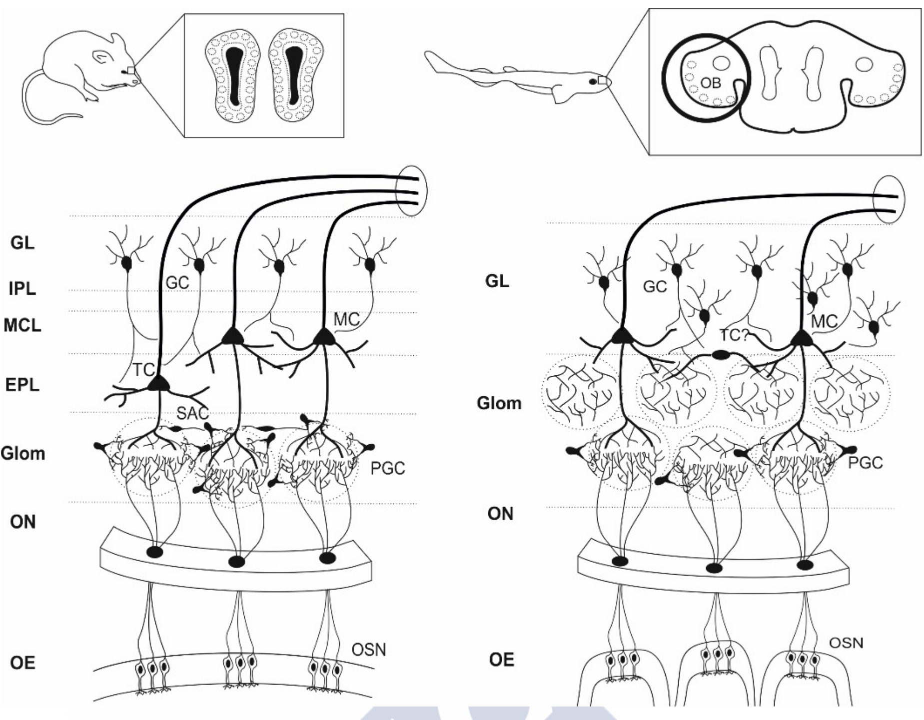


Figure 6. Schematic drawing showing the main anatomy of the OB of mice and catshark. Note the high lamination in mice compared to what is observed in the catshark. Abreviations: EPL, external plexiform layer; GC, granular cell; Glom, glomerular layer; GL, granular layer; IPL, inner plexiform layer; MC, mitral cell; MCL, mitral cell layer; OE, olfactory epithelium; ON, olfactory nerve (layer); OSN, olfactory sensory neuron; PGC, periglomerular cell; SAC, short axon cell; TC, tufted cell. **Elaborated from information about layers and connectivity as reported in Ennis et al. 2015 and Ferrando et al. 2012**.

A few developmental studies have been performed in the olfactory system of this species. First studies have analysed developmental genes such as Otx5, NeuroD or Pax3 in the olfactory placode in early embryos by in situ hybridization (Sauka-Splenger et al. 2001; O'Neill et al. 2007). Other studies have focused in a more advanced developmental period. In these studies, migrating cells have been observed that delaminate from the olfactory pit/epithelium and migrate along the developing olfactory axons which seemed to serve as tracks for their migration toward the telencephalic region where the

olfactory bulb primordium is going to emerge. Some of these cells express Pax6 or GFAP and mainly undergo apotosis at the OB entrance (Ferreiro-Galve et al. 2012, Quintana-Urzainqui et al. 2014, 2015). By studying the migration of these cells, and by using markers of proliferation and differentiation, three developmental periods have been stablished in the formation of the olfactory system (Quintana-Urzainqui et al. 2014). However, little attention has been paid to the OB itself and the neurogenic events that take place during development.

4. <u>Developmental sources of evolutive variation in the telencephalon and olfactory bulbs.</u>

As depicted above, changes in the number of neurons and the place where they migrate and differentiate are major factors driving evolutive variation in the morphology and function of the brain. Since the telencephalon is one of the brain components that shows most conspicuous variations throughout vertebrate evolution, we are interested in addressing similarities/differences in neurogenic patterns in the telencephalon of a basal vertebrate with respect to that reported in other vertebrate groups, which can contribute to gain understanding of the evolution of the telencephalon. We will also focus on the differentiation and final location of excitatory projection neurons, inhibitory interneurons and mature glial cells which could contribute to evolutive variation in the telencephalon.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

<u>4.1. Neurogenesis in the telencephalic hemispheres and the relevance of progenitor radial glial cells</u>

While studying the mechanisms underlying neurogenesis in the telencephalon and olfactory bulb of mammals and comparing them with the situation in other vertebrates, one main goal is to be able to differentiate the

mandatory (conserved) versus specific (diverged) components of the germinal niches and to understand the heterogeneity of the progenitor population, both during embryogenesis and in the adult life.

Apical progenitors during embryonic neurogenesis

Neurogenesis is the production of new neurons and glia from neural stem cells (Figure 7).

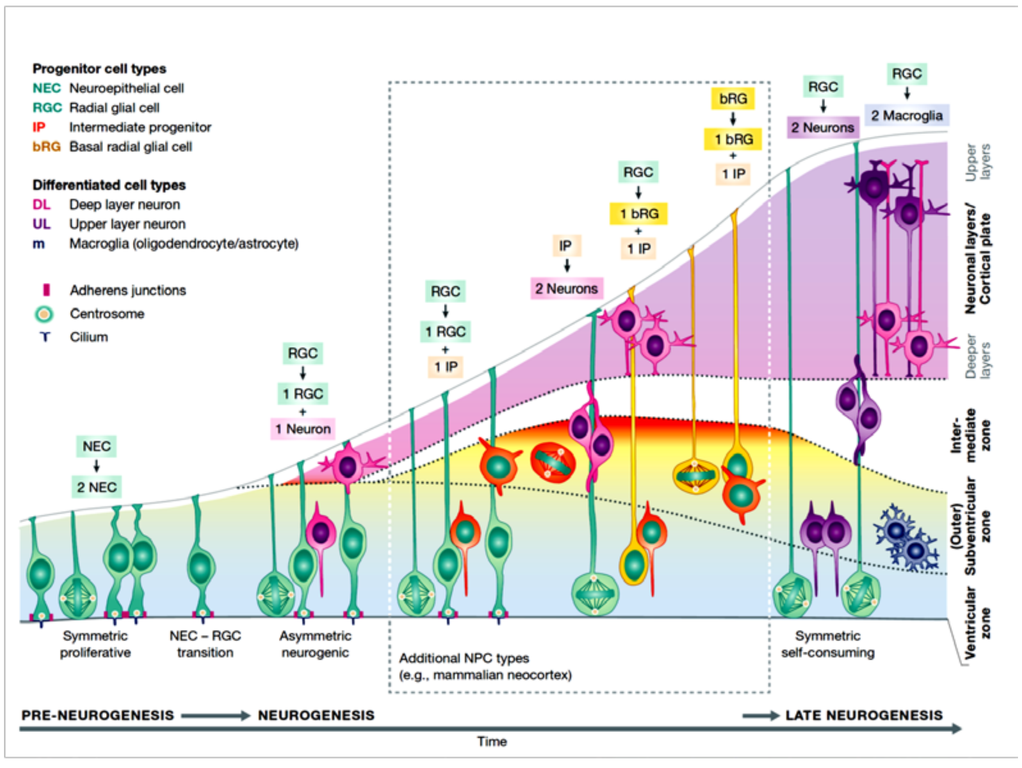


Figure 7. Schematic overview of neurogenesis in the embryonic vertebrate central nervous system. The principal types of progenitors with the progeny they produce are indicated by different colors. Additional progenitor types that are typically found in mammalian neocortex are indicated in the box; note that only some of the possible daughter cell outcomes are depicted. **Taken from Paridaen and Huttner, 2014. License Creative Commons.**

Two types of neural stem cells have been described: neuroepithelial cells (NECs) and progenitor radial glial cells (RGCs), both known also as apical

progenitors, since they divide at the apical (ventricular) side of the brain walls. During development of the mammalian telencephalon, the neural tube consists only of NECs organized in a pseudoestratificated neuroepithelium. These cells undergo symmetric divisions in order to expand this neuroepithelium. After a certain number of symmetric divisions, they start to divide asymmetrically, giving rise to one progenitor RGC and one postmitotic neuron (Haubensak et al. 2004; Noctor et al. 2004; Götz and Huttner, 2005). New-born neurons will follow a well-known transcription program until they become fully differentiated neurons, but progenitor RGCs retain proliferative and pluripotent properties, which imply that they would act as both neural and glial progenitors (Malatesta and Götz, 2013). While they initially give rise only to neurons, later in development, the neuronal production stops, progenitor RGCs reprogram themselves and start to produce cells of the glial system, such as astrocytes and oligodendrocytes, until they presumptively disappear by apoptosis or by generating two postmitotic cells (Martynoga et al. 2015).

Progenitor RGCs have been characterized by using antibodies against various glial markers during the telencephalic development of different vertebrate groups including mammals (Fox et al. 2004; Englund et al. 2006; Mamber et al. 2012), reptiles (Clinton et al. 2014; Martínez-Cerdeño et al. 2016), amphibians (Messenger and Warner, 1989; Moreno and González 2017) and teleost fishes (Thisse et al. 2001; Wulliman and Puelles, 1999; Wulliman and Knipp, 2000; Wulliman and Rink, 2002; Lyons et al. 2003; Mueller and Wulliman, 2003; Arochena et al. 2004; Alexandre et al. 2010). However, the existence of progenitor RGCs and their potential influence in the neurogenic process have not been investigated in the telencephalon of elasmobranch fishes. Analysing the expression pattern of different glial markers during the development of the telencephalon of *S. canicula* combined with proliferation

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

markers will be useful to know if, as in other vertebrates, radial glial cells act as progenitor cells during development of the telencephalon in basal vertebrates.

Basal progenitors during embryonic neurogenesis

During the neurogenic period, pallial-born neurons in mammals follow two neurogenic pathways: direct or indirect neurogenesis (Miyata et al. 2004). Despite the transcription program of both pathways is highly coincident, during indirect neurogenesis a new intermediate type of cells are generated termed intermediate progenitor cells (IPCs) or basal progenitors. IPCs are mitotically active cells derived from RGCs which migrate and become located subventricularly, creating a transient layer during development called subventricular zone (SVZ) (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004, Martinez-Cerdeño et al. 2006). IPCs in the SVZ will divide quickly in order to generate two new neuroblasts.

This indirect pathway might have served as an amplification mechanism of neurogenesis since it allows to generate increased numbers of neurons from a single RGC with respect to that obtained by the direct pathway (Smart et al. 2002). Indeed, it is believed that the SVZ appears in the evolution during the transition from reptiles to mammals (Martínez-Cerdeño et al. 2016; Moreno et al. 2017), and its emergence has been largely related with the increased complexity, size and gyrification of the mammalian cortex (Zecevic et al. 2005; Kriegstein et al. 2006; Martinez-Cerdeño et al. 2006; Cheung et al 2007; Striedter and Charvet, 2009; Reillo et al. 2011; Rash et al. 2013). However, the lack of studies in ancient vertebrates precludes any clear conclusion about the evolutionary origin of the SVZ.

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Postembryonic neurogenesis

In mammals, RGCs are lost in most brain regions at the end of the neurogenic period, either by symmetric self-consuming neurogenic divisions or by producing or becoming glial cells (Paridaen and Huttner, 2014; see Fig. 7). Therefore, the generation of new neurons from RGCs and other progenitor cells were believed to be restricted to the development. However, despite this general neurogenic decline, a subset of postnatal progenitor cells can be found in restricted regions of the brain (germinal niches) that maintain the potential to generate neurons in the adulthood, including the subventricular zone (SVZ) of the lateral walls of the telencephalic ventricles, the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Fig. 8) and the olfactory bulb (reviewed by Ming and Song, 2011; Bond et al. 2015; and Lim and Alvarez-Buylla, 2016; Lepousez et al. 2013).

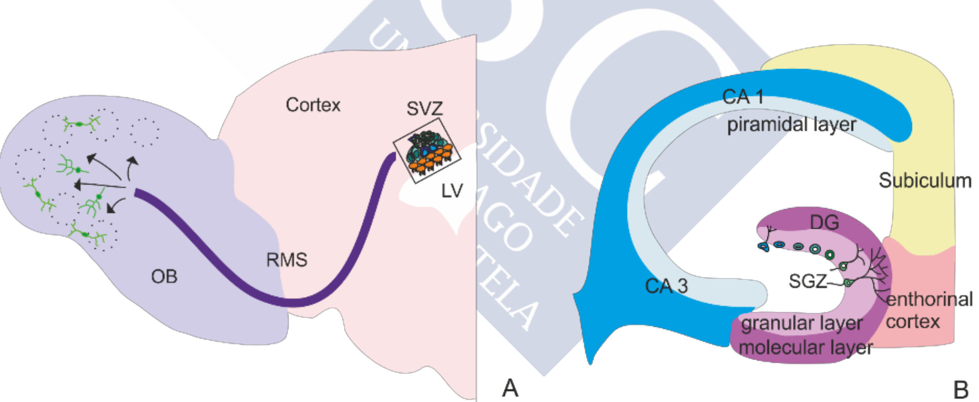


Figure 8. Main neurogenic niches in the adult mammalian brain. **(A)** Schema of the subventricular zone (SVZ) of the lateral walls of the telencephalic ventricles (LV) showing new olfactory bulb interneurons that reach the olfactory bulb (OB) through a particular tangential migration called the rostral migratory stream (RMS). **(B)** Schema of the organization of the hippocampus showing how the subgranular zone (SGZ) of the dentate gyrus (DG) genrate new granular cells. **Elaborated/Modified from Ming and Song, 2011, with permision of Elsevier.**

These postnatal progenitors, usually referred as B1 cells in the SVZ or Type-1 cells in the subgranular zone of the hippocampus (Fig. 9), displays features and markers of both progenitor RGCs and mature astrocytes, which prompted the hypothesis that postnatal progenitors could correspond to embryonic progenitors that persist into the adult life (see Fuentealba et al. 2015). Indeed, it has been recently shown that B1 cells are generated from a subpopulation of progenitor RGCs in the embryo, namely pre-B1 cells, which set aside from those generating neurons. These cells remain quiescent until they become reactivated at different ages in the postnatal brain to produce distinct cohorts of IPCs (C cells; expressing neuronal commitment markers), immature neurons (A cells; postmitotic), mature neurons and glial cells (Fig. 9; Kriegstein and Alvarez-Buylla, 2009).

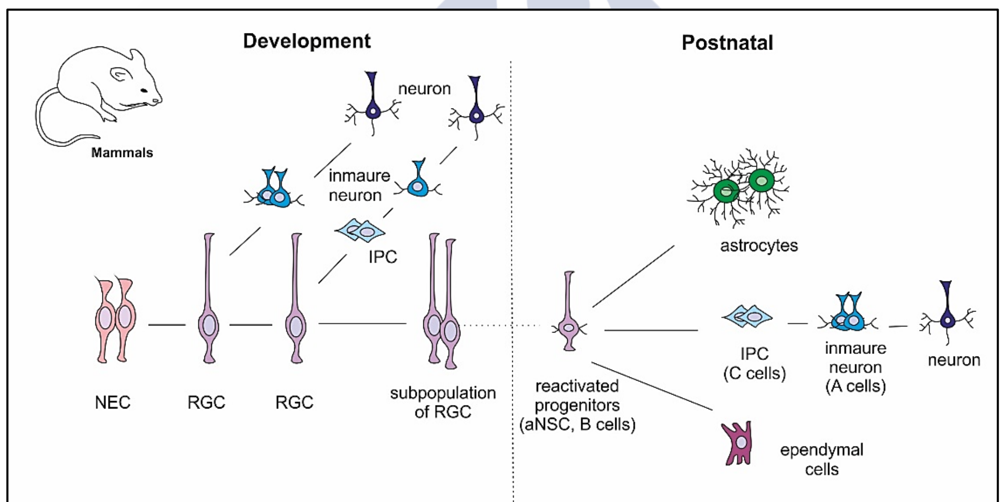


Figure 9. Schema showing the main cell types during developemntal and postanatal neurogenesis in mammals. **Elaborated from data of Kriegstein and Álvarez-Buylla, 2009**.

However, some controversy exists regarding the existence of adult neurogenesis in primates/humans (Sorrells et al. 2018; Boldrini et al. 2018; reviewed by Kempermann et al. 2018).

Adult neurogenesis has also been investigated in other species of vertebrates like birds (Goldman and Nottebohm 1983; Alvarez-Buylla et al, 1992; Alvarez-Buylla et al. 1998; Barnea and Pravasudov, 2011), reptiles (reviewed by González-Granero, 2011), amphibians (Simmons et al. 2008; Kirham et al. 2014) and teleost fishes (reviewed by Ganz and Brand, 2016). Comparisons across vertebrate taxa revealed that the capacity of adult neurogenesis has undergone a remarkable reduction throughout evolution (see Grandel and Brand, 2013; Lindsey and Tropepe, 2006). Both the number of neurogenic niches and the quantity of new cells is restricted to fewer and more anterior periventricular brain regions in more recent lineages of vertebrates (i.e. birds and mammals), when compared to more basal vertebrates (Grandel and Brand, 2013). In zebrafish, contrary to that found in mammals, progenitor RGCs persist in the adult brain (Than-Trong and Bally-Cuif, 2015) where they act as adult neural progenitors in constitutive and/or regenerative neurogenesis. Progenitor RGCs in the adult brain of zebrafish are morphologically similar to progenitor RGCs in the developing brain and fulfill similar roles in the generation of neurons and glia, and also as guide for the migration of young neurons along their radial processes.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Therefore, in vertebrates, adult neurogenesis depends on cells that express various glial markers, but differences are observed between mammals and fishes regarding the abundance and nature of adult progenitors (how different they are from embryonic progenitors). Strikingly, cartilaginous fishes, in which adult neurogenesis has been found (Leonard et al. 1978) are not typically used for this kind of analyses. Radial cells expressing glial markers have been found in adult cartilaginous fishes including ghostshark, skates, squalomorphs and *S. canicula* (Chiba, 2000; Kálmán and Gould, 2001; Ari and Kálmán, 2008a, b), but their relation to neurogenesis has not been investigated. Comparative investigations

must be extended to this group in order to unravel the extent of shared and/or divergent characteristics of adult neurogenesis among vertebrates.

Specific transcriptional programs for cell differentiation both in embryos and in adulthood

In the telencephalon of mammals, the transcriptional program involved in the differentiation of excitatory (glutamatergic) projection neurons appears to be rather conserved between development and adulthood (Hevner et al. 2006; Hodge et al. 2012). A rather similar sequence of transcription factors appears to operate in birds, reptiles, *Xenopus* and zebrafish (Wullimann and Rink, 2001; Martínez-Cerdeño et al. 2016; Mueller and Wullimann, 2016; Moreno and González, 2017; among others). Since the specification/differentiation of different neuronal types or the direction of cell migration are also major factors in evolutive changes, these studies need to be extended to cartilaginous fishes. As inhibitory (GABAergic) interneurons have already been investigated in the developing telencephalon (Carrera et al. 2008; Quintana-Urzainqui et al. 2015), we are going to focus on excitatory (glutamatergic) projection neurons.

4.2. Neurogenesis in the OB and the glial system

Numerous investigations in mammals have evidenced that the olfactory system represent a good model to study diverse aspects of neural development including neurogenesis, migration, differentiation, synaptogenesis, interactions glia-neuron and neural plasticity mechanisms.

The development of the OB begins when olfactory axons of olfactory receptor neurons (ORNs) located in the OE extend their axons along the mesenchyme into the central nervous system, where they induce the formation of the olfactory primordium (OP). This primordium will give rise to a macroscopically visible OB by E12.5 (Hinds, 1968). These axons are

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ensheathed by the processes of the olfactory ensheathing cells (OECs) which are a glial type exclusive of the olfactory system that play a key role in axonal growth and path-finding processes within embryonic and adult olfactory system (Su and He, 2010).

During embryonic development a specific battery of transcription factors defines progenitor territories and is related with the specification of diverse cell subtypes. Projection neurons of the OB (mitral cells), are born locally from apical progenitors located in the ventricular zone of the rostral pallium and migrate radially to their final destinations. These cells are the first to be born, whereas interneurons are generated later in development (Blanchart et al. 2006). In mammals, numerous transcription factors regulate the OB neurogenesis (for review see: Díaz-Guerra et al. 2013). The paired box gene Pax6 (Pax6), the T-box genes (Tbr1 and Tbr2) and the proneural basic helix-loop-helix gene (NeuroD) encode transcription factors involved in the neurogenesis of glutamatergic cells in the OB of mammals (Stoykova and Gruss, 1994; Bulfone et al. 1999; Nomura et al. 2007; Boutin et al. 2009; Brill et al. 2009; Osorio et al. 2010; Winpenny et al. 2011; Mizuguchi et al. 2012; Roybon et al. 2015), amphibians (Franco et al. 2001; Brox et al. 2004; Joven et al. 2013), and zebrafish (Mione et al. 2001; Mueller et al. 2008; Mueller and Wullimann, 2016). Curiously, this transcription factor program is quite similar to that operating in the neurogenesis of cortical glutamatergic neurons (Hevner et al. 2006).

After the establishment of the mitral cell layer, in the last embryonic periods of rodents, astrocyte cells colonize the OB. Different subtypes of GFAP-expressing astrocytes were found in all layers of the olfactory bulb (Bailey and Shipley, 1993; Chiu and Greer, 1996; Bailey et al. 1999; Olude et al. 2015). Several studies in mammals have shown that, besides the contribution of the

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

dendrites of mitral cells and ORN axons, the development of the olfactory glomeruli needs the interaction of glial cells (Bailey and Shipley, 1993; Bailey et al. 1999).

In the mammalian OB, neurogenesis continues postnatally during the whole life of individuals. The adult olfactory system continuously receives interneurons from the telencephalic subventricular zone that migrate along the RMS into the OB, where they integrate into the neural circuit network (see Fig. 8A; for review see: Díaz-Guerra et al. 2013). In addition, OECs perform a key role in supporting the continual turnover of olfactory receptor neurons (ORNs) throughout adult life (Doucette, 1993). New ORNs extend their axons along the peripheral olfactory nerve and into the central nervous system where they form appropriate connections within the olfactory nerve layer. Therefore, the olfactory bulb and the olfactory nerve are subject to lifelong plasticity.

Most of the studies about the olfactory system are focused on mammals. Cartilaginous fishes possess a well-developed sense of smell that is important for survival, localizing preys, avoiding predators, and chemosensory communication (for review see: Yopak et al. 2015). Cellular and molecular knowledge of the olfactory system in basal vertebrates, like the catshark will be useful for a deeper understanding of the neural processes that take place during development of the central and peripheral olfactory system, and how the olfactory system has evolved across vertebrates. Some studies have been performed in shark concerning OECs (Quintana-Urzainui et al. 2012) and related to Pax6 expression with the morphogenesis of the olfactory placode and the OB (Ferreiro-Galve et al. 2012). However, information about the transcriptional program ruling OB neurogenesis or the glial system in the central olfactory system during embryonic and postnatal development in the catshark is lacking.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

A comprehensive understanding of shark telencephalon development will shed new insight into the ancestral traits of the telencephalon of gnathostomes, and the possible changes in developmental programs that created the different types of telencephalic architecture during evolution.

References

Anadón R, Rodríguez-Moldes I, Adrio F (2013). Glycine-immunoreactive neurons in the brain of a shark *(Scyliorhinus canicula L.)*. *J Comp Neurol* 521:3057-82.

Anderson ML, Finlay BL (2014). Allocating structure to function: the strong links between neuroplasticity and natural selection. *Front Hum Neurosci* 7:918.

Alexandre P, Reugels AM, Barker D, Blanc E, Clarke JD (2010). Neurons derive from the more apical daughter in asymmetric divisions in the zebrafish neural tube. *Nat Neurosci* 13:673-9.

Álvarez-Buylla A, Ling CY, Nottebohm F (1992). High vocal center growth and its relation to neurogenesis, neuronal replacement and song acquisition in juvenile canaries. *J Neurobiol* 23:396-406.

Álvarez-Buylla A, García-Verdugo JM, Mateo AS, Merchant-Larios H (1998). Primary neural precursors and intermitotic nuclear migration in the ventricular zone of adult canaries. *J Neurosci* 18:1020-37.

Ari C, Kálmán M (2008a). Glial architecture of the ghost shark (Callorhinchus milii, Holocephali, Chondrichthyes) as revealed by different immunohistochemical markers. *J Exp Zool B Mol Dev Evo* 310:504-19.

Ari C, Kálmán M (2008b). Evolutionary changes of astroglia in Elasmobranchii comparing to amniotes: a study based on three immunohistochemical markers (GFAP, S-100, and glutamine synthetase). *Brain Behav Evol* 71:305-24.

Arochena M, Anadón R, Díaz-Regueira SM (2004). Development of vimentin and glial fibrillary acidic protein immunoreactivities in the brain of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

gray mullet (*Chelon labrosus*), an advanced teleost. *J Comp Neurol* 469:413-36.

Bachy I, Berthon J, Rétaux S (2002). Defining pallial and subpallial divisions in the developing *Xenopus* forebrain. *Mech Dev* 117:163-72.

Bailey MS, Shipley MT (1993). Astrocyte subtypes in the rat olfactory bulb: morphological heterogeneity and differential laminar distribution. *J Comp Neurol* 328:501-26.

Bailey MS, Puche AC, Shipley MT (1999). Development of the OB: evidence for glia-neuron interactions in glomerular formation. *J Comp Neurol* 415:423-48.

Ballard WW, Mellinger J, Lechenault (1993). A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). *J Exp Zoo* 267:318-336.

Barnea A, Pravosudov V (2011) Birds as a model to study adult neurogenesis: bridging evolutionary, comparative and neuroethological approaches. *Eur J Neurosci* 34:884-907.

Blanchart A, De Carlos JA, López-Mascaraque L (2006). Time frame of mitral cell development in the mice olfactory bulb. *J Comp Neurol* 496:529-43.

Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, Rosoklija GB, Stankov A, Arango V, Dwork AJ, Hen R, Mann JJ (2018). Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell* 22:589-599.

Bond AM, Ming GL, Song H (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell* 17:385-95.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Boutin C, Hardt O, de Chevigny A, Coré N, Goebbels S, Seidenfaden R, Bosio A, Cremer H (2010). NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. *Proc Natl Acad Sci USA* 107:1201-6.

Brox A, Puelles L, Ferreiro B, Medina L (2004). Expression of the genes Emx1, Tbr1, and Eomes (Tbr2) in the telencephalon of *Xenopus laevis* confirms the existence of a ventral pallial division in all tetrapods. *J Comp Neurol* 474:562-77.

Bulfone A, Martinez S, Marigo V, Campanella M, Basile A, Quaderi N, Gattuso C, Rubenstein JL, Ballabio A. (1999). Expression pattern of the Tbr2 (Eomesodermin) gene during mouse and chick brain development. *Mech Dev* 84:133-8.

Butler AB, Hodos W (2005). Comparative vertebrate neuroanatomy. Evolution and adaptation. 2nd ed. Wiley-Liss, Hoboken, NJ

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R and Rodríguez-Moldes I (2008a). Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. *Brain Res. Bull* 75:405-9.

Carrera I, Molist P, Anadón R, Rodríguez-Moldes I (2008b). Development of the serotoninergic system in the central nervous system of a shark, the lesser spotted dogfish *Scyliorhinus canicula*. *J Comp Neurol* 511:804-31.

Carrera I, Anadón R, Rodríguez-Moldes I (2012). Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain of the dogfish *Scyliorhinus canicula*: new perspectives on the evolution of the vertebrate catecholaminergic system. *J Comp Neurol* 520:3574-603.

Cheung AF, Pollen AA, Tavare A, DeProto J and Molnár Z (2007). Comparative aspects of cortical neurogenesis in vertebrates. *J Anat* 211:164-76.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Chiba A (2000). S-100 protein-immunoreactive structures in the brains of the elasmobranchs *Scyliorhinus torazame* and *Mustelus manazo*. *Neurosci Lett* 279:65-8.

Chiu K, Greer CA (1996). Immunocytochemical analyses of astrocyte development in the OB. *Brain Res Dev Brain Res* 95:28-37.

Clinton, BK, Cunningham CL, Kriegstein AR, Noctor SC Martínez-Cerdeño V (2014). Radial glia in the proliferative ventricular zone of the embryonic and adult turtle, *Trachemys scripta elegans*. *Neurogenesis* DOI:10.4161/23262125.2014.970905.

Compagno LJV. 1977. Phyletic relationships of living sharks and rays. *Am Zool* 17: 303-322.

Díaz-Guerra E, Pignatelli J, Nieto-Estévez V, Vicario-Abejón C (2013). Transcriptional regulation of olfactory bulb neurogenesis. *Anat Rec* 296:1364-82.

Doucette R (1993). Glial cells in the nerve fiber layer of the main olfactory bulb of embryonic and adult mammals. *Microsc Res Tech* 24:113-30.

Dryer L, Graziadei PPC (1993). A pilot study on morphological compartmentalization and heterogeneity in the elasmobranch olfactory bulb. *Anat Embryol* 188:41-51.

Ennis M, Puche AC, Holy T, Shipley MT (2015). The olfactory system. In: Paxinos G. The Rat Nervous System (4 ed) Academic Press: Elsevier. ISBN:978-0-12-374245-2.

Ferreiro-Galve S, Carrera I, Candal E, Villar-Cheda B, Anadón R, Mazan S, Rodríguez-Moldes I (2008). The segmental organization of the developing

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

shark brain based on neurochemical markers, with special attention to the prosencephalon. *Brain Res Bull* 75:236-40.

Ferrando S, Gallus L, Gambardella C, Amaroli A, Cutolo A, Masini MA, Vallarino M, Vacchi M (2012). Neuronal nitric oxide synthase (nNOS) immunoreactivity in the olfactory system of a cartilaginous fish. *J Chem Neuroanat* 43:133-40.

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012). Dynamic expression of Pax6 in the shark olfactory system: evidence for the presence of Pax6 cells along the olfactory nerve pathway. *J Exp Zool B Mol Dev Evol* 318:79-90.

Fox IJ, Paucar AA, Nakano I, Mottahedeh J, Dougherty JD, Kornblum HI (2004). Developmental expression of glial fibrillary acidic protein mRNA in mouse forebrain germinal zones--implications for stem cell biology. *Brain Res Dev Brain Res* 153:121-5.

Franco MD, Pape MP, Swiergiel JJ, Burd GD (2001). Differential and overlapping expression patterns of X-dll3 and Pax-6 genes suggest distinct roles in olfactory system development of the African clawed frog *Xenopus laevis*. *J Exp Biol* 204:2049-61.

Ganz J, Kroehne V, Freudenreich D, Machate A, Geffarth M, Braasch I, Kaslin J and Brand M (2015) Subdivisions of the adult zebrafish pallium based on molecular marker analysis *F1000Res* 3:308.

Ganz J, Brand M (2016). Adult Neurogenesis in Fish. *Cold Spring Harb Perspect Biol* DOI:10.1101/cshperspect.a019018.

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Goldman SA, Nottebohm F (1983). Neuronal production, migration and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci USA* 80:2390-2394.

González-Granero S, Lezameta M, García-Verdugo JM (2011). Adult neurogenesis in reptiles. In: Seki T, Sawamoto K, Parent JM, Alvarez-Buylla A (eds) Neurogenesis in the adult brain, vol. I. Springer, pp- 169-189.

Götz M, Huttner WB (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* 6(:777-88.

Götz M (2013). Radial glial cells. In: Kettenmann H, Ranson BR, editors. Neuroglia. New York: Oxford University Press. pp 50-6.1

Grandel H, Brand M (2013). Comparative aspects of adult neural stem cell activity in vertebrates. *Dev Genes Evol* 223:131-47

Haubensak W, Attardo A, Denk W and Huttner WB (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc Natl Acad Sci USA* 101:3196-201.

Hevner RF, Hodge RD, Daza RA and Englund C (2006). Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci. Res* 55:223-33

Hinds JW (1968). Autoradiographic study of histogenesis in the mouse olfactory bulb. I. Time of origin of neurons and neuroglia. *J Comp Neurol* 134:287-304.

Hodge RD, Kahoud RJ and Hevner RF (2012). Transcriptional control of glutamatergic differentiation during adult neurogenesis. *Cell Mo. Life Sci* 69:2125-2134.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Joven A, Morona R, González A, Moreno N (2013). Spatiotemporal patterns of Pax3, Pax6, and Pax7 expression in the developing brain of a urodele amphibian, *Pleurodeles waltl*. *J Comp Neurol* 521:3913-53.

Kálmán M, Gould RM. (2001). GFAP-immunopositive structures in spiny dogfish, *Squalus acanthias*, and little skate, *Raia erinacea*, brains: differences have evolutionary implications. *Anat Embryol (Berl)* 204:59-80.

Kempermann G, Gage FH, Aigner L, Song H, Curtis MA, Thuret S, Kuhn HG, Jessberger S, Frankland PW, Cameron HA, Gould E, Hen R, Abrous DN, Toni N, Schinder AF, Zhao X, Lucassen PJ, Frisén J (2018). Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell* DOI: 10.1016/j.stem.2018.04.004.

Khalaf-Nazzal R, Francis F (2013). Hippocampal development - old and new findings. *Neuroscience* 248:225-42.

Kirkham M, Hameed LS, Berg DA, Wang H, Simon A (2014). Progenitor cell dynamics in the Newt Telencephalon during homeostasis and neuronal regeneration. *Stem Cell Reports* 2:507-19.

Kriegstein A, Alvarez-Buylla A (2009). The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149-84.

Kriegstein A, Noctor S, Martínez-Cerdeño V (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat Rev Neurosci* 7:883-90.

Leonard RB, Coggeshall RE, Willis WD (1978). A documentation of an age related increase in neuronal and axonal numbers in the stingray, *Dasyatis sabina*, Leseuer. *J Comp Neurol* 179:13-21

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Lepousez G, Valley MT, Lledo PM (2013). The impact of adult neurogenesis on olfactory bulb circuits and computations. *Annu Rev Physiol* 75:339–63.

Lim DA, Alvarez-Buylla A (2016). The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. *Cold Spring Harb Perspect Biol* DOI: 10.1101/cshperspect.a018820.

Lindsey BW, Tropepe V (2006). A comparative framework for understanding the biological principles of adult neurogenesis. *Prog Neurobiol* 80:281-307.

Lyons DA, Guy AT, Clarke JD (2003). Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development* 130:3427-36.

Malatesta P, Götz M (2013). Radial glia - from boring cables to stem cell stars. *Development* 140:483-6.

Mamber C, Kamphuis W, Haring NL, Peprah N, Middeldorp J, et al. (2012). GFAPδ expression in glia of the developmental and adolescent mouse brain. *PLoS ONE* 7: e52659.

Marin O, Rubenstein JLR (2002). Patterning, Regionalization, and Cell Differentiation in the Forebrain. In: Rossant J, Tan PPL (ed) Mouse Development: Patterning, Morphogenesis and Organogenesis pp. 75–106. Academic Press.

Martínez-Cerdeño V, Noctor SC and Kriegstein AR (2006). The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb Cortex* 16 Suppl 1:152-61.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Martínez-Cerdeño V, Cunningham CL, Camacho J, Keiter JA, Ariza J, Lovern M and Noctor SC (2016). Evolutionary origin of Tbr2-expressing precursor cells and the subventricular zone in the developing cortex. *J Comp Neurol* DOI*:* 10.1002/cne.23879.

Martynoga B, Drechsel D, Guillermot F (2015) Molecular control of neurogenesis: a view from the mammalian cerebral cortex. *Cold Spring Harb Perspect Biol* DOI: 10.1101/cshperspect.a008359.

März M, Chapouton P, Diotel N, Vaillant C, Hesl B, Takamiya M, Lam CS, Kah O, Bally-Cuif L, Strähle U (2010). Heterogeneity in progenitor cell subtypes in the ventricular zone of the zebrafish adult telencephalon. *Glia* 58:870-88.

Medina L, Brox A, Legaz I, García-López M, Puelles L (2005). Expression patterns of developmental regulatory genes show comparable divisions in the telencephalon of *Xenopus* and mouse: insights into the evolution of the forebrain. *Brain Res Bull* 66:297-302.

Medina L, Abellán A (2009). Develpment and evolution of the pallium. *Semin. Cell Dev Biol* 20:698-711.

Medina L, Bupesh M, Abellán A (2011). Contribution of genoarchitecture to understanding forebrain evolution and development, with particular emphasis on the amygdala. *Brain Behav Evol* 78:216-36.

Medina L, Abellán A, Vicario A, Desfilis E (2014). Evolutionary and developmental contributions for understanding the organization of the basal ganglia. *Brain Behav Evol* 83:112-125.

Messenger NJ, Warner AE (1989). The appearance of neural and glial cell markers during early development of the nervous system in the amphibian embryo. *Development* 107:43-54.

Ming GL, Song H (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70:687-702.

Mione M, Shanmugalingam S, Kimelman D, Griffin K (2001). Overlapping expression of zebrafish T-brain-1 and eomesodermin during forebrain development. *Mech Dev* 100:93-7.

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T and Ogawa M (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131:3133-45.

Mizuguchi R, Naritsuka H, Mori K, Mao CA, Klein WH, Yoshihara Y (2012). Tbr2 deficiency in mitral and tufted cells disrupts excitatory-inhibitory balance of neural circuitry in the mouse olfactory bulb. *J Neurosci* 32:8831-44.

Moreno N, González A, Rétaux S (2009). Development and evolution of the subpallium. *Semin Cell Dev Biol* 20:735-43.

Moreno N, González A (2017). Pattern of Neurogenesis and Identification of Neuronal Progenitor Subtypes during Pallial Development in *Xenopus laevis*. *Front Neuroanat* 11:24.

Mueller T, Wulliman MF (2003). Anatomy of neurogenesis in the early zebrafish brain. *Brain Res Dev Brain Res* 140:137-55.

Mueller T, Wullimann MF, Guo S (2008). Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa), and GAD67 gene expression. *J Comp Neuro* 507:1245-57.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Mueller T, Wulliman MF (2016). Atlas of early zebrafish brain development. Elsevier ISBN: 978012417269.

Nieuwenhuys R (2009). The forebrain of actinopterygians revisited. *Brain Behav Evol* 73:229-52

Noctor SC, Martinez-Cerdeño V, Ivic L and Kriegstein AR (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* 7:136-44.

Nomura T, Haba H, Osumi N (2007). Role of a transcription factor Pax6 in the developing vertebrate olfactory system. *Dev Growth Differ* 49:683-90

Northcutt RG (1989). Brain variations and phylogenetic trends in elasmobranch fishes. *J Exp Zool Suppl* 2:83-100.

Northcutt RG (1995). The forebrain of gnathostomes: in search of a morphotype. *Brain Behav Evol* 46:275-318.

Olude MA, Mustapha OA, Aderounmu OA, Olopade JO, Ihunwo AO (2015). Astrocyte morphology, heterogeneity, and density in the developing African giant rat (*Cricetomys gambianus*). *Front Neuroanat* 26; 9:67.

O'Neill P, McCole RB, Baker CV (2007). A molecular analysis of neurogenic placode and cranial sensory ganglion development in the shark *Scyliorhinus canicula. Dev Biol* 304:156-181.

Osorio J, Mueller T, Retaux S, Vernier P, Wullimann MF (2010). Phylotypic expression of the bHLH genes Neurogenin2, Neurod, and Mash1 in the mouse embryonic forebrain. *J Comp Neurol* 518:851-71.

Paridaen JT, Huttner WB (2014). Neurogenesis during development of the vertebrate central nervous system. *EMBO Rep* 15:351-64.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Paredes RG (2003). Medial preoptic area/anterior hypothalamus and sexual motivation. *Scand J Psychol* 44:203-12.

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. *J Comp Neurol* 424:409-38.

Puelles L, Harrison M, Paxinos G, Watson C (2013). A developmental ontology for the mammalian brain based on the prosomeric model. *Trends in Neurosci* 36:570-8.

Purves D, Augustine GJ, Fitzpatrick D, Hall WC, Lamantia AS, McNamara JO, Williams M (2000). Neurociencia 3rd edition. Ed. Panamericana

Quintana-Urzainqui I, Sueiro C, Carrera I, Ferreiro-Galve S, Santos-Durán G, Pose-Méndez S, Mazan S, Candal E, Rodríguez-Moldes I (2012). Contributions of developmental studies in the dogfish *Scyliorhinus canicula* to the brain anatomy of elasmobranchs: insights on the basal ganglia. *Brain Behav Evol 80:127-41.*

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E (2014). Developmental, tract-tracing and immunohistochemical study of the peripheral olfactory system in a basal vertebrate: insights on Pax6 neurons migrating along the olfactory nerve. *Brain Struct Funct* 219:85-104.

Quintana-Urzainqui I, Rodríguez-Moldes I, Mazan S, Candal E (2015). Tangential migratory pathways of subpallial origin in the embryonic telencephalon of sharks: evolutionary implications. *Brain Struct Funct* 220:2905-26.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Rash BG, Tomasi S, Lim HD, Suh CY, Vaccarino FM (2013). Cortical gyrification induced by fibroblast growth factor 2 in the mouse brain. *J Neurosci* 33:10802-14.

Reillo I, de Juan Romero C, García-Cabezas MÁ, Borrell V (2011). A role for intermediate radial glia in the tangential expansion of the mammalian cerebral cortex. *Cereb Cortex* 21:1674-94.

Rodríguez-Moldes I, Manso MJ, Becerra M, Molist P, Anadon R (1993). Distribution of sustance P-like immunoreactivity in the brain of the elasmobranch *Scyliorhinus canicula*. *J Comp Neurol* 335:28-44.

Rodríguez-Moldes I (2009). A developmental approach to forebrain organization in elasmobranchs: new perspectives on the regionalization of the telencephalon. *Brain Behav Evol* 74:20-9.

Rodríguez-Moldes I, Carrera I, Pose-Méndez S, Quintana-Urzainqui I, Candal E, Anadón R, Mazan S and Ferreiro-Galve S (2011). Regionalization of the shark hindbrain: a survey of an ancestral organization. *Front. Neuroanat* 5:16.

Rodríguez-Moldes I, Santos-Durán GN, Pose-Méndez S, Quintana-Urzainqui I, Candal E (2017). The Brains of Cartilaginous Fishes. In: Kaas J (ed.), Evolution of Nervous Systems 2e. vol. 1, pp. 77–97. Oxford: Elsevier. ISBN: 9780128040423.

Roybon L, Mastracci TL, Li J, Stott SR, Leiter AB, Sussel L, Brundin P, Li JY (2015). The origin, development and molecular diversity of rodent olfactory bulb glutamatergic neurons iistinguished by expression of transcription factor NeuroD1. *PLoS One* DOI: 10.1371/journal.pone.0128035.

Sakura-Splenger T, Baratte B, Shi L, Mazan S (2001). Structure and expression of an Otx5-related gene in dogfish *Scyliorhinus canicula*. Evidence for a conserved role of Otx5 and Crxgenes in the specification of photoreceptors. *Dev Genes Evol* 211:533-544.

Simmons AM, Horowitz SS, Brown RA (2008). Cell proliferation in the forebrain and midbrain of the adult bullfrog, *Rana catesbeiana. Brain Behav Evo* 71:41-53.

Smart IH, Dehay C, Giroud P, Berland M, Kennedy H (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb Cortex* 12, 37–53.

Smeets WJAJ, Nieuwenhyus R, Roberts BL (1983). The central nervous system of cartilaginous fishes. Ed. Springer-Verlag.

Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, James D, Mayer S, Chang J, Auguste KI, Chang EF, Gutierrez AJ, Kriegstein AR, Mathern GW, Oldham MC, Huang EJ, Garcia-Verdugo JM, Yang Z, Alvarez-Buylla A (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555:377-381.

Strieder GF, Charvet C (2009). Telencephalon enlargement by the convergent evolution of expanded subventricular zones. *Biol Lett* 5: 134–137.

Stoykova A, Gruss P (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. J Neurosci 14:1395-412.

Su Z, He C (2010). Olfactory ensheathing cells: biology in neural development and regeneration. *Prog Neurobiol* 92:517-32.

Sueiro C (2003). Estudio immunohistoquímico de los sistemas gabaérgicos del sistema nervioso central de peces elasmobranquios y su relación con sistemas catecolaminérgicos y peptidérgicos. PhD

Than-Trong E, Bally-Cuif L (2015). Radial glía and neural progenitors in the adult zebrafish central nervous system, *Glia* 63:1406-28.

Thisse B, Pflumio S, Fürthauer M, Looping B, Heyer V, Degrave A, Woehl, Lux A, Steffan T, Charbonnier XQ, Thisse C (2001). Expression of zebrafish genome during embryogenesis. ZFIN direct Data 2001.

Uteshev VV (2014). Network Experimental Approaches: Ex vivo Recording. IN: Faingold CL, BlumenfeldH (ed) Neuronal Networks in Brain Function, CNS Disorders and Therapeutics pp: 67-75. Academic Press. ISBN: 978-0-12-415804-7.

Vallarino M, Danger JM, Fasolo A, Pelletier G, Saint-Pierre S, Vaudry H (1988). Distribution and characterization of neuropeptide Y in the brain of an elasmobranch fish. *Brain Res* 448:67-76.

Wulliman MF, Puelles L (1999). Postembryonic neural proliferation in the zebrafish forebrain and its relationship to prosomeric domains. *Anat Embryo* 329:329-348.

Wullimann MF, Knipp S (2000). Proliferation pattern changes in the zebrafish brain from embryonic thorugh early postembryonic stages. *Anat Embryol* 202:385-400.

Wullimann MF, Rink E (2001). Detailed immunohistology of Pax6 protein and tyrosine hydroxylase in the early zebrafish brain suggests role of Pax6 gene in development of dopaminergic diencephalic neurons. *Dev Brain Res* 131:173-191.

Wullimann MF, Rink E (2002). The teleostean forebrain: A comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. *Brain Res Bull* 57:363-370.

Wulliman MF (2009) Secondary neurogenesis and telencephalic organization in zebrafish and mice: a brief review. *Integr Zool* 4:123-33.

Yaghmaie F, Saeed O, Garan SA, Freitag W, Timiras PS, Sternberg H (2005). Caloric restriction reduces cell loss and maintains estrogen receptor-alpha immunoreactivity in the pre-optic hypothalamus of female B6D2F1 mice. *Neuro Endocrinol Lett* 26:197-203.

Yáñez J, Folgueira M, Köhler E, Martínez C, Anadón R (2011). Connections of the terminal nerve and the olfactory system in two galeomorph sharks: an experimental study using a carbocyanine dye. *J Comp Neurol* 519:3202-17.

Yopak KE, Lisney TJ, Darlington RB, Collin SP, Montgomery JC, Finlay BL (2010). A conserved pattern of brain scaling from sharks to primates. *Proc Natl Acad Sci U S A* 107:12946-51.

Yopak KE (2012). Neuroecology of cartilaginous fishes: the functional implications of brain scaling. *J Fish Biol* 80:1968-2023.

Yopak KE, Lisney TJ, Collin SP (2015). Not all sharks are "swimming noses": variation in olfactory bulb size in cartilaginous fishes. *Brain Struct Funct* 220:1127-43.

Zecevic N, Chen Y, Filipovic R (2005). Contributions of cortical subventricular zone to the development of the human cerebral cortex. *J Comp Neurol* 491:109-22.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Zielinski B, Hara TJ (2006). Fish Physiology: Sensory Systems Neuroscience. Ed Academic Press.

RATIONALE AND AIMS

RATIONALE AND AIMS OF THE THESIS

The developmental mechanisms underlying telencephalic evolutionary variation have been object of profound research. Comparative approach among different animal models are essential for gaining understanding of the evolution of the telencephalon, one of the brain areas that has suffered most drastic changes throughout vertebrate evolution. Developmental sources of such outcome include changes in the amount of cells, which in turn depend on variations (rate/duration) of the process of neurogenesis. For that, the knowledge and the characterization of the neurogenic process during development and in postnatal stages of a basal vertebrate as the catshark will help to increase our knowledge of the neurogenic process in an evolutive context. The knowledge of the main similarities and differences between mammals (low neurogenic potential) and basal vertebrates (high neurogenic potential), could be useful in the mid-term for improving therapies to increase or stabilize neurogenesis in pathological processes.

The lesser spotted dogfish (*Scyliorhinus canicula*) or catshark is considered as a model specie in evolutionary developmental (Evo-Devo) studies. First studies about the regionalization, cell migration and neurogenesis have been performed in the developing/adult telencephalon of this species, but more studies are needed in order to understand the neurogenic process and the heterogeneity of progenitor cell types. For that the aims of the present thesis are:

I. To explore the existence of potential apical radial glial progenitors and basal progenitors. We will focus on determining whether a subventricular zone, which has been largely related to the increased complexity of the telencephalon of mammals, is already present in basal vertebrates. We also aimed at investigating the presence of a glutamatergic lineage transcriptional program in the developing pallium. The results of this study are presented

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

in Chapter 1, entitled **Study of pallial neurogenesis in shark embryos and the evolutionary origin of the subventricular zone.**

II. To characterize different subtypes of radial glial cells in the telencephalon of *S. canicula* during development and in juveniles, and to evaluate the proliferative potential of radial glial cells during development. The results of this study are presented in Chapter 2, entitled **Expression of radial glial markers (GFAP, BLBP and GS) during telencephalic development in the catshark (*Scyliorhinus canicula*).**

III. To identify the main proliferating regions in the post-hatching telencephalon and to characterize the main types of cells that are present in adult neurogenic niches. The comparative analyses with other species could help to give light about the ancestral condition of adult neurogenesis. The results of this study are presented in Chapter 3, entitled **Characterization of neurogenic niches in the telencephalon of juvenile and adult sharks.**

IV. To investigate the development of the central olfactory system by characterizing different types of progenitor cells and studying the expression pattern of transcription factors related to the differentiation of glutamatergic cells. We also aimed at characterizing different subtypes of radial glial cells in the olfactory bulb of *S. canicula* during development and in juveniles, and to evaluate the proliferative potential of radial glial cells. The results of this study are presented in Chapter 4, entitled **Expression of neurogenic markers and radial glial markers in the developing olfactory bulb of catshark (*Scyliorhinus canicula*).**

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CHAPTER 1

STUDY OF PALLIAL NEUROGENESIS IN SHARK EMBRYOS AND THE EVOLUTIONARY ORIGIN OF THE SUBVENTRICULAR ZONE

Docampo-Seara A, Lagadec R, Mazan S, Rodríguez MA, Quintana-Urzainqui I, Candal E (2018). Study of pallial neurogenesis in shark embryos and the evolutionary origin of the subventricular zone*. Brain Structure and Function*. **https://doi.org/10.1007/s00429-018-1705-2**

Introduction

The telencephalon, as one of the most complex structures in the brain, has roles in decision making, memory, learning or higher cognitive behavior, and, thus, has been a major subject of interest for many researchers. One of its subdivisions is the mammalian cerebral cortex which is more generally called the vertebrate dorsal pallium. An important difference in the dorsal pallium/cortex among vertebrates is the absence of layers in amphibians and fishes in contrast to the three-layered structure observed in reptiles or the six-layered cortices present in mammals (Abdel-Mannan et al. 2008). Surprisingly, the neurogenic and migratory pattern operating during development of this pallial subdivision seems to show more similarities across vertebrates than expected (Medina and Abellan 2009; Wullimann 2009; Montiel et al. 2015; Quintana-Urzainqui et al. 2015).

Neurogenesis is the process by which new neurons are formed from stem cells through orderly sequenced events occurring both during development and in the adult life (Götz and Huttner, 2005; Espósito et al. 2005; Hevner et al. 2006). Neurogenesis involves a natural transition from a proliferating undifferentiated progenitor to a postmitotic and terminally differentiated neuron, which is usually accompanied by the migration and displacement of cells to their final position (Noctor et al. 2004; Hevner et al. 2006). During the course of pallial development, neuroepithelial cells, the primary neural stem/progenitor cells, undergo both symmetric and asymmetric types of cell division in order to maintain the progenitor pool. Neuroepithelial cells generate, by asymmetric division, secondary neural stem cells termed radial glial progenitors (apical progenitors) (for review see Götz and Huttner 2005 and Farkas and Huttner 2008). Radial glial progenitors express the proliferating cell nuclear antigen (PCNA) and transcription factors such as

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Sox2 and Pax6, as well as the glial fibrillary acid protein (GFAP) (Heins et al. 2002; Ferri et al. 2004; Englund et al. 2005; Favaro et al. 2009; Anthony and Heintz 2008; Götz et al., 2015; Martynoga et al. 2015). Radial glial progenitors either divide to generate immature neurons directly (direct pathway) or to generate intermediate (basal) progenitor cells (IPC) (indirect pathway) (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004; Götz and Huttner, 2005; Martínez-Cerdeño et al. 2006; Cheung et al. 2007; Marín and Müller, 2014; Martynoga et al. 2015). IPCs migrate out of the ventricular zone stablishing what is called a subventricular zone (SVZ). These cells are morphologically different to radial glial cells, they express different molecular factors and will divide in order to generate two new neuroblasts (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004; Englund et al. 2005; Götz and Huttner 2005). This indirect pathway might have served as an amplification mechanism of neurogenesis since it allows to generate increased numbers of neurons from a single division of a radial glial cell than the direct pathway (Smart et al. 2002). The transition of radial glia stem cells to IPCs and the beginning of the migratory process is characterized by Pax6 and Sox2 downregulation. In turn, the downregulation of Pax6 and Sox2 leads to the upregulation of different migratory factors in the IPCs. One of these factors is the stabilizing microtubule protein doublecortin (DCX), which acts regulating the organization and stability of microtubules in the IPCs and migrating neurons (Glesson et al. 1998, 1999). T-box gene *Tbr2* and the bHLH gene *NeuroD* are also expressed by IPCs (Hevner et al. 2006). The expression of Tbr2 is strongly associated with the IPC state and the beginning of the migratory process. Other than being expressed by basal progenitor cells (see above), Tbr2 expression is also considered to serve as marker for the glutamatergic lineage in the cortex (Englund et al. 2005; Hevner et al. 2006). The expression of NeuroD is associated not only with the migratory process

but also with the start of neuronal differentiation and glutamatergic phenotype in the cortex (Guillemot et al. 2006). As differentiation proceeds, Tbr2 is downregulated and another T box gene, *Tbr1*, is upregulated. Tbr1 is expressed in postmitotic neurons in many areas of the brain, and its expression is also related to the glutamatergic neuronal linage (Hevner et al. 2003; Hevner et al. 2006). Therefore, in mammals, the glutamatergic lineage transcriptional program has been characterized by the sequential expression of those transcription factors: Pax6-Tbr2-NeuroD-Tbr1. Glutamatergic cells of brain areas like the cortex, hippocampus, olfactory bulb or the cerebellum mainly follow this program of development (Englund et al. 2005; Hevner et al. 2006; Brill et al. 2009; Roybon et al., 2009; Hodge et al. 2012; Imamura and Greer 2013).

Analyzing data under an evolutionary point of view shows that radial glial cells acting as progenitor cells in the developing pallium are not exclusive of mammals. Studies in other vertebrates such as reptiles (Clinton et al. 2014; Martínez-Cerdeño et al. 2016), amphibians (Moreno and González 2017) or teleosts (Dong et al., 2012) have also identified proliferating radial glial cells during pallial development. In the same way, comparative studies about the existence of IPCs and the SVZ have been performed in different species of vertebrates. It is thought that the pallial SVZ appeared in evolution during the transition from reptiles to mammals (Cheung et al. 2007; Martínez-Cerdeño et al. 2016), though some elements of a mammalian SVZ are present in the reptilian cortices (reviewed in Montiel et al. 2015). Therefore, many authors have linked the evolutionary emergence of the pallial SVZ with the increased complexity, gyrification and the expansion of the mammalian cortex (Zecevic et al. 2005; Kriegstein et al. 2006; Martinez-Cerdeño et al. 2006; Cheung et al 2007; Rakic, 2009; Cheung et al 2010; Reillo et al. 2011; Rash et al. 2013). Despite a defined SVZ or IPCs have been found in the dorsal pallium of birds

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

and reptiles, recent studies suggest the existence of IPC-like cells in amphibians, opening the question about the evolutionary origin of IPCs and the SVZ (Moreno and González 2017). Regarding the transcription program for the specification of glutamatergic cells, studies in zebrafish also suggested a rather conserved transcriptional program in the pallium (Mione et al., 2001; Wullimann and Rink, 2001, 2002; Mueller and Wullimann, 2003, Mueller et al., 2008; Wullimann, 2009; Mueller and Wullimann, 2016). Whether this program of development is conserved in other species is not known. Extending these comparative studies to basal vertebrates will be key to understand the evolution of the pallial structures.

In this study, we have chosen the lesser spotted dogfish *Scyliorhinus canicula,* a basal vertebrate that belongs to the group of Chondrichthyans or cartilaginous fishes. They represent an ancient radiation of gnathostome (jawed) vertebrates and are currently considered the sister group of gnathostomes with a bony skeleton that gave rise to land vertebrates. This out-group position makes chondrichthyans essential in assessing the ancestral organization of the brain of jawed vertebrates (Rodríguez-Moldes et al. 2017 and references therein). In contrast to bony fishes, the chondrichthyan telencephalon develops, as in tetrapods, by evagination. In addition, the telencephalon of this species is especially large compared to the rest of the brain; in fact, it represents the 50% of the cerebral mass of these species (Yopak et al. 2015). This fact, added to its slow development and high neurogenic capacity (Quintana-Urzainqui et al. 2015) makes the telencephalon of *S. canicula* a valid model for studying neurogenesis and development.

The main purpose of this work is to gain knowledge about the evolutionary mechanisms of pallial neurogenesis by studying this process in cartilaginous fishes, an out-group of other jawed vertebrates. In particular, we

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

aimed at exploring the existence of potential basal progenitors (IPCs) and a SVZ and at investigating the presence of a glutamatergic lineage transcriptional program in the pallium. For this, we have performed a spatiotemporal analysis of the expression pattern of several markers such as *ScSox2* (pluripotent cells), GFAP (marker of radial glia), PCNA (proliferating cells), PH3 (mitotic cells), BrdU (S-phase cells after BrdU incorporation into the newly synthesized DNA), Pax6 (pallial marker and neurogenic regulator), *ScTbr2* (IPC and glutamatergic fate marker), DCX (migratory cells), *ScNeuroD* (postmitotic and migratory cells) and Tbr1 (immature cells and glutamatergic fate transcription factor), during key stages of pallial neurogenesis in *S. canicula*.

Materials and Methods

Experimental animals

We have analysed 22 embryos of *S. canicula* from stages 29 (S29) to 32 (S32). Embryos were provided by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France). Embryos were also kindly provided by the aquarium of O Grove (Galicia, Spain). Embryos were staged by their external features according to Ballard et al (1993). Eggs were raised in seawater tanks under standard conditions of temperature (15-16 ºC), pH (7.5-8.5) and salinity (35 g/L) and suitable measures were taken to minimize animal pain and discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by Spanish Royal Decree 53/2013 for animal experimentation and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS- 222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch's phosphate buffer [EPB: 0.1M phosphate buffer (PB) containing 1.75% of urea, pH 7.4] for 48-72 h depending on the stage of development. Sharks at S32 were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer´s solution (see Ferreiro-Galve et al. 2012a) followed by 4% PFA in EPB. Brains of S32 embryos were removed and postfixed in the same fixative for 24-48 h at 4 ºC. Subsequently, they were rinsed in PB saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (16 µm thick) were obtained in transverse or sagittal planes on a cryostat and mounted on Superfrost Plus slides (Menzel-Glasser, Madison, WI, USA).

Immunohistochemistry

Sections were pre-treated with 0.01M citrate buffer pH 6.0 for 30 min at 90 ºC for heat-induced epitope retrieval and allowed to cool for 20 min at room temperature (RT). Sections were rinsed in 0.05 M TBS pH 7.4 for 5 min and treated with 10 % H_2O_2 in TBS for 30 min at RT to block endogenous peroxidase activity. Sections were rinsed in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 5 min and incubated for 15h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate horseradish peroxidase coupled secondary antibody (see Table 1) for 1 hour at RT. All dilutions were made

with TBS containing 15 % normal goat serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Then, sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each. The immunoreaction was developed with 0.25 mg/ml diaminobenzidine (DAB) tetrahydrochloride (Sigma) in TBS pH 7.4 and 0.00075 % H_2O_2, or with SIGMAFAST™ 3.3-DAB tablets as indicated by the manufacturers. In some procedures, 2.5 mg/ml nickel ammonium sulphate was added. Finally, the sections were dehydrated and coverslipped.

Immunofluorescence

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 90 ºC and allowed to cool for 20 min at RT. Sections were rinsed in 0.05 M TBS pH 7.4 for 5 min and incubated approximately for 15 h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate fluorescent dye-labeled secondary antibodies (see Table 1) for 1 h at RT. All dilutions were made with TBS containing 15 % normal donkey serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % BSA (Sigma). All incubations were carried out in a humid chamber. Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each and in distilled water for 30 min. Sections were then allowed to dry for 30 min at 37ºC, and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany). Information about the primary and secondary antibodies is included in Table 1.

BrdU labelling

BrdU (5-bromo-2'-deoxyuridine, a synthetic nucleoside analog of thymidine; Sigma B5002) was administrated to embryos at S30 of development by immersion for 2,5 h in 10 mg/ml BrdU in oxygenated artificial sea water after opening the egg shell. Embryos were sacrificed at 24 h (S30) and 10 days (S31) after BrdU incorporation. For detection of BrdU, sections were incubated in 2N HCl for 30 min at 50 ºC to denature DNA strands. HCl reaction was stopped by addition of 0.1M sodium tetraborate and sections were then rinsed in TBS for 10 min before antibody incubation. Sections were incubated with the monoclonal mouse anti-BrdU antibody (Millipore, Billerica, MA; 1:200) at RT overnight and processed for immunofluorescence as described above.

In situ hybridization

We applied in situ hybridization (ISH) for *S. canicula Sox2, Tbr2/Eomes* and *NeuroD1 (ScSox2, ScTbr2, and ScNeuroD1)* genes. These probes were selected from a collection of *S. canicula* embryonic cDNA library (mixed stages S9 to 22) and submitted to high throughput EST sequencing (coordinated by Dr. Sylvie Mazan). Sense and antisense digoxigenin-UTP-labeled *ScSox2, ScTbr2* and Sc*NeuroD1* were synthesized directly by transcription *in vitro*. ISH was performed on cryostat sections of S29 to S31 embryos following standard protocols (Coolen et al. 2009). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65 ºC and incubated with the alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Roche Applied Science, Manheim, Germany) overnight at 4 ºC. The colour reaction was performed in the presence of BM-Purple (Roche). Finally, sections were dehydrated and coverslipped. Control sense probes did not produce any detectable signal. For combined ISH-immunohistochemistry, after colorimetric detection of probes,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

sections were rinsed three times in 1M PBS for 10 min each and immunohistochemistry was performed as described above.

Control and specificity of antibodies

The polyclonal antibody against GFAP has been widely used as marker of glial cells in the brain, retina and olfactory system of *S. canicula* (Wasowicz, 1999; Quintana-Urzainqui et al. 2014; Quintana-Urzainqui et al. 2015; Sánchez-Farías and Candal 2016). The PCNA antibody has been previously used to label progenitor cells in the brain, retina and olfactory system of the lesser spotted dogfish (Ferreiro-Galve et al., 2010; Quintana-Urzainqui et al. 2014; Quintana-Urzainqui et al. 2015; Sanchez-Farías and Candal 2015, among others). The PCNA is present in proliferating cells along the entire cell cycle, though its expression is stronger during the S phase of the cell cycle (Zerjatke et al. 2017). The specificity of the antibody against Pax6 has been tested by preabsortion test (Ferreiro-Galve et al., 2012b) and the specificity of the DCX has also been tested by western blot (Pose-Mendez et al. 2014). The anti-PH3 antibody has been used previously as a marker of mitotic cells in the brain and retina of *S. canicula* (Ferreiro-Galve et al. 2010; Quintana-Urzainqui et al. 2015). Tbr1 has been widely used as a marker of pallial/cortical neurons in the developing cortex of mammals and birds (Ceci et al. 2012; Pedraza and DeCarlos 2012; Ballout et al. 2016). The two polyclonal anti-Tbr1 antibodies used in this study revealed a similar pattern of distribution of immunoreactive (-ir) cells and, therefore, descriptions of Tbr1-immunoreactivity in next section apply to both.

Imaging

Fluorescent sections were photographed with the Leica TCS-SP2 scanning microscope with a combination of blue and green excitation lasers.

Confocal images were acquired separately for each laser channel with steps of 2 μm along the z-axis, and collapsed images were obtained with the LITE software (Leica). Also some sections were photographed with an Olympus AX70 epifluorescence photomicroscope fitted with an Olympus DP70 colour digital camera. Light field images were obtained with an Olympus BX51 microscope equipped with an Olympus DP71 colour digital camera. Both fluorescent and light field photographs were adjusted for contrast, brightness and intensity using Corel Draw X3.

Results

During the development of the telencephalon in *S. canicula*, a neurogenic period has been established by Quintana-Urzainqui et al. (2015) from stage S28 to S31 of development. This period of development is roughly comparable to stages E10.5 to E14.5 in mouse, stages HH19 to HH30 in chick (defined by Hamburger and Hamilton 1951) and to stages 24-48 hpf in zebrafish (reviewed in Rodríguez-Moldes 2017). From S31 to S32, key morphogenetic events take place, when the *S. canicula* telencephalon fuses at medial pallial levels and the lateral ventricles emerge. Main embryonic territories are clearly recognizable by S31 while the basic mature structure of the telencephalon is progressively achieved from S32 on (Quintana-Urzainqui et al. 2015). In the present study we have analysed the pallium of embryos from S29 to S32 using the expression of different neurogenic markers.

Early stages of development: Characterization of pallial progenitor cells

In the telencephalon of *S. canicula*, S28 and S29 can be considered early neurogenic stages (Quintana-Urzainqui et al. 2015). In this study we chose S29 as the representative stage of this period, during which the telencephalon is broadly subdivided into a pallial domain and a subpallial

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

domain on the basis of the complementary expression pattern of Pax6 and GAD65/67 (Carrera et al. 2008; Ferreiro-Galve et al. 2008; Quintana-Urzainqui et al. 2015). At this stage, the cytoarchitecture of the pallium is neuroepithelial (Fig. 1) and pallial subterritories are not yet recognizable (Fig. 1A). We used various cell markers with the aim of characterizing radial glial progenitors within the pallium. Sox2 has been used as a marker of stem cells and pluripotency (Hagey and Muhr, 2014; Shimozaki, 2014; Zhang and Cui, 2014). *In situ* hybridization for *ScSox2* showed that it is expressed across the entire telencephalic walls (Fig. 1B). Immunohistochemistry against the proliferating marker PCNA was also observed throughout the whole telencephalon. Both *ScSox2* and PCNA markers seemed to expand throughout most of the thickness of the telencephalic walls (Fig. 1B', C), with the exception of some cells located at very marginal positions (arrowheads in Fig. 1B', C), probably corresponding with the first cells exiting the cell cycle. Two different morphologies have been observed in PCNA-immunoreactive (-ir) nuclei: round (mainly close to the ventricular and pial surfaces; yellow arrows in Fig. 1C) and oval (as in cells located at intermediate positions; white arrows in Fig. 1C). We then analysed the expression of glial acid fibrilar protein (GFAP), a marker of radial glial cells, which has been widely reported in radial glial progenitors during development (Malatesta et al. 2008; Malatesta and Götz 2013). GFAP immunoreactivity has been observed in radial cell processes from the ventricular to the pial surface throughout the telencephalon (Fig. 1D), as well as in endfeet-like structures at the pial surface (arrows in Fig. 1D). In order to investigate if GFAP-ir cells exhibit characteristics of proliferative progenitors in sharks, we analysed double immunofluorescence for GFAP and PCNA (Fig. 1E). Although GFAP immunoreactivity does not allow us to clearly differentiate cell bodies, numerous PCNA-ir nuclei were bordered by GFAP immunoreactivity (Fig. 1E'). Therefore, it is highly

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

probable that GFAP and PCNA are expressed in the same cells since, at this stage, PCNA is expressed by virtually all cells in the ventricular surface of the telencephalic wall. Immunohistochemistry against Pax6, which has been previously used as a marker of pallial progenitors in the brain of vertebrates (Götz et al. 1998; Hevner et al. 2006; Curto et al. 2015), was also analysed. Pax6 can be detected in the telencephalon of *S. canicula* in S29 as a band of cells restricted to the pallial domain ventricular zone (Fig. 1F). In addition, double immunofluorescence for Pax6 and PCNA revealed colocalization between both factors in pallial cells in the ventricular border (Fig. 1G).

In summary, the shark pallium at S29 expresses proliferation and pluripotency markers which expand most of the thickness of the telencephalic wall.

Pallial neurogenesis in S. canicula: exploring the existence of a SVZ in sharks

The first signs of a zonation in the pallium were observed at S30. At this stage, *ScSox2* expression became restricted to a narrow band close to the ventricular surface, which we identified as the ventricular zone (VZ in Fig. 2A). On the other hand, DCX, a marker for newly differentiating and migrating neurons, was expressed from intermediate (weak) to marginal (strong), in what we have identified as different areas of the intermediate zone (inner intermediate zone (iIZ) and marginal intermediate zone (mIZ), Fig. 2B). A combination of ISH for *ScSox2* and immunohistochemistry for DCX can be observed in Fig. 2C showing the complementarity of their expression and how they clearly define the three areas mentioned above. In addition, double immunofluorescence for DCX and GFAP revealed DCX-ir cell bodies in the intermediate zone in close apposition to radial GFAP-ir processes (Figs. 2D,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

D’, D’’, D’’). This suggests that, by S30, active radial migration occurs in the pallium of *S. canicula*.

To further characterize this intermediate zone, we have analysed proliferation by means of PCNA immunohistochemistry. PCNA immunoreactivity can still be observed covering most of the pallial territory, with the exception of some marginal portions in this territory (Fig. 3A). We observed a band of cells showing particularly intense PCNA-immunorreactivity at intermediate positions (arrows in Fig. 3A), in what it would correspond to the limit between the VZ and IZ. Since intense PCNA-ir cells (in the S phase of the cycle) are committed to undergo mitosis, we suspected that actively mitotic cells might be present outside the ventricular surface, in a subventricular position. In order to confirm this, we performed immunohistochemistry against the mitotic marker PH3. This revealed that most mitotic cells are, as expected, located lining the ventricular surface. However, some positive cells were detected subventricularly, expanding from medial to lateral portions of the pallium, at positions compatible with the intermediate band of intense PCNA immunoreactivity (arrows in Fig. 3B).

The existence of an intermediate band of cells located at the interface between the VZ and IZ (areas defined, respectively, by *ScSox2* expression and DCX immunoreactivity), which contains proliferative and mitotically active cells, led us to consider the existence of a SVZ in the shark pallium. By definition (see Discussion), proliferating cells in this SVZ should be identified as IPCs. To investigate the existence of these cells, we studied the expression pattern of a well-known marker of IPCs in mammals, the transcription factor Tbr2. *ScTbr2* expression was detected also as a subventricular band along the pallial domain (Fig. 3C-C’’), located at the same position as both the intense PCNA-ir band and the PH3-ir subventricular cell bodies (compare Figs. 3A, B

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

and C). Moreover, PH3 immunohistochemistry combined with *in situ* hybridization for *ScTbr2* showed double labelled cells in subventricular positions (Fig. 3D, E). At S31 of development, subventricular mitoses (arrows in Fig. 3F) and the band of *ScTbr2* expressing cells were still very evident (Fig. 3G, H). However, the relative number of mitotic PH3 positive cell bodies appeared to decrease compared to the previous stage.

To further investigate the presence of IPCs (subventricularly dividing) cells we have administered BrdU at S30 for 2,5 h (which becomes incorporated into DNA as it replicates in dividing cells) and then analysed BrdU incorporation at 24 h (S30) and 10 days (S31) after administration (Fig. 4A). Our results show that, 24 h post administration, BrdU-immunoreactive cells are located not only at the VZ, but also at abventricular positions (Fig. 4B, C). Similar results were found at 10 days after administration, though the number of cells containing BrdU has largely increased (Fig. 4D). Interestingly, cells showing weaker levels of BrdU are located at positions where cells undergo mitosis, i.e., at the ventricular surface and at the SVZ. Moreover, some of these weakly BrdU-immunoreactive cells were double labelled for the mitosis marker pH3 (Fig. 4B-D'').

At later stages (S32 onwards) we found no evidence of subventricular PH3-ir or *ScTbr2*-expressing cells (data not shown), which indicates that this subventricular population might be transitory.

The observation of a transient population of proliferative cells expressing *ScTbr2* and located at a subventricular position suggests that the shark pallium may contain basal progenitors forming a SVZ-like structure.

Study of neurogenic transcription factors in S31 and S32

S31 represents the mid developmental neurogenic period and, as it proceeds, the basic mature structure of the telencephalon is progressively acquired (Quintana-Urzainqui, et al. 2015). We have taken it as reference for studying the rostral pallial subdivision since it constitutes a well identified part of the telencephalon of *S. canicula* and it can be easily followed from S31 to S32. Since our and previous results have shown that the expression pattern of most transcription factors is highly conserved between mammals and sharks (Ferreiro-Galve et al. 2008; Rodríguez-Moldes et al. 2008, 2011; Rodríguez-Moldes 2009; Quintana-Urzainqui et al. 2015; Santos-Durán et al. 2015, 2016; Pose-Méndez et al. 2016a, b, 2017; Rodríguez-Moldes et al. 2017) we have selected some neurogenic transcription factors known to be sequentially expressed during pallial neurogenesis in other vertebrates (Pax6, Tbr2, NeuroD and Tbr1) and we studied their expression in the shark pallium.

First we studied the transcription factor Pax6 by means of immunohistochemistry. Its expression pattern was mainly found in the VZ of the pallium, which is notably narrower than at previous stages (Fig. 5A). In addition, numerous Pax6 positive cells can be found outside the ventricular layer in the presumptive dorsal pallial territory which has been already described as a divergent and distinctive trait of sharks (Ferreiro-Galve 2010; Rodríguez-Moldes et al. 2017). Right above and adjacent to the ventricular zone defined by Pax6, we observed the *ScTbr2* expressing band of cells described before in a subventricular position (Fig. 5B). *ScNeuroD* and Tbr1-positive cells seem to occupy very similar positions (compare Fig. 5C and D), suggesting that they could be expressed by the same population of pallial cells at S31. This observation holds also true at later stages (Fig. 5G and H). Although we have not determined by co-expression analysis in the same

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

sections whether there is some extent of overlapping between *ScTbr2* and *ScNeuroD*/Tbr1-*positive* populations, it seems clear that *ScNeuroD*/Tbr1-positive band is consistently thicker than the *ScTbr2*-positive band and that it expands further towards the intermediate pallial zone. The expression pattern of these four markers suggest that they are expressed sequentially as shark pallial progenitors exit the cell cycle, migrate radially and differentiate (first Pax6, followed by *ScTbr2* and finally *ScNeuroD/*Tbr1)

At stage 32, Pax6 immunoreactivity is no longer observed in the ventricular zone (Fig. 5E), which was already described by Ferreiro-Galve 2010. Only a few (one or two per section) Pax6 positive cells could be detected in contact with the ventricular surface (arrows in Fig. 5E). However, abundant Pax6 positive cells were observed throughout the telencephalon, specially concentrated close to the midline of the pallium (Fig. 5E).

On the other hand, the transcription factors *ScTbr2*, *ScNeuroD* and Tbr1 were no longer observed close to the ventricular surface but more restricted to the marginal positions of the pallium (Figs. 5F, G, H). Contrary to what we observed at the previous stage, positive cell bodies for the three transcription factors were apparently located in the same band of cells (Figs. 5F, G, H). This coincidence in their distribution pattern suggest that they could be expressed by the same population. To test this, we performed double ISH-immunohistochemistry for *ScTbr2*-Tbr1 and *ScNeuroD*-Tbr1. Most (if not all) neurons in this population seem to express *ScNeuroD* and most of them co-express Tbr1 (Fig. 5I). Only a few scattered cells expressed only Tbr1 (Fig. 5I). We detected certain degree of colocalization between *ScTbr2* and Tbr1-positive populations (see double labelled cells in Figs. 5J, J´), but also many cells that expressed only one of these markers (Fig. 5J´´, J´´´). In summary, at stage 32, differentiated *ScNeuroD*-positive neurons are found in a band at

more marginal positions than at stage 31, possibly as a result of radial migration. This *ScNeuroD* population seems to be heterogeneous in terms of marker expression, being a mixture of Tbr1-positive, *ScTbr2*-positive, double Tbr1-*ScTbr2*-positive.

Discussion

In this work we have investigated the neurogenic process underlying the generation of pallial neurons in the cartilaginous fish *S. canicula*. Cartilaginous fishes are the only taxa available to study evolution of the early vertebrate pallium since they occupy a key phylogenetic position as a monophyletic group external to all other living jawed vertebrates (gnathostomes; Coolen et al. 2009; Rodríguez-Moldes et al. 2017). Indeed, recent whole-genome analysis showed that the genome of cartilaginous fishes evolved significantly slower than that of bony vertebrates including mammals, and even slower than the genome of the “living fossil” the coelacanth (Venkatesh et al. 2014), confirming that cartilaginous fishes must have retained many characteristics of the common ancestor of jawed vertebrates and therefore they must be good representatives of the ancestral condition of vertebrates. Consequently, it seems that cartilaginous fishes, because of their ancient evolutionary origin, may have remained closer to the stem of vertebrates than many other more divergent and derived animal groups (like bony fishes, amphibians or birds). Supporting this hypothesis are the astonishing similarities between the shark and mammalian developing telencephalon. Although the telencephalon, specially its dorsal part, is one of the structures that underwent more changes through mammalian evolution, the developing telencephalon of sharks shows more similarities to that of mammals than those of other animal groups mentioned above. For example, sharks possess evaginated telencephalons (instead of everted, like ray-finned

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

fishes); most tangential migratory routes of subpallial interneurons towards the pallium are very similar to those reported in mammals (Quintana-Urzainqui et al. 2015; Alzu'bi et al. 2017), while these have not been found to date in other vertebrate groups like amphibians; and all four pallial sectors have been characterized in shark embryos (Rodríguez-Moldes et al. 2017). Although the telencephalon of sharks also shows some specific (possibly derived) characteristics like the expression of Pax6 in postmitotic pallial neurons (Rodríguez-Moldes et al. 2017), they do not exhibit big divergent telencephalic fields, like the dorsal ventricular ridge (DVR) observed in birds/sauropsids.

With that in mind, we studied pallial neurogenesis in sharks with the aim of investigating to what extent this process has changed throughout gnathostome brain evolution. Are apical progenitors present in basal vertebrates as sharks? Is the SVZ an exclusive structure of mammals? Is the transcription program of pallial-born cells a conserved feature throughout evolution? These questions are discussed below.

The pallium of S.canicula contains radial-glia like apical progenitors and presents radial migration like other vertebrates.

Neurons in the pallium (developing cortex in mammals) are initially generated from a pool of progenitors in the early pseudostratified epithelium and divide at the ventricular (also named apical) surface. These are called apical progenitors (or radial glial progenitors), they express Pax6 and GFAP and they extend their processes to the pial surface while also maintaining contact with the apical surface (Englund et al. 2005; Malatesta y Götz, 2013; Götz et al., 2015). During the different phases of their cell cycle, apical progenitors undergo periodic apico-basal movements of their nuclei through a process called interkinetic nuclear migration (Sauer 1935; Götz and Huttner

2005). In this work we describe the existence of a similar kind of progenitors in the shark pallium expressing *ScSox2* and Pax6 in their nuclei and GFAP in their radial processes. PCNA immunoreactivity revealed two different nuclear morphologies: round and close to the ventricular surface *versus* elongated and located at various levels across the pallial wall above the ventricular surface. Round and ventricular nuclei probably correspond to progenitors in M phase while elongated nuclei correspond to progenitors in G1/S/G2 phases which typically undergo interkinetic nuclear migration (Sauer 1935; Götz and Huttner 2005). These observations support the existence of apical progenitors in sharks exhibiting the main features of radial glial precursors.

Developmental studies show the presence of apical progenitors/radial glial cells during the development of the central nervous system of turtles (Clinton et al. 2014; Martínez-Cerdeño et al. 2016), amphibians (Moreno and González 2017) and teleosts (Wulliman and Puelles, 1999; Wulliman and Knipp, 2000; Wulliman and Rink, 2002; Lyons et al. 2003; Mueller and Wulliman, 2003; Alexandre et al. 2010), that would act as neural progenitor cells. The fact that radial glial progenitors are present in the developing brain of sharks and in other species of vertebrates suggests that they are an ancient trait of vertebrates that has been highly conserved along vertebrate evolution. Therefore, we can conclude that apical/radial glial-like progenitors appeared very early in evolution. Unfortunately, there are no developmental studies available in lampreys that pointed to the existence of radial glial progenitors in this vertebrate group (positioned at the base of vertebrate evolution), which prevents us from assessing if the emergence of radial glial progenitors occurred at the transition from agnathans to jawed vertebrates or even earlier in evolution.

At a certain point in development of the mammalian pallium, some cells in the highly proliferative pseudostratified epithelium begin to exit the cell cycle and migrate radially along the processes of the radial glial progenitors (Corbin et al. 2001; Campbell and Götz 2003; Götz and Huttner 2005; Kosodo and Huttner, 2009; Rakic 2009; Rakic et al., 2009; Aboitiz 2011). Eventually they stop their radial movement and adopt a new position in the developing pallium away from the ventricular surface, what give rise to the formation a stratified structure. Cells still cycling (progenitors) remain in contact with the ventricular surface constituting the ventricular zone (VZ). Radially migrating cells and new settled neurons form the intermediate zone, which in turn can be subdivided in different subzones (iIZ, mIZ). In this study we have observed the formation of this basic zonation in embryos of *S. canicula* by stage S30. The shark ventricular zone expressed proliferating (PCNA and PH3), stem cell (*ScSox2*) and pallial progenitor markers (Pax6) while intermediate zone expressed markers of postmitotic and newly generated neurons like DCX or *ScNeuroD*. In addition, we observed DCX-positive cell bodies outside the VZ and closely apposed to GFAP-positive radial processes. We interpret those cells as newly generated neurons migrating along the radial processes of apical progenitors, indicating that in the shark pallium the process of radial migration takes place in a similar way to that described in other vertebrates. Radial migration was thought to be a unique trait in mammals and birds (Hatten 2002). However, recent studies in different species of vertebrates clearly revealed that it is also a highly conserved trait of other vertebrates (zebrafish: Mueller et al. 2011; amphibians: Moreno and González 2017; reptiles: Montiel et al. 2015). Our observations in sharks support this idea. However, whether radial migration appeared before the emergence of jawed vertebrates is still unknown. Studies in the adult brain of lampreys and mixins have revealed radial glia–like cells (ependymoglia), immunoreactive to anti-GFAP

antibodies in mixins and to anti-citokeratins antibodies in lampreys (Wicht et al. 1994; Merrick et al. 1995; Nieuwenhuys and Nicholson 1998). Whether they play a role in neuronal migration during embryonic development is not known. In fact, most neurons in the brain of lampreys remain close to the ventricular zone (Nieuwenhuys and Nicholson 1998) and, therefore it is possible that radial migration is not necessary in this animal group.

The shark pallium contains Tbr2 intermediate progenitors and a basic subventricular zone. Evolutionary implications

The pallial SVZ was until recently thought to appear in the transition from reptiles to mammals (Martínez-Cerdeño et al. 2006, 2016) and it has been pointed as one of the main evolutionary events leading to the high complexity of mammalian cortices (see Introduction). Although several studies have described the existence of subventricular zones in other vertebrates (Montiel et al. 2015), very few observed them in the dorsal pallium, which is the homologous territory to the mammalian developing cortex (reviewed by Medina and Abellán 2009).

But, what defines a pallial SVZ? In 1970, the Boulder Committee Report defined it as a zone located at the junction between the ventricular and intermediate zones, containing round or oval cells distinguished from young neurons of the intermediate zone by their proliferative activity and the lack of interkinetic nuclear migration during the cell cycle. These neurons come to position soon after the intermediate zone begins to form (Angevine et al. 1970). Other two criteria have been incorporated in the last decades. It is now widely accepted that the subventricular zone contains intermediate progenitors. Unlike apical progenitors, basal (intermediate) progenitors are characterized by their subventricular position (away from the ventricular surface) and they do not undergo interkinetic nuclear migration (Götz and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Huttner 2005; Martinez-Cerdeño et al. 2016). Intermediate progenitors are also characterized by their differential gene expression, typically expressing the non-coding RNA *Svet1* and the genes that encode the transcription factors Tbr2, Cux1 and Cux2 (Englund et al. 2005; Götz and Huttner 2005 and references therein; Hevner et al. 2006). The presence of the transcription factor Tbr2 has been proposed as a criterion to identify SVZ cells and define the boundaries of the SVZ itself (Martinez-Cerdeño et al. 2016). Besides, Tbr2 is transiently expressed in cortical SVZ progenitor cells (Arnold et al. 2008; Martínez-Cerdeño et al. 2016). Therefore, a well-defined pallial SVZ must satisfy at least the following criteria: (1) subventricular position throughout the area of the dorsal cortex, (2) presence of mitotically active cells (3) presence of Tbr2-expressing cells dividing at this location that (4) are transiently found during development. According to the above criterion, a clearly defined and organized SVZ has been observed in the ventral telencephalon of birds (subpallium) and some regions of the pallium like the dorsal ventricular ridge (DVR) (Cheung et al. 2007; Montiel et al. 2015). However, its presence in the hyperpallium, which is considered by most as the homologous of the developing cortex of mammals (reviewed by Medina and Abellan 2009), is still a matter of debate (Striedter and Keefer 2000; Martínez-Cerdeño et al. 2006; Striedter and Charvet 2008; Charvet and Striedter, 2009; Charvet et al. 2009; Montiel et al. 2015; Martinez-Cerdeño et al. 2016). The first study concerning the existence of a SVZ in the pallium of birds dates from 1999, where Bulfone et al. (1999) found Tbr2 positive cells organized subventricularly in a HH28 chicken. However, neither their proliferation status nor their exact position within the pallium were specified. Nomura et al. (2016) recently demonstrated the existence of different types of basal progenitors in the chicken pallium and that some of them did not express Tbr2. In this work, the authors proposed that all intermediate progenitors (both Tbr2-

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

positive and negative) could derive from a primitive population of scattered abventricular mitotic cells that might be already present in ancestral amniotes (Nomura et al. 2016).

Turtles have been pointed as the most closely related living animal to the common ancestor of mammals and reptiles (Goffinet 1983; Nieuwenhuys 1994). Studies in *Trachemys scripta elegans*, a specie of turtle, reported a very similar scenario than the one described in birds by Montiel et al. (2015), presenting an anatomically defined SVZ in the DVR but not in the dorsal cortex homologous region (Martinez Cerdeño et al. 2006; 2016). It is important to highlight that both, birds and turtles exhibit clear expression of Tbr2 in the hyperpallium/dorsal pallium, however these Tbr2-positive cells are not clustered in a subventricular position but scattered through the ventricular zone. All evidences to date point to the lack of a SVZ in the pallium of other reptiles (Charvet et al. 2009; Martinez-Cerdeño et al. 2016).

In amphibians, very recent work from Moreno and González (2017) explored the existence of a pallial SVZ in a detailed study of *Xenopus* pallial neurogenesis. They detected clusters of Tbr2-positive cells away from the ventricular zone (especially in the ventral pallium) and some scarce abventricular mitoses. However, they reported that all Tbr2 pallial neurons were postmitotic and the occasional PH3-positive mitotic cells did not express Tbr2. Concerning teleost fishes, Mueller et al. (2008) described the existence of Tbr2 cells in the pallium of developing zebrafish as a tier of cells close or within the ventricular zone (see also Wullimann, 2009; Mueller and Wulliman, 2016). Unfortunately, the lack of further characterization precludes a clear identification of these cells as potential intermediate progenitors in bony fishes.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Most of the evolutionary hypotheses derived from the studies mentioned above consider that the first intermediate progenitors expressing Tbr2 in an organized and anatomically defined SVZ appeared during mammalian evolution (Cheung 2007, 2010; Montiel et al. 2015). They postulate that the SVZ might have represented a key evolutionary milestone leading to the emergence of a 6-layered cortex. Other authors proposed that pallial SVZ and basal progenitors might have appeared independently in birds and mammals leading to the emergence of different basal progenitor modalities in these animal groups (Charvet et al. 2009; Nomura et al. 2016). As far as we are concerned, Martínez-Cerdeño et al. (2016) were the only authors so far postulating an earlier origin for Tbr2-related SVZ in the common ancestor of mammals and sauropsids. They based their conclusions on a comparative study in two species of birds (chick and dove), a species of lizard and a species of turtle. Contrary to other studies in birds, they reported a subventricular band of Tbr2-positive cells in the hyperpallium (dorsal cortex homolog). Unfortunately, whether these cells expressed mitotic markers was not specified. Very few studies considered the scenario in anamniotes, suggesting that intermediate progenitors might be present in a primitive form, mainly as scattered abventricular mitoses that do not express Tbr2 (Moreno and González 2017).

Thus, the current view about pallial SVZ evolution to date hypothesizes that the first abventricular mitoses appeared at some point during anamniote evolution (probably anterior to a tetrapod ancestor) representing the first step and primitive form of basal progenitors and that only mammals, and perhaps some birds, secondarily acquired higher complexity in their organization accompanied by the expression of Tbr2-related genetic program. These considerations were mainly based on observations in *Xenopus* where no

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

double labelled Tbr2-PH3 have been reported so far (Moreno and González, 2017). However, information about non-tetrapod vertebrates was lacking.

In this work we present evidence of the existence of an anatomically defined SVZ in the pallium of sharks. First, we identified the interface between the VZ and IZ (areas defined, respectively, by *ScSox2* expression and DCX immunoreactivity). Second, we observed proliferating, mitotically-active (PH3 positive) band of neurons at a subventricular position aligned parallel to the ventricular surface. Third, this band of neurons strongly expresses *ScTbr2* and colocalization between PH3 and *ScTbr2* was confirmed. Importantly, this population of cells has been observed at all medio-lateral levels of the developing pallium, including therefore the potential dorsal pallium (see Figs. 3 and 5, present work). BrdU assays have been additionally used to investigate proliferation behaviour of cells dividing in this band. Dividing cells incorporate BrdU during the S-phase of the cell cycle. BrdU detection reveals where these dividing cells (or their daughters) are located at the time of fixation. When embryos are fixed after 24 hours, cells that undergo an only mitosis after BrdU incorporation retain high levels of BrdU while dividing cells dilute the incorporated BrdU by a factor of two with each cell division (see Striedter 2015). Under this rationale, in short time windows, weaker levels of BrdU indicate when progenitors have divided, at least once (therefore weaker levels can be correlated to higher proliferation rates). In our hands, weaker BrdU levels were present either at the VZ (where apical progenitors are found, mitosis occurring at the ventricle) or at the SVZ (where basal, intermediate progenitors are found, mitosis occurring subventricularly since they do not undergo interkinetic nuclear migration; see above). Moreover, the mitotic marker PH3 was found in weakly BrdU-immunoreactive cells at these locations even when fixed 10 days after BrdU incorporation, which indicates that these progenitors are still capable of cell division.

Finally, in agreement with the Boulder Committee Report description (Angevine 1970), we observed that these cells are placed right after the ventricular and intermediate zones are formed (S30 in *S. canicula*). This population is very evident at S30 and S31 of development (equivalent to stages E13.5 and E14.5 in mouse approximately) and it disappears by S32, when the basic mature morphology of the shark brain is achieved and Pax6 associated ventricular proliferation seems to cease (Ferreiro-Galve, 2010; Quintana-Urzainqui et al. 2015; Rodríguez-Moldes et al., 2017 and present results). The transient nature of these type of cells also fits with the description of the SVZ dynamics in mammals (Arnold et al. 2008). Moreover, it has been described that the SVZ serves as a route of migration for GABAergic neurons originated in the subpallium and migrating through the pallium during development (Del Rio et al. 1992; reviewed in Montiel et al. 2015). This kind of tangential migration was recently identified in shark embryos by *ScDlx2* and GAD expression analysis. The position of the *ScTbr2* and PH3-positive nuclei described in the present work fits precisely the position of the stream of tangentially migrating cells throughout the pallium described by Quintana-Urzainqui et al (2015). However, the quantity of mitotic cells found at this subventricular position seemed to be much lower than in mammals. It seems plausible that what we are observing in the brain of *S. canicula* are the first steps towards the formation of intermediate progenitors organized in a defined zone in the pallium of vertebrates. Sharks and skates represent a significant step in pallial organization when compared to agnathans such as the lamprey (the only extant vertebrate with a more ancient origin than sharks). The pallium of lampreys is simpler and significantly smaller when compared to the shark pallium (Pombal et al. 2011). Cartilaginous fishes are the first vertebrates to develop evaginated palliums with clear and big ventricles. It is conceivable that the expansion of the pallium in sharks with respect to

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

lampreys might be due to the appearance of a new type of amplifying progenitor. However, the existence of intermediate progenitors in more ancient vertebrates (lampreys) remains to be explored. A summary of all the available data about the existence of a SVZ in the dorsal pallium along the scale of vertebrates is represented in Figure 5.

Our observations led us to propose that basal progenitors exhibiting the basic features of a SVZ, including the expression of the basal progenitor marker Tbr2, might have been already present in the common ancestor of mammals and cartilaginous fishes, that is, in the common ancestor of all gnathostomes. There is no doubt that the increase in complexity of the pallial SVZ across different mammals (including the appearance of different types of basal progenitors and the compartmentalization in an inner and outer SVZ) correlates with increasing complexity in mammalian cortices. Nevertheless, we propose here that the existence of a basic pallial SVZ with all its components can be traced back to the origin of all jawed vertebrates. Consequently, we do not contemplate intermediate progenitors and their genetic program of specification (Tbr2-related) as a novelty of mammals or even amniotes but the ancestral condition of pallial neurogenesis in vertebrates. We also postulate that the evolutionary appearance of basal progenitors might have been crucial for the first big increase in size of the pallium (in cartilaginous fishes, the telencephalon represent the 50% of the size of the encephalon; Yopak et al. 2015). In the light of our results we postulate a scenario in which basal progenitors expressing Tbr2 appeared in the common ancestor of all gnathostomes and were secondary lost in many groups of vertebrates (see Fig. 6). The functional reasons and structural consequences of this potential loss remain to be explored.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Sequence of transcription factor expression in pallial cells. Comparison with other vertebrates.

During development, the mammalian cortex glutamatergic cells, represented by pyramidal cells, are born locally from radial glial and Pax6-expressing progenitor cells located in the ventricular zone of the pallium (Englund et al. 2005). As new-born neurons differentiate and migrate radially to their final destination in the cortex, they successively express a well stablished sequence of transcription factors, namely Tbr2, NeuroD and Tbr1 (reviewed by Hevner et al. 2006). These factors show a partially overlapping expression pattern that perfectly differentiates the VZ (Pax6), SVZ (Tbr2), IZ (NeuroD) and cortical plate (CP) (Tbr1) in the mouse developing cortex. Despite the exact role of these transcription factors is not well understood, it is accepted that they are part of the transcriptional program that establishes the glutamatergic phenotype (Englund et al. 2005; Abdel-Mannan et al. 2008; Roybon et al. 2009; Boutin et al. 2010).

Despite this sequence of factors has not been studied in the analogous cortex of sauropsids, Tbr2 and Tbr1 expression has been studied in birds (Cheung et al. 2007; Martínez-Cerdeño et al. 2016; Nomura et al. 2016) and Tbr2 in reptiles (Clinton et al. 2014; Martínez-Cerdeño et al 2016). Tbr2 expression, both in birds and reptiles, has been observed in ventricular or subventricular positions, and a marginal band of Tbr1 in birds, which is highly coincident with the expression pattern in the mammalian cortex. On the other hand, as far as we know, there are no studies concerning NeuroD in the developing telencephalon of this species. In amphibians (*Xenopus*), some studies have detected Tbr2, NeuroD and Tbr1 expression in postmitotic neurons in the developing/juvenile pallium (D'amico et al. 2013; Moreno and González 2017). However, up to date, the expression of this factors in

Xenopus has not been analyzed together. In teleost fishes, the expression of Pax6, the T-box transcription factors and NeuroD have been studied during development and larvae stages in zebrafish (Mione et al., 2001; Wullimann and Rink, 2001, 2002; Mueller and Wullimann, 2003; Mueller et al., 2008; Wullimann, 2009; Mueller and Wullimann, 2016). These studies suggest that a rather similar neurogenic sequence to that found in mammals might be present in the developing pallium of zebrafish, except for the absence of a Pax6 expression domain in the ventricular proliferative layer in this species (Wullimann and Rink, 2001).

Here we present full evidence that the transcription factors involved in the development of pallial-born neurons in mammals are present in sharks in analogous stages of development. At S31 Pax6 immunoreactivity was detected in the ventricular zone while *ScTbr2, ScNeuroD* and Tbr1 codistribute in a subventricular band. However, *ScTbr2* positive cells are located closer to the VZ compared to *ScNeuroD* or Tbr1 positive cells, which show overlapping expression patterns. At S32, *ScTbr2* expression close to the VZ of the dorsal pallium disappears and it is now observed in more marginal positions. In the same way, *ScNeuroD* and Tbr1 are detected closer to the pial surface, suggesting a radial migration of these cells, though the emergence of *ScTbr2-, ScNeuroD* and Tbr1-expressing cells at marginal positions cannot be discarded. The expression pattern of these four transcription factors has been summarized in in Fig. 7. The expression pattern of these markers is a conserved feature of many glutamatergic neurons in the mammalian brain like in the cortex, olfactory bulb, hippocampus or cerebellum, both during development and, concerning the hippocampus, in adult neurogenesis (Englund et al. 2005; Hevner et al. 2006; Hodge et al. 2012; Imamura and Greer 2013; Bond et al. 2015). Here we show that these markers are conserved along evolution at least in the pallial structure, concluding that the main

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

molecular machinery/ transcriptional program that gives rise to pallial-born neurons is an ancestral condition of vertebrates. Whether they are conserved in other brain areas in sharks is unknown.

The fact that Pax6 and *ScTbr2* are present in the VZ and SVZ respectively is similar to that reported in mammals. However, we found that *ScTbr2, ScNeuroD* and Tbr1 codistribute in many cells in the same territory (present results). This suggest that, despite in sharks apical progenitors (Pax6) and newborn neuroblasts are clearly differentiated, *ScTbr2, ScNeuroD* and Tbr1 cell populations do not show a zonal distribution pattern similar to that observed in mammals. In addition, Tbr2 and Tbr1 do not colocalize in the cortex of mammals, contrary to what we have found in the pallium of *S. canicula*, suggesting that the expression of Tbr2-NeuroD-Tbr1 do not take place as sequentially as in mammals, but rather overlapped. This scenario is similar to that found with mitral cells in the olfactory bulb of mammals, where Tbr2 and Tbr1 double labeled cells can be found (Imamura and Greer 2013).

It has been suggested that the segregation of transcription factors in zones in mammals might be related to layer-related fate specification (Hevner et al. 2006). In addition, cortical layering in mammals is also controlled by a subset of marginal/subpial neurons called Cajal-Retzius cells (C-R). These cells secrete signaling molecules like Reelin, an extracellular matrix glycoprotein, related with the formation of cortical layers (Hevner et al. 2003; D'Arcangelo 2006). C-R cells have been found in species with layered cortices (reptiles: Goffinet et al. 1999; Tissir et al. 2003; Cabrera-Socorro et al. 2007; birds: Bernier et al. 2000; mammals: Cabrera-Socorro et al. 2007). Interestingly, in the developing pallium of *S canicula* a marginal population positive to Reelin has been found during the neurogenic period (Quintana-Urzainqui et al. 2015). Whether they are similar to C-R cells needs further

investigation. If that is the case, the lack of segregation of transcription factors in the pallium of *S. canicula* and the absence of layering in the adult would support the hypothesis that C-R-dependent layering might need a previous segregation of transcription factors in zones.

Our results clearly suggest that the main machinery of transcription factor orchestrating neurogenesis of pallial-born neurons in mammals is present in sharks. However, as depicted above, a clear segregation in layers (VZ; SVZ; IZ; CP) of all the transcription factors as the one observed in mammals, is not found in *S. canicula*, which might result in cytoarchitectonic organization differences between the pallium of mammals and fishes.

Conclusions

In this work we describe the presence of the main two types of pallial progenitors (apical and basal) in the telencephalon of sharks as well as the existence of a rudimentary pallial SVZ. The evolutionary implications of this observation are not trivial, moving the hypothetical origin of this structure back to the common ancestor of all jawed vertebrates. We also report that the main transcriptional program specifying the glutamatergic lineage in the pallium (Pax6, Tbr2, NeuroD, Tbr1) was already present in cartilaginous fishes. However, the segregation of these transcription factors in different cell populations was not observed at later stages of embryonic development indicating that this non-zonal distribution pattern might be the ancestral condition that lead to a non-laminated pallium. Comparative studies are a powerful tool to address evolutionary changes. Studying a basal jawed vertebrate like *S. canicula* has proven to be very useful, especially in regard to the development and evolution of the telencephalon. Insights in this field contribute to our knowledge of basic mechanisms of neurogenesis and ultimately will help to unveil how our brains evolved.

References

Abdel-Mannan O, Cheung AF and Mölnár Z (2008). Evolution of cortical neurogenesis. *Brain Res. Bull* 75:398-404.

Aboitiz F (2011). Genetic and developmental homology in amniote brains. Toward conciliating radical views of brain evolution. *Brain Res. Bull* 84:125-136.

Alexandre P, Reugels AM, Barker D, Blanc E, Clarke JD (2010). Neurons derive from the more apical daughter in asymmetric divisions in the zebrafish neural tube. *Nat Neurosci* 13:673-9.

Alzu'bi A, Lindsay S, Kerwin J, Looi SJ, Khalil F, Clowry GJ (2017). Distinct cortical and sub-cortical neurogenic domains for GABAergic interneuron precursor transcription factors NKX2.1, OLIG2 and COUP-TFII in early fetal human telencephalon. *Brain Struct Funct* 222:2309-2328.

Anthony TE, Heintz N (2008). Genetic lineage tracing defines distinct neurogenic and gliogenic stages of ventral telencephalic radial glial development. *Neural Dev* 3:30

Angevine JB, Bodian D, Coulombre AJ, Edds MV, Hamburger V, Jacobson M, Lyser KM, Prestige MC, Sidman RL, Varon S, Weiss PA. (1970). Embryonic vertebrate central nervous system: revised terminology. *Anatomical Record* 166:257–261.

Arnold SJ, Huang GJ, Cheung AF, Era T, Nishikawa S, Bikoff EK, Molnár Z, Robertson EJ, Groszer M (2008). The T-box transcription factor Eomes/Tbr2 regulates neurogenesis in the cortical subventricular zone. *Genes Dev*. 22:2479-84.

Ballard WW, Mellinger J and Lechenault (1993). A series of normal stages for development of *Scyliorhinus canicula*, the lesser spoted dogfish (Chondrichthyes: Scyliorhinidae). *J exp zoo* 267:318-336.

Ballout N, Frappé I, Péron S, Jaber M, Zibara K, Gaillard A (2016). Development and Maturation of Embryonic Cortical Neurons Grafted into the Damaged Adult Motor Cortex. *Front Neural Circuits* 10:55.

Bernier B, Bar I, D'Arcangelo G, Curran T, Goffinet AM (2000). Reelin mRNA expression during embryonic brain development in the chick. *J Comp Neurol* 422:448-63.

Bond AM, Ming GL, Song H (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell* 17:385-95.

Boutin C, Hardt O, de Chevigny A, Coré N and Goebbels S (2010). NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. *Proc Natl Acad Sci U S A* 107:1201-6.

Brill MS, Ninkovic J, Winpenny E, Hodge RD, Ozen I, Yang R, Lepier A, Gascón S, Erdelyi F, Szabo G, Parras C, Guillemot F, Frotscher M, Berninger B, Hevner RF, Raineteau O and Götz M. (2009). Adult generation of glutamatergic olfactory bulb interneurons. *Nature Neurosci* 12:1524-33

Bulfone A, Martinez S, Marigo V, Campanella M, Basile A, Quaderi N, Gattuso C, Rubenstein JL, Ballabio A (1999). Expression pattern of the Tbr2 (Eomesodermin) gene during mouse and chick brain development. *Mech Dev* 84:133-8.

Cabrera-Socorro A, Hernandez-Acosta NC, Gonzalez-Gomez M, Meyer G (2007). Comparative aspects of p73 and Reelin expression in Cajal-Retzius cells and the cortical hem in lizard, mouse and human. *Brain Res* 1132:59-70.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Campbell K, Götz M (2003). Radial glia: multi-purpose cells for vertebrate brain development. *Trends Neurosci* 25:235-8.

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R and Rodríguez-Moldes I (2008). Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. *Brain Res. Bull.* 75:405-9.

Ceci ML, Pedraza M, de Carlos JA (2012). The embryonic septum and ventral pallium, new sources of olfactory cortex cells. *PLoS One*.7:e44716.

Charvet CJ, Stiedter GF (2009). Developmental origins of mosaic brain evolution: morphometric analysis of the developing zebra finch brain. *J Comp Neurol* 514:203-213.

Charvet CJ, Owerkowicz T, Striedter GF (2009). Phylogeny of the telencephalic subventricular zone in sauropsids: evidence for the sequential evolution of pallial and subpallial subventricular zones. *Brain Behav Evol* 73:28-94.

Cheung AFP, Pollen AA, Tavare A, Deproto J, Molnár Z (2007). Comparative aspects of cortical neurogenesis in vertebrates. *J of Anat* 211:164–176.

Cheung AF, Kondo S, Abdel-Mannan O, Chodroff RA, Sirey TM, Bluy LE, Webber N, DeProto J, Karlen SJ, Krubitzer L, Stolp HB, Saunders NR, Molnár Z (2010). The subventricular zone is the developmental milestone of a 6-layered neocortex: comparisons in metatherian and eutherian mammals. *Cereb Cortex* 20:1071-81.

Clinton BK, Cunningham CL, Kriegstein AR, Noctor SC, Martínez-Cerdeño V (2014). Radial glia in the proliferative ventricular zone of the embryonic and adult turtle, *Trachemys scripta elegans*. *Neurogenesis* (Austin). doi: 10.4161/23262125.2014.970905.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Corbin JG, Nery S, Fishell G (2001). Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. *Nat Neurosci* 4 Suppl:1177–1182.

Coolen M, Menuet A., Chassoux D, Compagnucci C, Henry S, Lévèque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodriguez- Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009). The dogfish Scyliorhinus canicula, a reference in jawed vertebrates. In: Behringer, R.R., Johnson, A.D., Krumlauf, R.E. (Eds.), Emerging Model Organisms, A Laboratory Manual, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 431–446.

Curto GG, Gard C, Rives V (2015). Structures and properties of PAX linked regulatory networks architecting and pacing the emergence of neuronal diversity. *Semin Cell Dev Biol* 44:75-86.

D'Amico LA, Boujard D, Coumailleau P (2013). The neurogenic factor NeuroD1 is expressed in post-mitotic cells during juvenile and adult *Xenopus* neurogenesis and not in progenitor or radial glial cells. *PLos One* . doi: 10.1371/journal.pone.0066487.

D'Arcangelo G (2006). Reelin mouse mutants as models of cortical development disorders. *Epilepsy & Behavior* 8, 81-90.

Del Río JA, Soriano E, Ferrer I (1992). Development of GABA immunoreactivity in the neocortex of the mouse. J Comp Neurol 326:501-526

Dong Z, Yang N, Yeo SY, Chitnis A, Guo S (2012). Intralineage directional Notch signaling regulates self-renewal and differentiation of asymmetrically dividing radial glia. Neuron 74: 65–78.

Englund C, Flink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T and Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci* 25(1):247-51.

Espósito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, Pitossi FJ, Schinder AF (2005). Neuronal differentiation in the adult hippocampus recapitulates embryonic development. *J. Neurosci*. 25:10074–10086.

Farkas LM, Huttner WB (2008). The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. *Curr Opin Cell Biol* 20:707-15.

Favaro R, Valotta M, Ferri ALM, Latorre E, Mariani J, Giachino C, Lancini C, Tosetti V, Ottolenghi S, Taylor V and Nicolis SK (2009) Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. *Nat Neurosci* 12:1248–1256.

Ferreiro-Galve S, Carrera I, Candal E, Villar-Cheda B, Anadón R, Mazan S, Rodríguez-Moldes I (2008). The segmental organization of the developing shark brain based on neurochemical markers, with special attention to the prosencephalon. Brain Res Bull 75:236-40.

Ferreiro-Galve S (2010). Brain and retina regionalization in sharks. Study based on the spatiotemporal expression pattern of Pax6 and other neurochemical markers. Ph.D. thesis, Universidade de Santiago de Compostela, Santiago de Compostela.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Ferreiro-Galve S, Rodríguez-Moldes I, Anadón R, Candal E (2010). Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas. *J Chem Neuroanat* 39:1-14.

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012a). Dynamic expression of Pax6 in the shark olfactory system: evidence for the presence of Pax6 cells along the olfactory nerve pathway. *J Exp Zool B Mol Dev Evol* 318:79-90.

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012b). Pax6 expression during retinogenesis in sharks: comparison with markers of cell proliferation and neuronal differentiation. *J Exp Zool B Mol Dev Evol* 318:91-108.

Ferri ALM, Cavallaro M, Braida D, Di Cristofano A, Canta A, Vezzani A, Ottolenghi S, Pandolfi PP, Sala M, DeBiasi S, Nicolis SK (2004) Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development* 131:3805–3819.

Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME and Walsh CA (1998). Doublecortin, a brain-specific gen mutated in human X-linked Lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* 92:63-72.

Gleeson JG, Lin PT, Flanaga LA and Walsh CA (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* 23:257-71.

Goffinet AM (1983). The embryonic development of the cortical plate in reptiles: a comparative study in Emys orbicularis and Lacerta agilis. *J Comp Neurol* 215:437-52.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Goffinet AM, Bar I, Bernier B, Trujillo C, Raynaud A, Meyer G (1999). Reelin expression during embryonic brain development in lacertilian lizards. *J Comp Neurol* 414:533-50.

Götz M, Stoykova A, Gruss P (1998). Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* 21:1031-44.

Götz M and Huttner WB (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* 6:777-88.

Götz M, Sirko S, Beckers J, Irmler M (2015). Reactive astrocytes as neural stem or progenitor cells – *in vivo* lineage, *in vitro* potential, and genome-wide expression analysis. *Glía* 63:1452–1468.

Guillemot F, Molnár Z, Tarabykin V and Stoykova A (2006). Molecular mechanisms of cortical differentiation. *Eur J Neurosci* 23:857-68.

Hagey DW, Muhr J (2014). Sox2 acts in a dose-dependent fashion to regulate proliferation of cortical progenitors. *Cell Rep* 9:1908-20.

Hamburger V, Hamilton HL (1951). A series of normal stages in the development of the chick embryo. *J Morphol*. 88:49-92.

Hatten ME (2002). New directions in neuronal migration. *Science* 297:1660-3.

Haubensak W, Attardo A, Denk W and Huttner WB (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc Natl Acad Sci U S A* 101:3196-201.

Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapuoton P, Barde YA and Götz M (2002). Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci* 5:308-15.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Hevner RF, Neogi T, Englund C, Daza RA, Fink A (2003). Cajal-Retzius cells in the mouse: transcription factors, neurotransmitters, and birthdays suggest a pallial origin. *Brain Res Dev Brain Res* 141:39-53.

Hevner RF, Hodge RD, Daza RA and Englund C (2006). Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci. Res*. 55:223-33.

Hodge RD, Kahoud RJ, Hevner RF (2012). Transcriptional control of glutamatergic differentiation during adult neurogenesis. Cell Mol Life Sci 69:2125-34.

Imamura F and Greer CA (2013). Pax6 regulates Tbr1 and Tbr2 expressions in olfactory bulb mitral cells. *Trends Neurosci.* 37:77-84.

Kosodo Y, Huttner WB (2009). Basal process and cell divisions of neural progenitors in the developing brain. *Dev Growth Differ* 51:251-61.

Kriegstein A, Noctor S, Martínez-Cerdeño V (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat Rev Neurosci* 7:883-90.

Lyons DA, Guy AT, Clarke JD (2003). Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development* 130:3427-36.

Malatesta P, Appolloni I, Calzolari F (2008). Radial glia and neural stem cells. *Cell Tissues Res* 331:165-75.

Malatesta P, Götz M (2013). Radial glia - from boring cables to stem cell stars. *Development* 140:483-6.

Marín O and Müller U (2014). Lineage origins of GABAergic versus glutamatergic neurons in the neocortex. *Curr Opin Neurobiol* 26:132-41.

Martínez-Cerdeño V, Noctor SC and Kriegstein AR (2006). The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb Cortex.*16 Suppl 1:152-61.

Martínez-Cerdeño V, Cunningham CL, Camacho J, Keiter JA, Ariza J, Lovern M and Noctor SC (2016). Evolutionary origin of Tbr2-expressing precursor cells and the subventricular zone in the developing cortex. *J Comp Neurol.* doi: 10.1002/cne.23879.

Martynoga B, Drechel D and Guillemot F (2015). Molecular Control of Neurogenesis: A view from the mammalian cerebral cortex. *Cold Spring Harvor Lab Press*.

Medina L and Abellán A (2009). Development and evolution of the pallium. *Semin. Cell Dev. Biol.* 20:698-711.

Merrick SE, Pleasure SJ, Lurie DI, Pijak DS, Selzer ME, Lee VM (1995). Glial cells of the lamprey nervous system contain keratin-like proteins. *J Comp Neurol*. 355:199-210.

Mione M, Shanmugalingam S, Kimelman D, Griffin K (2001). Overlapping expression of zebrafish T-brain-1 and eomesodermin during forebrain development. *Mech Dev* 100:93-7.

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T and Ogawa M (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131:3133-45.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Montiel JF, Vasistha NA, Garcia-Moreno F, Molnár Z (2015). From sauropsids to mammals and back: New approaches to comparative cortical development. *J Comp Neurol.* 524:630-45.

Moreno N, González A (2017). Pattern of Neurogenesis and Identification of Neuronal Progenitor Subtypes during Pallial Development in *Xenopus laevis*. *Front Neuroanat* 11:24.

Mueller T, Wulliman MF (2003). Anatomy of neurogenesis in the early zebrafish brain. *Brain Res Dev Brain Res* 140:137-55.

Mueller T, Wullimann MF, Guo S (2008). Early teleostean basal ganglia development visualized by zebrafish *Dlx2a, Lhx6, Lhx7, Tbr2 (eomeasa)* and *GAD67* gene expression. *J Comp Neurol* 507:1245-1257.

Mueller T, Wulliman MF (2016). Atlas of early zebrafish brain development. Elsevier ISBN: 978012417269.

Mueller T, Dong Z, Berberoglu MA, Guo S (2011). The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). *Brain Res* 1381:95-105.

Nieuwenhuys R (1994). The neocortex. An overview of its evolutionary development, structural organization and synaptology. *Anat Embryol (Berl)*190:307-37.

Nieuwenhuys R, Nicholson C (1998). Lampreys, Petromyzontoidea. In: Nieuwenhuys R, Donkelaar T, Nicholson C (eds). The central nervous system of vertebrates, vol. 1. Springer-Verlag, Berlin, pp. 397-495.

Noctor SC, Martinez-Cerdeño V, Ivic L and Kriegstein AR (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci.* 7:136-44.

Nomura T, Ohtaka-Maruyama C, Yamashita W, Wakamatsu Y, Murakami Y, Calegari F, Suzuki K, Gotoh H, Ono K. (2016). The evolution of basal progenitors in the developing non-mammalian brain. *Development (Cambridge, England),* 143:66–74.

Pedraza M, De Carlos JA (2012). A further analysis of olfactory cortex development. *Front Neuroanat* 6:35

Pombal MA, Alvarez-Otero R, Pérez-Fernández J, Solveira C, Megías M (2011). Development and organization of the lamprey telencephalon with special reference to the GABAergic system. *Front Neuroanat*. 5:20.

Pose-Méndez S, Candal E, Adrio F, Rodríguez-Moldes I (2014). Development of the cerebellar afferent system in the shark Scyliorhinus canicula: insights into the basal organization of precerebellar nuclei in gnathostomes. *J Comp Neurol* 522:131-68.

Pose-Méndez S, Candal E, Mazan S, Rodríguez-Moldes I (2016a). Genoarchitechture of the rostral hindbrain of a shark: basis for understanding the emergence of the cerebellum at the agnathan-gnatosthome transition. *Brain Struct Funct* 221:132135.

Pose-Méndez S, Candal E, Mazan S, Rodríguez-Moldes I (2016b). Mophogenesis of the cerebellum and cerebellum-related structures in the shark Scyliorhinus canicula: insights on the ground pattern of the cerebellar ontogeny. *Brain Struct Funct* 221:1691-717.

Pose-Méndez S, Rodríguez-Moldes I, Candal E, Mazan S, Anadón R (2017). A developmental study of the cerebellar nucleus in the catshark, a basal gnathostome. *Brain Behav Evol* DOI: 10.1159/000453654.

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E (2014). Developmental, tract-tracing and immunohistochemical study of the peripheral olfactory system in a basal vertebrate: insights on Pax6 neurons migrating along the olfactory nerve. *Brain Struct Funct* 219:85-104.

Quintana-Urzainqui I, Rodríguez-Moldes I, Mazan S and Candal E (2015). Tangential migratory pathways of subpallial origin in the embryonic telencephalon of sharks: evolutionary implications. *Brain Struct. Funct* 220:2905-26.

Rakic P (2009). Evolution of the neocortex: a perspective from developmental biology. *Nat. Rev. Neurosci*. 10:724–735.

Rash BG, Tomasi S, Lim HD, Suh CY, Vaccarino FM (2013). Cortical gyrification induced by fibroblast growth factor 2 in the mouse brain. *J Neurosci* 45(3):306-12.

Reillo I, de Juan Romero C, García-Cabezas MÁ, Borrell V (2011). A role for intermediate radial glia in the tangential expansion of the mammalian cerebral cortex. *Cereb Cortex* 21:1674-94.

Rodríguez-Moldes I (2009). A developmental approach to forebrain organization in elasmobranchs: new perspectives on the regionalization of the telencephalon. *Brain Behav Evol.* 74:20-9.

Rodríguez-Moldes I, Ferreiro-Galve S, Carrera I, Sueiro C, Candal E, Mazan S, Anadón R (2008). Development of the cerebellar body in sharks: spatiotemporal relations of Pax6 expression, cell proliferation and differentiation. Neurosci Letters 432:105-110.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Rodríguez-Moldes I, Carrera I, Pose-Méndez S, Quintana-Urzainqui I, Candal E, Anadón R, Mazan S and Ferreiro-Galve S (2011). Regionalization of the shark hindbrain: a survey of an ancestral organization. *Front. Neuroanat.* 5:16.

Rodríguez-Moldes I, Santos-Durán GN, Pose-Méndez S, Quintana-Urzainqui I, Candal E (2017). The Brains of Cartilaginous Fishes. In: Kaas J (ed.), Evolution of Nervous Systems 2e. vol. 1, pp. 77–97. Oxford: Elsevier. ISBN: 9780128040423.

Roybon L, Deierborg T, Brundin P and Li JY (2009). Involvement of Ngn2, Tbr and NeuroD proteins during postnatal olfactory bulb neurogenesis. *Eur J Neurosci*. 29:232-43.

Sánchez-Farías N, Candal E (2015). Doublecortin is widely expressed in the developing and adult retina of sharks. *Exp Eye Res* 134:90-100.

Sánchez-Farías N, Candal E (2016). Identification of Radial Glia Progenitors in the Developing and Adult Retina of Sharks. *Front Neuroanat* 10:65.

Santos-Durán GN, Menuet A, Lagadec R, Mayeur H, Ferreiro-Galve S, Mazan S, Rodríguez-Moldes I, Candal E (2015). Prosomeric organization of the hypothalamus in an elasmobranch, the catshark S*cyliorhinus canicula*. *Front Neuroanat* 9:37.

Santos-Durán GN, Ferreiro-Galve S, Menuet A, Quintana-Urzainqui I, Mazan S, Rodríguez-Moldes I, Candal E (2016). The Shark Alar Hypothalamus: Molecular Characterization of Prosomeric Subdivisions and Evolutionary Trends. *Front Neuroanat* 10:113.

Sauer FC (1935). Mitosis in the neural tube. *J Comp Neurol* 62:377-405.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Shimozaki K (2014). Sox2 transcription network acts as a molecular switch to regulate properties of neural stem cells. *World J Stem Cells*. 6:485-90.

Smart IH, Dehay C, Giroud P, Berland M, Kennedy H (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb. Cortex* 12, 37–53.

Stiedter GF (2015). Neurobiology: A Functional Approach. Oxford and New York: Oxford University Press.

Striedter GF, Keefer BP (2000). Cell migration and aggregation in the developing telencephalon: pulse-labeling chick embryos with bromodeoxyuridine. *J Neurosci* 20:8021-30

Striedter GF, Charvet CJ (2008). Developmental origins of species differences in telencephalon and tectum size: morphometric comparisons between a parakeet (Melopsittacus undulatus) and a quail (Colinus virgianus). *J Comp Neurol.* 507:1663-75

Striedter GF, Charvet CJ (2009). Telencephalon enlargement by the convergent evolution of expanded subventricular zones. *Biol Lett*. 5:134-7.

Tissir F, Lambert De Rouvroit C, Sire JY, Meyer G, Goffinet AM (2003). Reelin expression during embryonic brain development in Crocodylus niloticus. *J Comp Neurol* 457:250-62.

Venkatesh B, Lee AP, Ravi V, Maurya AK, Michelle M, Swann JB, Ohta Y, Flajnik MF, Sutoh Y, Hoon S, Gangu V, Roy SW, Irimia M. (2014) doi: 10.1038/nature12826.Elephant.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Wasowicz M, Ward R, Repérant J (1999). An investigation of astroglial morphology in torpedo and scyliorhinus. *J Neurocytol*. 28:639-53.

Wicht H, Derouiche A, Korf HW (1994). An immunocytochemical investigation of glial morphology in the Pacific hagfish: radial and astrocyte-like glia have the same phylogenetic age. *J Neurocytol* 23:565-76.

Wulliman MF (2009). Secondary neurogenesis and telencephalic organization in zebrafish and mice: a brief review. *Integr Zool* 4:123-33.

Wulliman MF, Puelles L (1999). Postembryonic neural proliferation in the zebrafish forebrain and its relationship to prosomeric domains. *Anat Embryo* 329:329-348.

Wullimann MF, Knipp S (2000). Proliferation pattern changes in the zebrafish brain from embryonic thorugh early postembryonic stages. *Anat Embryol* 202:385-400.

Wullimann MF, Rink E (2001). Detailed immunohistology of Pax6 protein and tyrosine hydroxylase in the early zebrafish brain suggests role of Pax6 gene in development of dopaminergic diencephalic neurons. *Dev Brain Res* 131:173-191.

Wullimann MF, Rink E (2002). The teleostean forebrain: A comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. *Brain Res Bull* 57:363-370.

Yopak KE, Lisney TJ, Collin SP (2015). Not all sharks are "swimming noses": variation in olfactory bulb size in cartilaginous fishes. *Brain Struct Funct.* 220:1127-43.

Zhang S, Cui W (2014). Sox2, a key factor in the regulation of pluripotency and neural differentiation. *World J Stem Cells* 6:305-11.

Zecevic N, Chen Y, Filipovic R (2005). Contributions of cortical subventricular zone to the development of the human cerebral cortex. *J Comp Neurol* 491:109-22.

Zerjatke T, Gak IA, Kirova D, Fuhrmann M, Daniel K, Gonciarz M, Müller D, Glauche I, Mansfeld J (2017). Quantitative Cell Cycle Analysis Based on an Endogenous All-in-One Reporter for Cell Tracking and Classification. *Cell Rep.* 19:1953-1966.

Table 1. Primary and secondary antibodies used.

Table 1. **Primary and secondary antibodies used.**

Primary Antibody	Source	Working dilution	**Secondary Antibody**	Source	Working dilution
GFAP	Policlonal rabbit anti-GFAP Dako (Cat. nº Z0334)	1:500	**Goat anti-rabbit HRP coupled**	Dako, Glostrup, Denmark	1:200
PCNA	Monoclonal mouse anti-PCNA Sigma (Cat. nº P8825)	1:500	**Goat anti-mouse** **HRP coupled**	Dako, Glostrup, Denmark	1:200
Pax6	Policlonal rabbit anti-Pax6 Covance (Cat nº PRB-278P)	1:300	**488-cojugated donkey anti-rabbit**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
DCX	Policlonal rabbit anti-DCX Cell Signalling (Cat. nº 4604)	1:300	**488-cojugated donkey anti-mouse**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
PH3	Policlonl rabbit anti-PH3 Bio Technology (Cat. nº 06-570)	1:400	**546-conjugated donkey anti-rabbit**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
Tbr1	Policlonal rabbit anti-Tbr1 Chemicon (Cat. nº AB9616) Millipore (Cat. nº AB10554)	1:200	**546-conjugated donkey anti-mouse**	Alexa Fluor Molecular Probes, Eugene, OR	1:200

Fig. 1 Photomicrographs of transverse sections of the telencephalon of S29 of development showing the expression pattern of different progenitor cell markers. **(A)** Sagittal and transversal schemas showing gross anatomy of a S29 embryo. **(B-B')** Transversal sections showing the expression of *ScSox2* in the telencephalic wall. B' is a magnification of the region squared in B. Black arrowheads point to cells at marginal positions that do not express *ScSox2*. **(C)** High magnification of the pallium showing PCNA immunoreactivity. Black arrowheads at marginal positions point to PCNA negative cells. Note the existence of two different morphologies of cells: round (yellow arrows) and oval (white arrows). **(D)** High magnification of the pallium showing radial processes stained with GFAP that reach the pial surface forming endfeet structures (arrows). **(E-E')** Double immunofluorescence for PCNA and GFAP in the pallium of *S. canicula* showing some double labelled cells (asterisks). **(F)** Panoramic view of the telencephalon of a S29 embryo showing immunoreactivity for Pax6 restricted to the pallial domain. **(G)** Double immunofluorescence for Pax6 and PCNA in the pallium of a S29 embryo showing double labelled cells. Scale bars 500µm (B, F); 50 µm (B', C, D, E); 25 µm (E', G).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

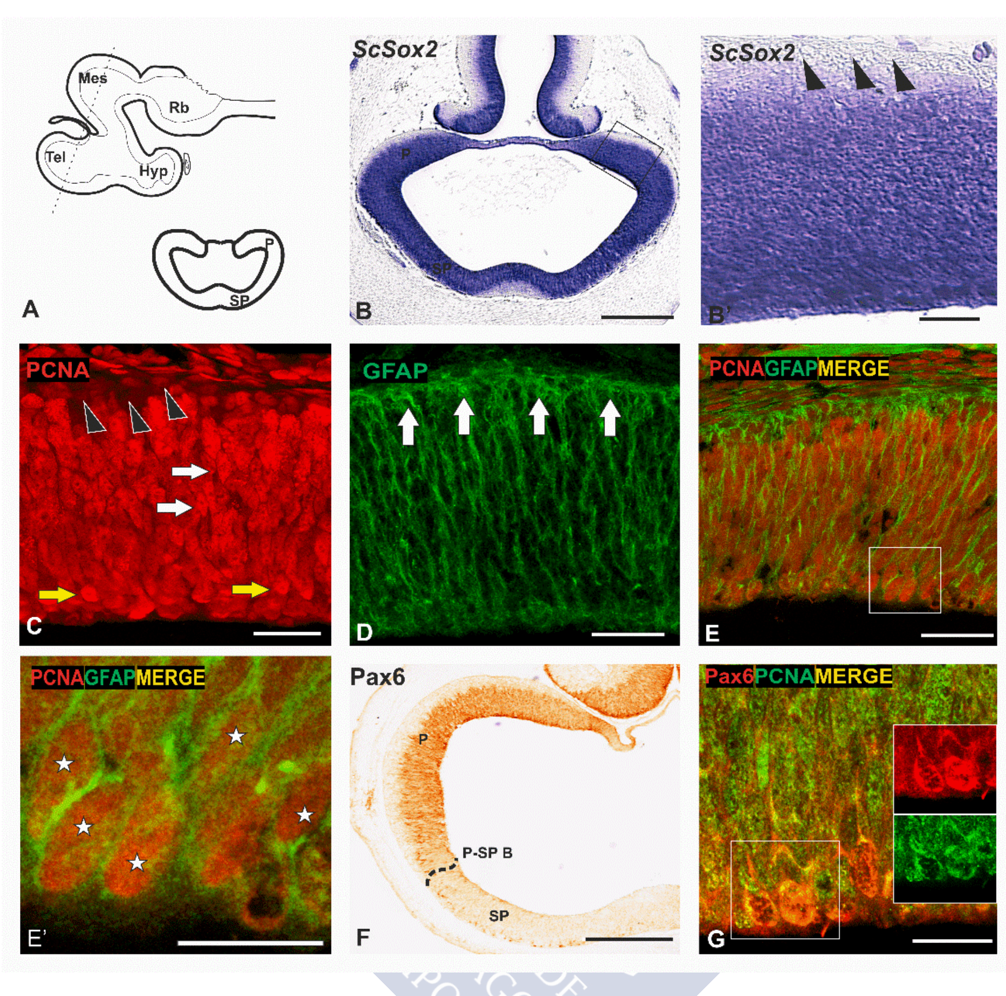
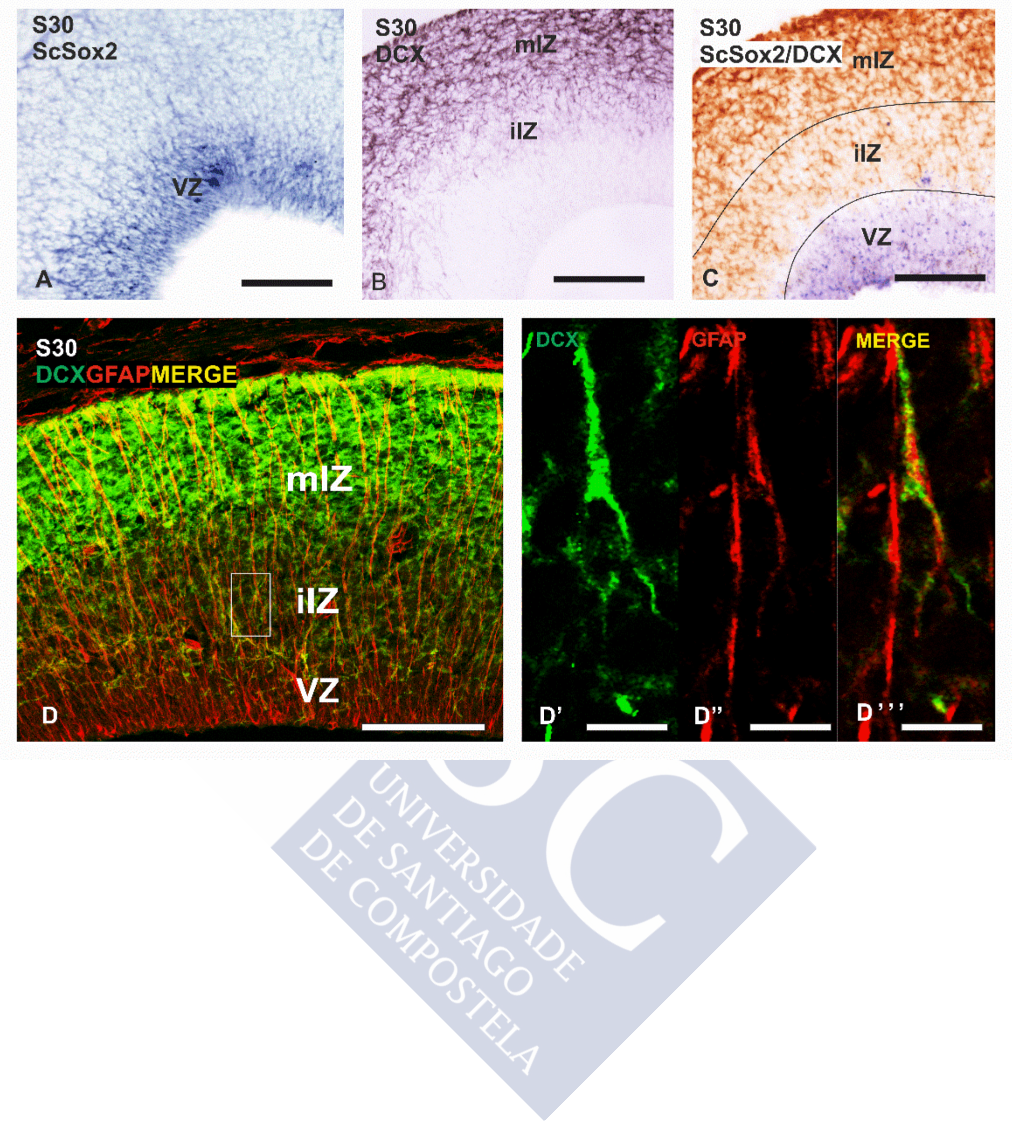


FIGURE 1

Fig. 2 Transversal sections of S30 and S31 embryos showing different neurogenic markers. **(A-C)** Transverse sections showing the differential expression of *ScSox2* and DCX in three different layers. **(D-D''')** Double immunofluorescence for DCX and GFAP showing DCX immunoreactive cells attached to GFAP positive cell processes in the IZ. Scale bars 200 µm (A-C), 100 µm (D), 10 µm (D', D'', D''').

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

FIGURE 2

Fig. 3 Transverse sections showing the expression pattern of SVZ markers in S30 and S31. **(A)** Transverse section of a S30 embryo showing PCNA immunoreactivity in the telencephalon. Note the presence of an intermediate band of intense PCNA-immunoreactivity (arrows and box). **(B)** Transverse section of a S30 embryo showing PH3 immunoreactivity in the telencephalon. **(C-E)** Transverse section at different magnification showing the expression pattern of *ScTbr2* **(C-C'')** and colocalization between *ScTbr2* and PH3 **(D-E).** **(F-H)** Transverse sections of the pallium of S31 embryos showing positivity for PH3, *ScTbr2* and double labelled cells for both markers defining the existence of a presumptive SVZ. Scale bars 200 µm (A, B, C, F, G, H), 100 µm (C'), 50 µm (C'', D, E).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

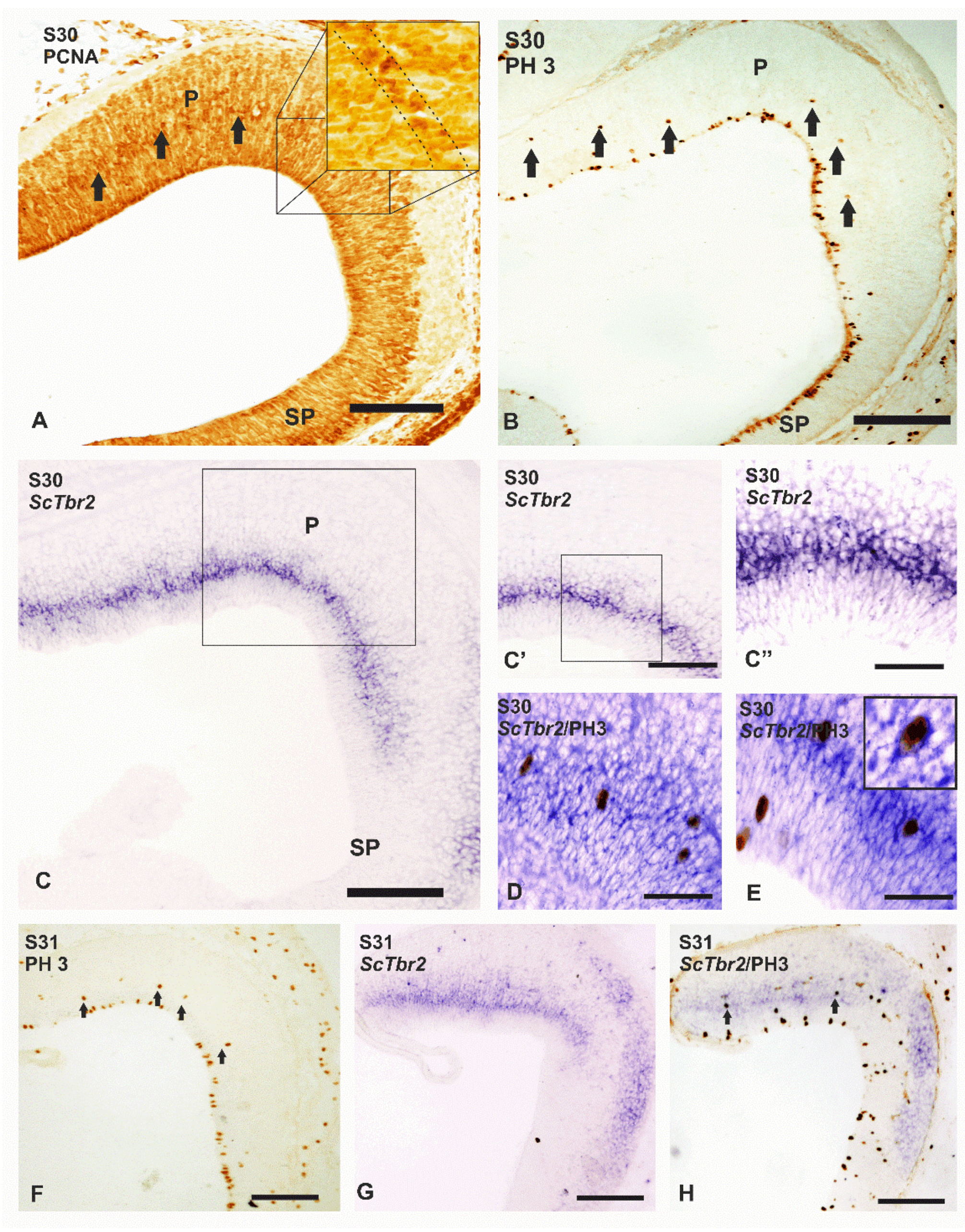


FIGURE 3

Fig. 4 Schema and transverse sections showing BrdU incorporations combined with PH3 in the pallium of *S. canicula*. **(A)** BrdU was administrated to embryos at S30 of development. Embryos were sacrificed at 24 h (S30) and 10 days (S31) after BrdU incorporation. **(B-C'')** Transverse sections at different magnifications showing double BrdU/PH3 labelling 24 h after BrdU administration. Double BrdU/PH3 positive cells can be observed in subventricular positions (arrowheads). **(D-D'')** Transverse section of a S31 of development 10 days after BrdU administration showing double immunolabeled cells for BrdU and PH3 in subventricular positions (arrowheads). Scale bars: 100 µm (D, D', D''), 50 µm (B, B', B'', C, C', C'').

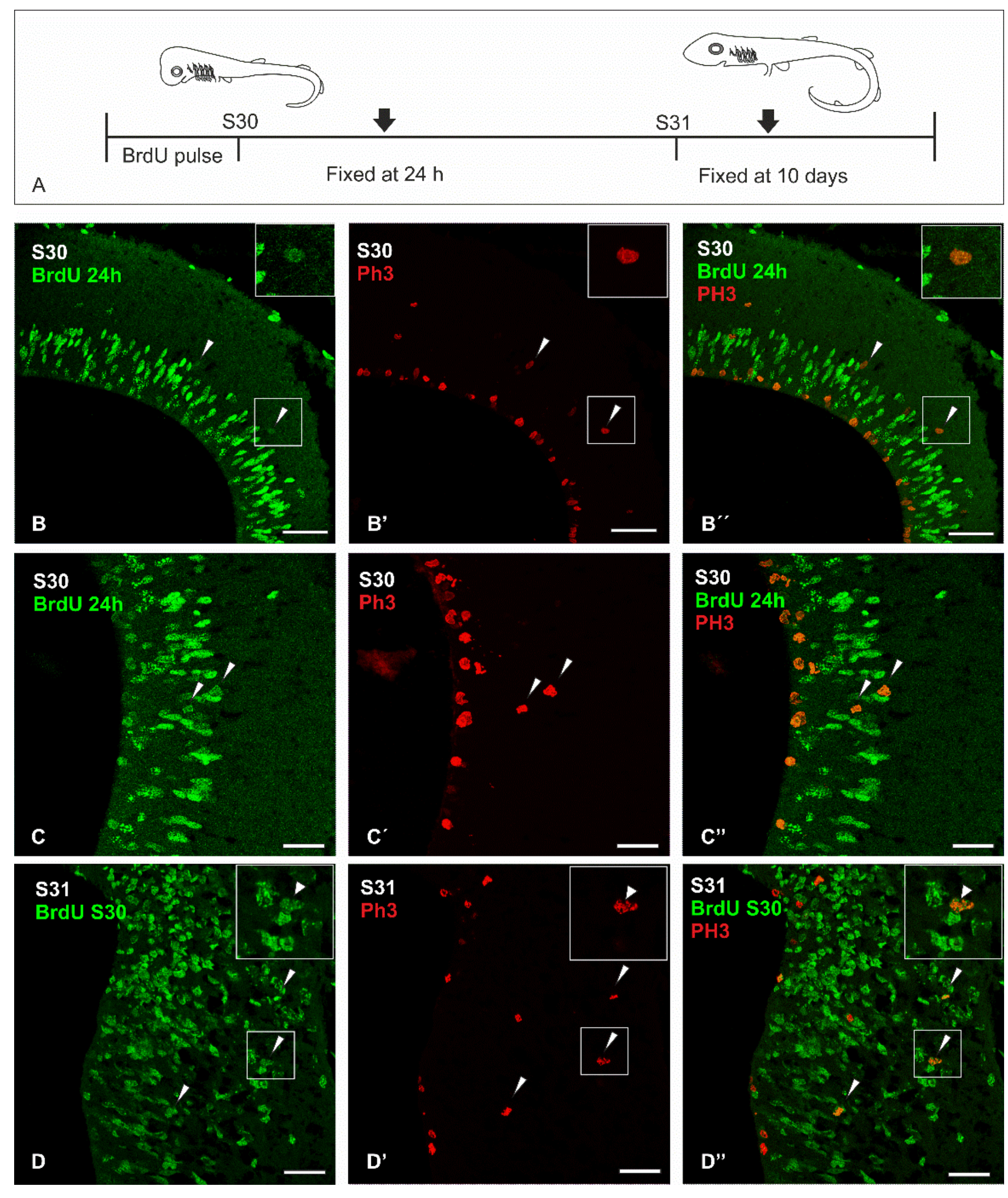


FIGURE 4

Fig. 5 Transverse sections of S31 and S32 embryos showing the expression pattern of the neurogenic markers Pax6, *ScTrb2*, *ScNeuroD* and Tbr1 in the telencephalon of *S. canicula*. **(A)** At S31, Pax6 immunoreactivity was detected in many disperse cells in the IZ of the dorsal pallium and in an intense band of cells at the VZ of the pallium. *ScTbr2* is expressed in subventricular positions of the pallium **(B)**. *ScNeuroD* **(C)** and Tbr1 **(D)** were not detected in the VZ but also in the IZ. **(E)** Photomicrograph of the telencephalon of a S32 showing barely no expression of Pax6 in the VZ (arrows). In addition, *ScTbr2* **(F)**, *ScNeuroD* **(G)** and Tbr1 **(H)** can be observed in more marginal positions of the pallium respect to the previous stage. **(I)** Photomicrograph of the rostral telencephalon showing *ScNeuroD-expression* and Tbr1-immunoreactivity. **(J-J''')** Photomicrograph of the rostral telencephalon showing *ScTbr2-expression* and Tbr1-immunoreactivity, showing double labelled cells **(J')** and single labelled cells for *ScTbr2* **(J'')** and for Tbr1 **(J''')**. Scale bars 200 µm (A-H), 100 µm (I, J).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

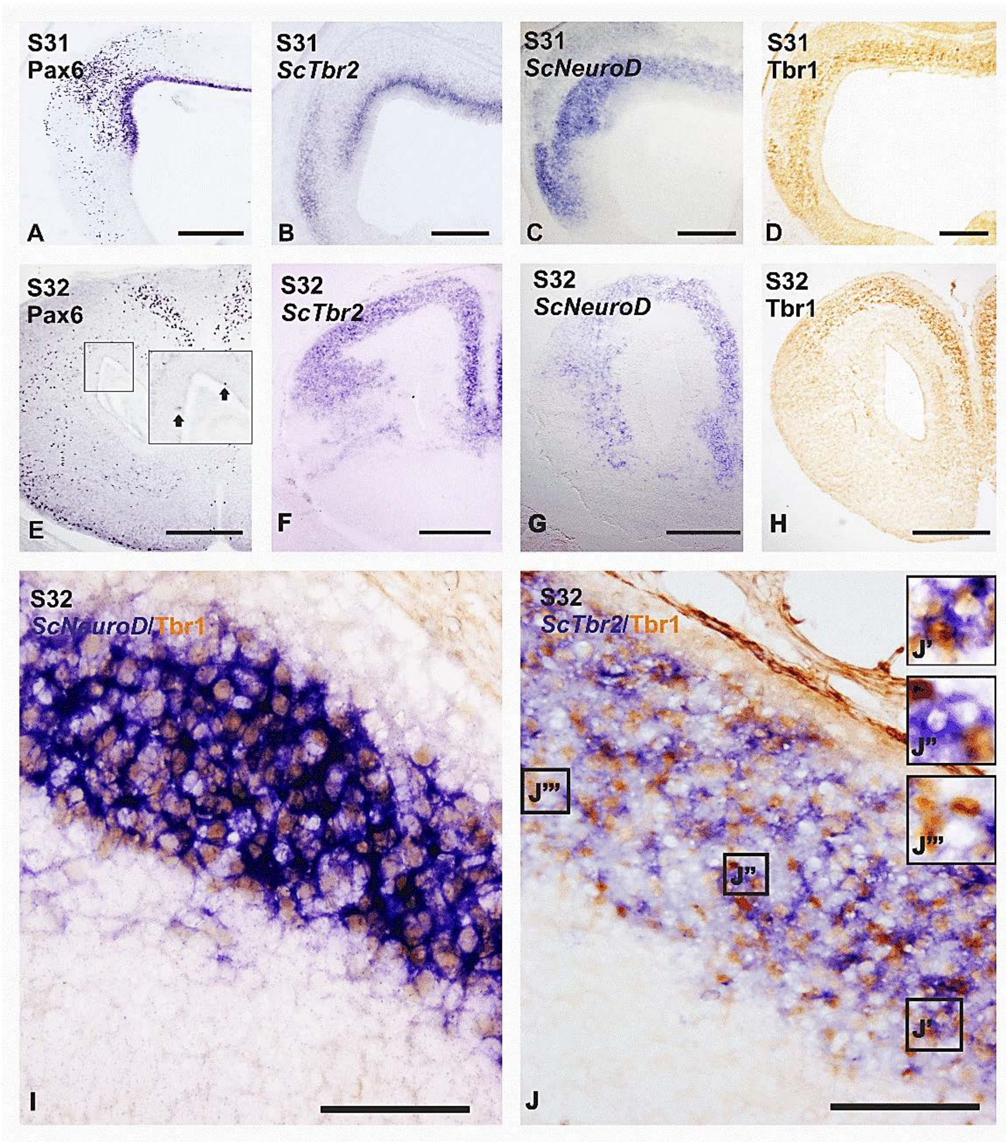


FIGURE 5

Fig. 6 Summary of published data about the existence of a SVZ in the dorsal pallia subdivision along the scale of vertebrates

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

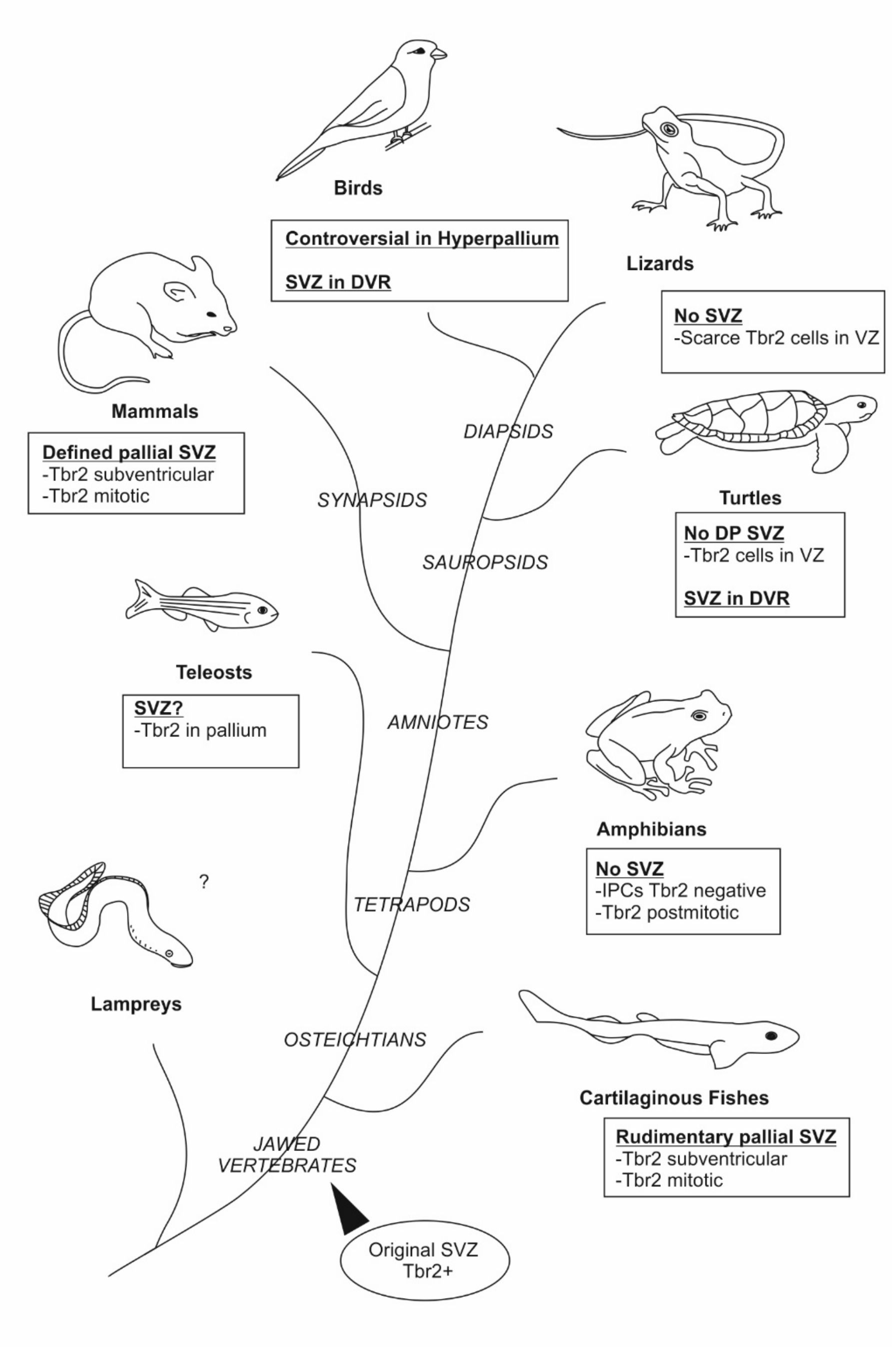
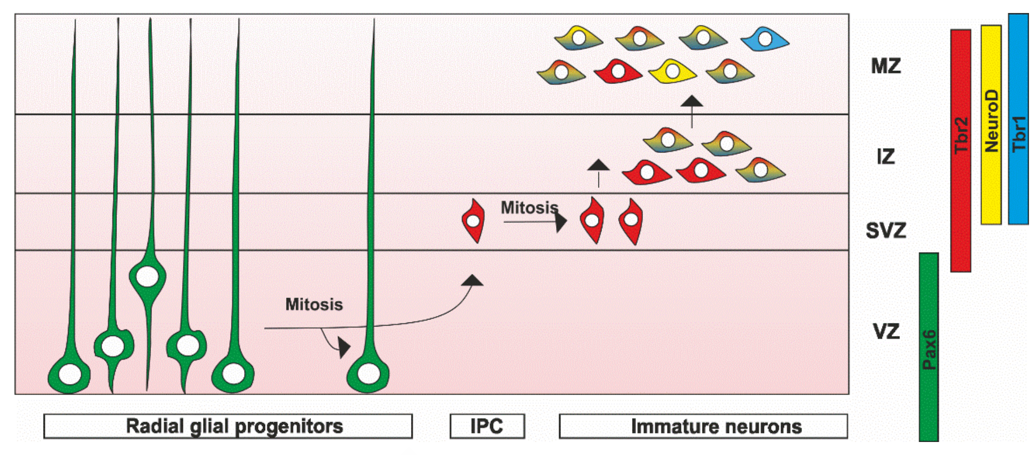


FIGURE 6

Fig. 7 Schema of the development of pallial born neurons in the developing pallium of *S. canicula*.



USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

FIGURE 7

CHAPTER 2

EXPRESSION OF RADIAL GLIA MARKERS (GFAP, BLBP AND GS) DURING TELENCEPHALIC DEVELOPMENT IN THE CATSHARK (*Scyliorhinus canicula*).

Docampo-Seara A, Santos-Duran GN, Candal E, Rodríguez MA (2018). Expression of radial glial markers (GFAP, BLBP and GS) during telencephalic development in the catshark (*Scyliorhinus canicula*). *Brain Structure and Function.* **Pending on second revision**.

Introduction

The telencephalon is one of the most studied areas in the entire central nervous system (CNS) since it develops into various structures that are responsible of complex functions such as cognition, memory or development of social skills. Neurodegenerative diseases affecting these areas have become a concern, since the mechanisms for neuronal loss are intricate and effective treatments for these diseases do not exist. In the last decades, the identification of different types of neural stem cells in the brain together with the comprehension of how they produce the impressive diversity of brain cells has become crucial for developing cellular therapies for brain repair (Kriegstein and Álvarez-Buylla, 2009).

During development, neuroepithelial cells (NECs) are the primary neural stem cells in the CNS (see Falk and Götz, 2017 and references therein). In the embryonic mouse forebrain, with the onset of neurogenesis (E9-E10), NECs present in the neural tube give rise to radial glial cells (RGCs), the first cell type of glial nature arising in the developing central nervous system (for review see: Götz and Huttner, 2005; Alvarez-Buylla and Kriesgstein, 2013; Götz, 2013; Turrero and Harwell, 2017). These cells were first described in the 19th century in the embryonic spinal cord and cerebral cortex by Camillo Golgi and Giuseppe Magini, respectively (for review see: Bentivoglio and Mazzarelo, 1999; Kriegstein and Götz, 2003; Farkas and Huttner, 2008; Götz, 2013). They are characterized by a bipolar morphology with a ventricular contacting body and a radial process ending in a subpial endfoot. Ultrastructural studies during corticogenesis carried out by Rakic (1972) showed that radial processes of RGCs act as a scaffold for migration of new-born neurons to upper cortical layers. Later studies have shown that RGCs are involved in the correct patterning of the central nervous system (for review see: Götz, 2013).

Importantly, numerous studies on the developing mammalian telencephalon have additionally shown that RGCs also act as neural stem cells since they are the origin of all neurons and the different subtypes of differentiated glial cells (astrocytes, oligodendrocytes and ependymal cells), either directly or through other neural progenitor cell types subsequently derived from them (Götz et al. 1998; Marshall et al. 2003; Spassky et al. 2005; Alvarez-Buylla and Kriesgstein, 2013; Götz, 2013; Taverna et al. 2014; Beattie and Hippenmeyer, 2017; Turrero and Harwell, 2017). RGCs also act as progenitor cells in the developing telencephalon of non-mammalian vertebrate groups including reptiles (Clinton et al. 2014; Martínez-Cerdeño et al. 2016) amphibians (D´Amico et al. 2011; Moreno and González, 2017) and teleosts (Lyons et al. 2003, Alexandre et al. 2010).

In mammals, the vast majority of RGC disappear in postnatal stages, except for a few locations such as the lateral telencephalic ventricles, hypothalamus and cerebellum where radial glia persists throughout life (Malatesta et al. 2008; Götz, 2013). In non-mammalian vertebrates RGC that act as progenitors are also persistent in some areas of the brain. Proliferating RGC have been detected in the telencephalon of birds (Alvarez-Buylla et al. 1990, 1998), reptiles (reviewed by González-Granero, 2011, Clinton et al. 2014), amphibians (Kirkham et al. 2014) and teleost fishes (März et al. 2010). RGC (named ependymoglia) has been studied in the brain of lampreys (Merrick et al. 1995). However, no proliferation has been detected in the adult telencephalon (Villar-Cheda et al. 2006)

The term glial cell can therefore result puzzling to some extent since it includes both this progenitor population as well as a differentiated population of parenchymal astrocytes, oligodendrocytes, and ependymal cells, with which it shares some properties (Kriegstein and Alvarez-Buylla, 2009). In this context,

various markers have been commonly used to identify RGCs, including the brain lipid binding protein (BLBP), the intermediate filament glial fibrillary acidic protein (GFAP), and the enzyme glutamine synthase (GS). BLBP is a protein involved in lipid metabolism and membrane synthesis. Its expression has been related with the neurogenic and/or gliogenic potential of radial glia (Hartfuss et al. 2001; Pinto and Götz, 2007; Podgornyi and Aleksandrova, 2009; Marz et al. 2010; Li et al. 2011; Diotel et al. 2016). Some studies have referred BLBP expression in the embryonic and adult brain in non-mammalian vertebrates. This protein has been detected in RGCs in the telencephalon of amphibian embryos (D´Amico et al. 2011; Moreno and González, 2017) and adult zebrafish (Grupp et al. 2010; März et al. 2010; Diotel et al. 2016). However, BLBP does not allow to clearly distinguish RGCs from NECs since it is expressed by both cell types (Pinto and Götz, 2007; Götz, 2013; Than-Trong and Bally-Cuif, 2015; Diotel et al. 2016). On the other hand, in mammals, glial markers like GFAP and GS are expressed in RGCs but not in NECs, allowing distinguishing between these types of neural stem cells. However, these markers are also expressed by differentiated glia including astroglia and ependymal cells (for review see: Götz, 2013).

In summary, in mammals and other non-mammalian vertebrates, different types of progenitor cells express similar markers that are additionally shared with several types of differentiated glial cells (Thang-Trong and Bally-Cuif, 2015). Therefore, a good comprehension of the emergence/development of cells with hallmarks of radial glial and a deep characterization of these cells is necessary. Cartilaginous fishes occupy a key phylogenetic position in the tree of jawed vertebrates as the sister group of bony vertebrates, which include bony fishes and tetrapods (Coolen et al. 2009). Our aim is to investigate and characterize progenitor RGCs in the telencephalon of the lesser spotted dogfish (catshark) *Scyliorhinus canicula*, a model species of cartilaginous fishes for evo-

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

devo studies (Coolen et al. 2009; Rodríguez-Moldes et al. 2017). With this aim, we analysed the expression of three RGC molecular markers, GFAP, BLBP and GS in the telencephalic hemispheres in embryos and early juveniles using immunohistochemical and double immunofluorescence techniques. We additionally used double immunofluorescence techniques combining these glial markers with the proliferating cell nuclear antigen (PCNA) to evaluate the proliferative potential of different subtypes of RGCs.

Materials and Methods

Experimental animals

We used 15 embryos of *S. canicula* from stages 25 (S25) to 33 (S33) and 3 posthatching juveniles. Most embryos were provided by the Marine Biological Model Supply Service of the CNRS, UPMC Roscoff Biological Station (France) and some embryos and juveniles were kindly provided by the aquarium of O Grove (Galicia, Spain). Embryos were staged by their external features according to Ballard et al. (1993). Eggs were raised in seawater tanks under standard conditions of temperature (15-16ºC), pH (7.5-8.5) and salinity (35 g/L) and suitable measures were taken to minimize animal pain and discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS- 222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch's phosphate

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

buffer [EPB: 0.1M phosphate buffer (PB) containing 1.75% of urea, pH 7.4] for 48-72 h depending on the stage of development. Sharks from stage 32 (S32) onwards were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer´s solution (see Ferreiro-Galve et al. 2012) followed by 4% PFA in EPB. Brains were removed and postfixed in the same fixative for 24-48 h at 4ºC. Subsequently, they were rinsed in PB saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (16-18µm thick) were obtained in transverse or sagittal planes on a cryostat and mounted on to Superfrost Plus (Menzel-Glasser, Madison, WI, USA) slides.

Immunohistochemistry

Sections were pre-treated with 0.01M citrate buffer pH 6.0 for 30 min at 90 ºC for heat-induced epitope retrieval and allowed to cool for 15 min at room temperature (RT). Sections were rinsed in 0.05M Tris-buffered saline (TBS; pH 7.4) for 5 min and treated with 10% H_2O_2 in TBS for 30 min at RT to block endogenous peroxidase activity. Sections were rinsed in 0.05 M TBS pH 7.4 for 5 min, and incubated approximately for 15 h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate HRP-coupled secondary antibody (see Table 1) for 1 h at RT. All dilutions were made with TBS containing 15% normal goat serum (Millipore, Billerica, MA) 0.2% Triton X-100 (Sigma) and 2% bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Then, sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each. The immunoreaction was developed with 0.25 mg/ml diaminobenzidine tetrahydrochloride (DAB, Sigma) in TBS pH 7.4 and 0.00075% H_2O_2, or with SIGMAFAST™ 3.3-DAB tablets as indicated by the

manufacturer. To enhance the GFAP immunoreaction in sections of early developmental stages, 2.5 mg/ml nickel ammonium sulphate was added. Finally, the sections were dehydrated and coverslipped. Additional information about the primary and secondary antibodies is included in Table 1.

Control and specificity of antibodies

The monoclonal antibody against the proliferating cell nuclear antigen (PCNA) has been previously used in our lab to label progenitor cells in the brain, retina and olfactory system of the catshark or lesser spotted dogfish (Quintana-Urzainqui et al. 2014; Quintana-Urzainqui et al. 2015; Sánchez-Farías and Candal 2015, among others). PCNA is present in proliferating cells along the entire cell cycle, though its expression is stronger during the S phase (Zerjatke et al. 2017). The anti-PH3 antibody has been used previously as a marker of mitotic cells in the telencephalon of *S. canicula* (Quintana-Urzainqui et al. 2015). The polyclonal antibody against GFAP has been previously used as marker of glial cells in the brain and retina of *S. canicula* (Quintana-Urzainqui et al. 2014; Quintana-Urzainqui et al. 2015; Sánchez-Farías and Candal, 2016). The monoclonal antibody against GS has been previously used as a marker of mature Müller cells in the retina of *S. canicula* (Bejarano-Escobar et al. 2012; Sánchez-Farías and Candal, 2016). On the other hand, the BLBP antibody was never characterized in sharks. The specificity of all antibodies against glial markers used in this work was tested by Western Blot analysis of brain protein extracts of adult catshark by using standard procedures (for further information of methods see Anadón et al. 2000). ProSieve proteins standards (Lonza, Rockland, ME) were used as molecular weight markers (Fig. 1 lane 1). As positive controls, protein extracts of adult mouse brain were run in parallel to assess the specificity of antibodies.

According to the manufacturer, the GFAP antibody recognizes a 50kDa intracytoplasmic filamentous protein characteristic of astrocytes. In blots of shark protein extracts, it recognizes a single band around 49 kDa (Fig. 1 lane 2). With regard to BLBP, according to the manufacturer the antibody labels a band about 15kDa in western blot analysis in mouse brain protein extracts. In the present blot, a band about 15 kDa can be recognizable (Fig. 1 lane 3). In the case of GS, in mouse brain extracts of proteins, a band of approximately 44 kDa can be detected by western blot. In this blot a band close to 49 kDa can be observed.

Image acquisition and analysis

Light field images were obtained with an Olympus BX51 microscope equipped with an Olympus DP71 colour digital camera (Olympus, Tokyo, Japan). Fluorescent sections were analysed and photographed with a spectral laser scanning microscope (TCS-SP2, Leica, Wetzlar, Germany) using a combination of blue and green excitation lasers. Stacks of confocal images were acquired separately for each laser channel with steps of 0.8 µm or 2 µm along the z-axis. Z-projections of an average of 12 optical sections were done with LITE software (Leica). Photographs were adjusted for brightness and contrast, and plates were prepared using Corel Draw X3.

Cell counting

Cell counting was performed attending to immunoreactivity both in cell bodies and processes. In order to estimate the amount of single and double-labelled cells for glial markers (GFAP/GS and BLBP/GS), cells were counted in two regions of the pallium (dorsomedial and presumptive ventral pallium) and in the subpallium. For quantification, confocal images from central stacks of 3-4 µm were taken and cells from areas of 50x50 µm of the ventricle were counted manually. One area in each region was counted in two different sections

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

from two different S32/33 embryos (4 area/region/experiment). Average, standard deviations and bar representations and the proportion of each cell type were determined using Microsoft Excel 2016. For further information about numbers and graphics see Supp. Fig. 1.

Results

In this study, we investigated the expression pattern of GFAP, BLBP and GS in a series of catshark embryos representative of the early (S25 to S26), middle (S27 to S31) and late (S32 to S34) periods of telencephalic development (Ballard et al. 1993). In the telencephalon, the middle period corresponds with early (S28) to middle (S31) stages of neurogenesis. In S29 embryos, the telencephalic walls mainly consist of a pseudostratified neuroepithelium. Pallial and subpallial regions are already recognizable but no further subdivisions or differences along the rostro-caudal axis of the telencephalon are appreciable. By S31, the zonation of the telencephalic walls into ventricular (neuroepithelial), intermediate and marginal zones (from the ventricle to the meningeal surface) is already recognizable and the major pallial regions are observed. From S32 onwards, important morphological changes take place and the basic mature structure of the telencephalon is progressively achieved. The cytoarchitectonic organization of the telencephalon during this late period is highly similar to that observed in juvenile and adults. The mature telencephalon of *S. canicula* consists of the telencephalic hemispheres and the olfactory bulb. According to the classical view (Smeets et al. 1983) the telencephalic hemispheres can be subdivided in an evaginated portion (rostral and medial telencephalon) and in a non evaginated portion (caudal telencephalon). For further information of the development of telencephalon of dogfish see Quintana-Urzainqui et al. (2015) and Rodríguez-Moldes et al. (2017).

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The aims of the present study are to investigate the development of the radial glial system in basal vertebrates and examine the proliferative potential of these cells. The expression pattern of the three glial markers (GFAP, BLBP and GS) and the coexpression study is described below. Schematic drawings about the distribution of GFAP, BLBP and GS expressing cells in the catshark telencephalon during embryonic development and in juveniles are presented in Figures 2 and 3, respectively.

Expression pattern of GFAP in the telencephalic hemispheres of embryonic stages and juveniles.

At S25, the telencephalic walls consist of a pseudostratified neuroepithelium that presents numerous cells that show weak GFAP immunolabeling in the cytoplasm. At this stage, a band of intense GFAP-immunoreactive processes is observed subpially (Fig. 4A).

At stage S29, GFAP immunoreactivity is present in numerous cells distributed from rostral to caudal regions of the telencephalic hemispheres (Fig. 2, 4B). GFAP immunoreactivity is more intense in the medial regions (Figs. 4C, D) than in lateral regions of the telencephalon (Fig. 4E). Numerous cells show GFAP immunoreactive processes running radially, with the proximal regions near the ventricular surface and the apical region enlarging subpially (resembling the radial glia of mammals). The shape of cells is ill-defined with this glial marker, and only in some cells the nucleus appears outlined by GFAP immunoreactivity. At these stages an intense immunoreactivity has also been detected in epithelial cells of the choroid plexus (Fig. 2F).

In the telencephalon of S30 and S31 embryos, the walls become thickened with the appearance of a thick mantle formed of neurons, whereas the ventricular zone is formed by one or two rows of radial glial cells that show

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

GFAP immunoreactivity around the cell nucleus (Fig. 4G, G'). Numerous GFAP-immunoreactive radial processes originated from these cells are arranged as a palisade, crossing the mantle zone and extending to the pial surface (Fig. 2A´ at S31; Fig. 4G), where their hollow conical endfeet show an intense GFAP immunoreactivity (Fig. 4G). The thickness of the telencephalic wall and the length of radial glial processes vary among regions. In the rostral telencephalon, no differences in the intensity of immunoreactivity have been detected between medial and lateral territories. Caudally, a more intense immunoreactivity can be visualized in medial telencephalic territories than in lateral regions. Scarce GFAP-immunoreactive cell bodies are also observed alone or in small groups in the mantle zone. In the subpallium, GFAP-immunoreactive processes converge toward the midline in the medial ganglionic eminence homologue (Fig. 2B´, C´ at S31; Fig. 4H). Moreover, in this region, radial processes show a more complex, branched appearance in the marginal layer, which is in contrast with that observed in more immature pallial regions. Like in the previous embryonic stages, epithelial cells of the choroid plexus show intense GFAP immunoreactivity (asterisk in Fig. 4G). At these stages, GFAP-immunoreactive endfeet-like structures have also been observed around blood vessels.

In S32 embryos, the number of rows of GFAP-immunoreactive cells lining the lateral telencephalic ventricles increases and a few immunoreactive cells are observed in the mantle zone (Fig. 2A´-D´at S32). In the dorsal telencephalic walls, faint GFAP-immunoreactive radial processes were appreciable in the rostral pallium. In the subpallium, numerous GFAP-immunoreactive processes converge toward the midline and show a more complex, branched appearance in the middle of this area, which also contains some interspersed intense GFAP-immunoreactive cells (Fig. 2A´, B´ at S32; Fig. 4I). More caudally, in the pallium, arched GFAP-immunoreactive processes form a curved palisade from the ventricular zone to the meningeal

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

surface (Fig. 2C´at S32; Fig. 4J) and numerous GFAP-immunoreactive cells are observed in the ventricular zone (Fig. 4J'). At this level, in the medial pallium, numerous GFAP-immunoreactive processes with an intensely stained endfeet are observed the pallial midline (Fig. 4K).

In lateral regions of the subpallium, no GFAP-immunoreactive processes are observed, although GFAP-positive subpial endfeet are present. In the caudal telencephalon, numerous GFAP-immunoreactive radial processes appear to course longitudinally owing to the tangential orientation of the section with regard to the dorsal pallium, although in the adjacent lateral pallium numerous radial processes are appreciable (Fig. 2D´at S32). In the caudal subpallium only scarce and weakly GFAP-immunoreactive glial processes were observed. In the telencephalon of S32 embryos, GFAP-positive endfeet are also observed around blood vessels.

In posthatching juveniles, the thickness of the walls of the telencephalic hemispheres increases with respect to the embryonic period while that of the ventricular zone decreases. We have observed GFAP-immunoreactive ependymal cells (or tanycytes, Hortsmann, 1954) with a stained radial process lining the ventricle, their number being higher in the pallium than in the subpallium (Fig. 3A´-D´; Figs. 4L, M). Numerous GFAP-immunoreactive subpial endfeet, as well as perivascular endfeet surrounding blood vessels are observed in the pallium and subpallium (Figs. 4N, O). The dorsal and medial pallium showed numerous GFAP-immunoreactive processes, while in the presumptive ventral pallium and the subpallium such processes were barely stained (Fig. 4L). As in embryos, numerous GFAP-immunoreactive processes converge in the midline of the telencephalon and also toward the external sulcus between the presumptive ventral pallium and the olfactory bulbs (Fig. 3C´). In caudal telencephalon, a few GFAP-immunoreactive cells with round perikarya

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

were scattered in the centromedial region of the dorsal pallium; these cells exhibit an immunoreactive process ending on blood vessels (Fig. 4P). Numerous GFAP-immunoreactive subpial endfeet are observed below the pial surface, and in the ventricular roof of the caudal telencephalic ventricle (Fig. 4Q). In addition, "longitudinal" (rostrocaudal) GFAP-immunoreactive processes are also observed in the most caudal pallium, like in embryos (Fig. 3D´), and numerous GFAP-immunoreactive endfeet establish contact with blood vessels.

Expression pattern of BLBP in the telencephalic hemispheres of embryos and juveniles.

BLBP immunolabeling is detected in the telencephalon of *S. canicula* from S29 embryos onwards. At S29, BLBP immunoreactivity is restricted to dorsolateral regions of the pallium and the presumptive homolog of the medial ganglionic eminence (MGE-h) of the subpallium (Fig. 2A´´ at S29; Figs. 5A, B), where numerous ventricular cells and cells away the ventricular surface are observed. BLBP immunoreactivity is more intense in cells near the ventricle, which frequently show a bipolar morphology (Fig. 5B'). Numerous BLBP-immunoreactive subpial endfeet are also observed. In addition, strong BLBP immunoreactivity is detected in epithelial cells of the choroid plexus adjacent to the walls of telencephalic hemispheres. At the S31 stage, BLBP immunoreactivity is localized in the same regions of the telencephalon than in S29 and S30, but the amount of cells and fibbers is higher and the intensity of staining was stronger. (Fig. 2A´´-C´´ of S31).

At the stage S32, strong BLBP immunoreactivity is observed in the whole ventricular border of the lateral ventricle at rostral and medial telencephalic levels (Fig. 2A´´-B´´ at S32). Immunoreactivity is observed in the apical region of cell bodies that line the ventricle and in radial processes that present dilatations away the ventricular zone, giving a dotted appearance to the

paraventricular region. These dilatations are not uniformly distributed, being mostly located in dorsal and lateral pallial regions, as well as in the ventricular zone of the subpallium (Figs. 5C, C´, C''). In the caudal subpallium, BLBP immunoreactivity is mostly observed in the ventricular zone of lateral subpallial regions (Fig. 2C´´ at S32; Fig. 5D). Groups of BLBP-positive cells are observed in the caudal part of the dorso-medial pallium (Fig. 2D´´ at S32). These round and large cells have an eccentric nucleus and some of them show a thick process (Fig. 5E). In the pallium and subpallium numerous BLBP-immunoreactive radial processes are observed emerging from the ventricular cells and coursing to the pial surface or converging in the telencephalic midline. Caudally, the number of immunoreactive processes coursing to the pial surface is higher in the pallium than in the subpallium (Fig. 2A´´-D´´ at S32). Numerous, strongly BLBP-immunoreactive subpial endfeet are observed in the telencephalon from rostral to caudal levels (Fig. 5F).

In juveniles, from rostral to caudal levels of the telencephalon, intensely BLBP-immunolabeled ependymocytes (or tanycytes) are observed along the surface of the lateral ventricles, which seem to be more numerous in the dorsal, lateral and ventral ventricular zones than in the medial ventricular zone (Fig. 3A´´-C´´; Fig. 5G). These cells show oval-shaped perikarya from which a basal BLBP-immunoreactive radial process extend, perpendicularly in some of them (Fig. 5G'-G''). In addition, groups of a few BLBP-immunoreactive cells are observed in the pial surface of the dorsolateral telencephalic region (Fig. 3B´´). These cells show a rounded cell body with a BLBP-immunoreactive process oriented perpendicularly to the pial surface (Fig. 5H). BLBP immunoreactivity decreases in the caudal telencephalon. At this level, BLBP-expressing ependymal cells (tanycytes) are observed in the subpallial ventricle, but not in the choroid plexus (Fig. 3D´´; Figs. 5I, I´). Numerous BLBP-immunoreactive subpial endfeet are observed, and weakly BLBP-immunoreactive radial glial

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

processes course from the ventricular zone to the pia forming subpial endfeet (Figs. 5I''). Also, BLBP-immunoreactive endfeet are observed on the surface of blood vessels.

Expression pattern of GS in the telencephalic hemispheres of embryonic stages and juveniles.

No GS immunolabelling is detected in the telencephalon before S32. At this stage, intense GS immunoreactivity is mainly observed in the ventricular pole of glial cells, in dilatations along radial processes near the ventricular zone and in subpial endfeet. Other regions of the cell body and the radial processes appear faintly stained. The pattern of GS immunoreactivity in the ventricular zone of S32 embryos is similar to the pattern of BLBP immunoreactivity observed at the same stage (Fig. 2A´´´-D´´´ at S32). The GS-immunoreactive dilatations of radial processes are more numerous in the subpallium than in the pallium, where less immunoreactive cells are present; differences in the number of immunoreactive cells between the dorsal, medial and lateral ventricular zones can also be appreciated (Figs. 6A-A'').

In pallium and subpallium of S32 embryos, some GS-immunoreactive cells are observed in adventricular regions (Fig. 2B´´´-D´´´ at S32; Figs. 6B, C). Numerous large and GS-immunoreactive cells are seen in the medial and dorsomedial pallium, these cells resembling those immunolabelled with BLBP (Fig. 6C); a few smaller GS-immunoreactive cells are observed in the medial subpallium near the ventricular zone (Fig. 6B). In the pallium, wavy GS-immunoreactive processes are observed coursing from the ventricle to the pial surface, where intensely immunoreactive endfeet are seen (Fig. 6D), whereas in the subpallium most of the positive processes are straight (Fig. 2C´´´ at S32). In transverse sections of the caudal telencephalon, radial processes appear to run longitudinally through the dorsal pallium (Fig. 2D´´´ at S32).

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The expression pattern of GS in juveniles was similar to that observed in embryos (Fig. 3A´´´-D´´´). Numerous GS-immunoreactive ependymal cells (or tanycytes) are seen, and GS- positive cells are also observed away the ependymal region. There are no evident differences between rostral and caudal telencephalic regions, but immunoreactive ependymal cells are more numerous in the dorsal and lateral pallium than in the medial pallium or the subpallium (Figs. 6E-I). In rostral and middle levels of the pallium, GS-positive cells are observed near the ventricular zone. These GS-immunoreactive cells exhibit a round cell body and a radial process (Fig. 6J). GS-immunoreactive cells with bipolar morphology are seen in the subpallium away the ependyma (Fig. 3A´´´-D´´´; Fig. 6K). In the caudal dorsal pallium, GS-immunoreactive cells similar those observed in S32 are observed (Fig. 3D´´´; Fig. 6L). From the rostral to the caudal telencephalon, GS-immunoreactive radial processes are observed both in the pallium and the subpallium (Figs. 6M, N), but they are especially abundant in the dorsal pallial subdivision, where the immunoreactive processes show a parallel and wavy arrangement. In the caudal telencephalon, specifically in the dorsal centromedial pallium, processes appear cross-sectioned in transverse sections (Fig. 3A´´´-D´´´).

Double immunofluorescence of GFAP/GS and BLBP/GS.

The similar pattern of GFAP, BLBP and GS immunoreactivities observed in the ventricular zone in latter stages of development led us to explore the possibility that the three glial markers were labelling the same glial populations. For this goal, double immunofluorescence combinations of GS and GFAP or BLBP were carried out and the cells of the ventricular zone have been analysed in detail for the presence of these markers.

The ventricular zone of the telencephalic regions exhibits different degrees of thickness from S32 embryos onwards. The ventricular zone of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

pallium is wider than the subpallial one. Meanwhile, the subpallial ventricular zone seems to be homogenous in thickness, in contrast to the pallium, where differences can be appreciated between the different pallial territories. The ventricular zone of the dorsal and medial pallium is clearly thinner than the ventricular zone of the presumptive ventral pallium. Moreover, the ventricular zone of the pallial/subpallial border seems to be substantially thicker than the adjacent ventricular zones.

Double immunofluorescence against GFAP/GS was performed in the S32/33 stage. Results of this GFAP/GS double labelling show co-expression of both markers in most glial cells bordering the telencephalic ventricles. Numerous GFAP/GS-positive cells are observed in the dorsal, medial and presumptive ventral pallial ventricular zone (white stars Figs. 7A, A', A''), and in the subpallial ventricular zone. Although cells expressing both markers represent the majority of cells, a few cells express only one glial marker (yellow stars in Figs. 7A, A', A''). Cell counting approach in the pallium revealed that around 84% of cells where double-labelled, while the rest expressed only one glial marker (see graphics in Figs. 7B, C). Cells expressing GFAP or GS only represent similar percentages (around 8%). In the subpallium, all cells coexpress both markers. For an integrated graphic of all regions see Suppl. Fig. 1C.

Double immunofluorescence against BLBP/GS was performed in the S32/33 stage. Similarly to GFAP/GS double immunofluorescence, many double-labelled cells can be observed in different regions of the pallial ventricular zone (white stars in Figs. D, D', D''). However, overall co-expression of both glial markers is observed in the subpallium. Cell counting in the pallium have shown that around 80% of cells where double-labelled cells, while the rest expressed or BLBP or GS alone (see graphics in Fig. 7E, F). In contrast to GFAP/GS, the percentage of cells expressing BLBP or GS alone

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

shows differences between different portions of the pallium. In the subpallium all cells coexpress both markers. For an integrated graphic of all regions see Suppl. Fig. 1D.

Double immunofluorescence to PCNA and GFAP or BLBP.

At later embryonic stages, PCNA immunofluorescence shows numerous cells with PCNA-positive nuclei lining the ventricles. These proliferating cells are widely distributed along the pallial subdivisions, in contrast with the subpallium, where few PCNA-positive cells are appreciable. PCNA-immunoreactive cells are numerous in rostral levels of the telencephalon and its number decreases toward caudal levels. In middle sections of the telencephalon, where the pallium can be subdivided into dorsal, medial and presumptive ventral pallium, differences among these subdivisions are found as regards PCNA immunoreactivity. The dorsal and medial ventricular zones show scarcer PCNA-immunoreactive cells compared to the presumptive ventral pallial ventricular zone and the pallial-subpallial boundary. Faint immunolabelled cells are observed close to the ventricle, while PCNA-immunoreactive nuclei more intensely stained are located in the basal portions of the ventricular zone. For panoramic views see Figs. 8A or 8D. On the basis of this distribution pattern we have analysed the co-expression of PCNA and the RGC markers GFAP and BLBP in S33 and S34 of development (final stages before hatching).

At S33 of development, double immunofluorescence against GFAP and PCNA reveals wide colocalization of both markers in cells of the telencephalic ependyma, especially in the pallial subdivision (Fig. 8A). In the dorsal (Figs. 8B, B’, B’’) and presumptive ventral pallium (Figs. 8C, C’, C’’) three populations of cells are distinguishable: GFAP +/PCNA+ cells (Figs. 6B-C’’), GFAP+/PCNA- cells and GFAP-/PCNA+ cells. The most abundant cell population is that consisting of GFAP+/PCNA+ cells. Interestingly, most of

these cells show fainter PCNA immunoreactivity than those that do not co-express GFAP. The immunoreactivity against GFAP/PCNA in the ventricular zone of the medial pallium exhibits the same distribution than in other pallial subdivisions. Numerous double labelled cells are also detected along the pallial-subpallial boundary, where the thick ventricular zone shows a high number of proliferating cells.

Double immunofluorescence against BLBP and PCNA in S33 reveals wide codistribution in the telencephalic ventricular zone, with a high level of co-expression of both factors in the pallial subdivision (Fig. 8D). This distribution is similar to that of GFAP/PCNA double immunofluorescent cells. In the dorsal (Figs. 8E, E', E'') and ventral (Figs. 8E, E', E'') pallium, three different populations of cells are distinguishable: BLBP+/PCNA+ cells, BLBP+/PCNA- cells and BLBP-/PCNA+ cells. Numerous PCNA immunoreactive cells co-expressed BLBP but, as for GFAP and PCNA immunofluorescence, double labelled cells use to present fainter PCNA-immunoreactive nuclei compared to cells that do not co-express BLBP. Abundant double labelled cells are also detected in the boundary between the medial pallium and the subpallium. The expression of BLBP and PCNA in the ventricular zone of the medial pallium shows the same pattern than in other pallial subdivisions.

In addition, numerous scattered cells showing PCNA-positive nuclei are distributed away from the ventricular zone. A few of these cells co-express GFAP and show a different morphology from that of the PCNA-positive cells of the ventricular zone. Many BLBP-positive cells located away the ventricular regions also showed PCNA immunoreactivity.

Double immunofluorescence against BLBP/PCNA and GFAP/PCNA has also been performed in S34 embryos (Fig. 9A, B). Besides, double

immunofluorescence between GS and PH3 has been performed (Fig. 9C). As in S33 embryos, both BLBP/PCNA and GFAP/PCNA immunofluorescence allows to observe numerous double labelled cells in the ventricular zone of the telencephalon (Figs. 9D-E''). In addition, double immunofluorescence GS/PH3 evidences many double-positive cells (not as much as PCNA), in the ventricular zone of the pallium (Figs. 9F-F``). In this case, no differences between the telencephalic subdivisions have been appreciated.

Discussion

The present work represents the first study of the glial cytoarchitecture during the embryonic development of a cartilaginous fish using markers of radial glia and cell proliferation. We analysed the expression of GFAP, BLBP, GS, and PCNA by means of immunohistochemistry in the developing telencephalon of *S. canicula* in S25 to S34 embryos, as well as in posthatching (early) juveniles. On the basis of major morphological characteristics, Ballard et al. (1993) established 3 periods during development of *S. canicula*: (1) early development, from the day of fertilization until S26 of development; (2) intermediate development, from S27 to S31; (3) and late development, from S32 to hatching. More recently, equivalences among brain developmental stages of *S. canicula* and mouse, birds and zebrafish have also been established (Rodríguez-Moldes et al. 2017). From the day of fertilization until hatching, the catshark embryogenesis takes around 6 months (depending on water temperature), which is a lengthy period compared to the 72-hours of zebrafish embryogenesis. Therefore, sharks provide a good time window for studying certain developmental processes that might be ignored by a rapid development (Quintana-Urzainqui et al. 2015; Rodríguez-Moldes et al. 2017). In addition, sharks exhibit an evaginated telencephalon, instead of an everted telencephalon

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

as in teleost fishes, which ease comparisons between developmental periods and developmental events in sharks and other tetrapods.

We show that the expression pattern of the three glial markers studied follows a sequential expression pattern along development: early (GFAP), intermediate (GFAP and BLBP) and late (GFAP, BLBP and GS). We found that these markers mainly label RGCs in the ventricular zone of the developing telencephalon and that their expression remains in juveniles. In addition, some non-ventricular positive cells were found during development (BLBP- and GS-immunoreactive cells) and in juveniles (GS-inmunoreactive cells) which do not have morphology of RGCs. Ventricular zone RGCs usually co-express all glial markers studied in late development, but exhibit some degree of heterogeneity in the dorsal telencephalon. In addition, double immunofluorescence experiments have shown a high degree of colocalization between the proliferation marker (PCNA) and glial markers such as GFAP or BLBP. The comparative aspects across vertebrates and the developmental implications regarding the appearance of these cells will be discussed below.

GFAP, BLBP and GS are expressed in cells with morphology of RGC during embryonic development and in postnatal individuals.

In the telencephalic hemispheres of the lesser spotted dogfish, GFAP immunoreactivity is detected in embryos from early developmental stages (S25) until the end of embryonic period (S32-S34), as well as in posthatching juveniles. In mouse embryos, GFAP mRNA was first detected in the germinal zones of the forebrain on E15 and it increases on E17 (Fox et al. 2004), which correspond to late embryonic stages (Rodríguez-Moldes et al., 2017). Mamber et al. (2012) showed that GFAPδ transcripts are expressed from E12 in radial glia, but GFAP-immunoreactive structures are only detected from E18 onwards. RGCs expressing GFAP were also detected in the forebrain of late embryonic

stages of *Xenopus laevis* (S36) (Messenger and Warner, 1989). Studies in zebrafish embryos have shown that expression of GFAP in the central nervous system (CNS) begin at the time of formation of the first somite (Thisse et al. 2001) but, contrary to that observed in *S. canicula*, mouse and *Xenopus* its expression decreases during the embryonic period (Marcus and Easter, 1995). The early developmental expression of GFAP in chondrichthyes and teleosts contrasts with its later expression in mammals. In mammals, the establishment of the RGC scaffold during forebrain development necessary for neuronal migration occurs previously to the neurogenic period (Martynoga et al. 2012). Quintana-Urzainqui et al. (2015) established that neurogenesis and neuronal migration in the catshark telencephalon mainly occurs during the intermediate developmental period (S27-S31). Like in mammals, GFAP expression in the telencephalon of the catshark occurs before neurogenesis; therefore, the presence and distribution pattern of GFAP-positive radial processes in the telencephalic hemispheres at these stages indicate that they could act as guide-posts during neuronal migration, as do RGC in the mammalian cortex (Rakic, 1972). Regarding the distribution of GFAP within glial cells, in early embryos GFAP is mainly located in subpial endfeet. As embryonic development progresses, GFAP is observed in cell bodies lining the ventricle and its radial processes and subpial endfeet. These GFAP-immunoreactive cells morphologically resemble glial cells described in the developing cerebral cortex of mammals (for review see: Alvarez-Buylla and Kriegstein, 2013). The distribution pattern of GFAP in catshark embryos also resembles that shown in studies of amphibians (Messenger and Warner, 1989) and teleosts (Marcus and Easter, 1995; Arochena et al. 2004). A similar distribution pattern is found in early juveniles, though GFAP expression decreases with respect to that observed in embryos: the thickness of the ependyma decreases with respect to the ventricular zone of embryonic stages, and less GFAP immunoreactive

ependymocytes or tanycytes are visualized in contact with the ventricle. In any case, radial glial processes and subpial endfeet still contain GFAP.

The distribution pattern of GFAP observed in the telencephalon of posthatching juveniles is in agreement with the GFAP distribution pattern reported in the adult brain of reptiles (Lazzari and Franceschini, 2006), amphibians (Kirkham et al. 2014) and diverse bony fishes (carp: Kálmán, 1998; trout: Diaz-Regueira and Anadón, 1998; grey mullet: Diaz-Regueira and Anadón, 1998; Arochena et al. 2004; zebrafish: Grupp et al. 2010; Than-Trong and Bally-Cuiff, 2015; african lungfish: Lazzari and Franceschini, 2004) and chondrichthyes (sharks, rays and skates: Wasowicz et al. 1999; Kálmán and Gould, 2001; Ari and Kálmán, 2008b; and chimaeras: Ari and Kálmán, 2008a), in which it has been shown that ependymal and radial glial cells are the predominant astroglial cell type. However, GFAP positive cells are found in the ventral telencephalon of catshark posthatching juveniles, which is not the case in teleost fish, such as zebrafish (Ganz et al., 2010; März et al., 2010), where GFAP expression is essentially absent. Zebrafish and catshark show many differences in their embryonic development; zebrafish is fast developmental specie, while catshark is slow developmental specie, and the telencephalon morphogenesis process is also different between both species. In addition, galeomorph sharks have relatively large brains, similar in size to those of the mammal and bird brains with similar body weights (Northcutt, 1981) and its size brain increases from posthaching period until adulthood. During mammalian embryonic development the length of radial glia processes has been related with the radial growing of the neural tube, in addition, the number of radial glial cells determine the size of a brain region, and at the same time radial glia cells act as guides in the neuronal migration process (for review see Götz, 2013). The presence of GFAP in the ventral telencephalon of catshark may be related with the continuous growth of the brain.

In the adult brain of skates, GFAP-positive cells with stellate morphology typical of astrocytes were also found (Kálmán and Gould, 2001). Although we have not observed astrocyte-like cells in the telencephalic hemispheres of juveniles, GFAP-immunoreactive cells with astrocytic morphology can be visualized in other brain areas (see Chapter 4).

BLBP-immunoreactivity in RGCs of the catshark telencephalic hemispheres is detected after GFAP-immunoreactivity, around S28 (present results). At this developmental stage BLBP-immunoreactivity is weak and restricted to ventricular cells, but as development proceeds (S31-S32) its expression increases considerably and can also be observed in cells away of the ventricular zone (present results). In mammals, BLBP is a marker of radial glia and its expression is firstly visualized in RGCs present in the caudolateral regions of the forebrain on E12.5 and, by E14.5-E16.5, BLBP expression expands to medial regions of the forebrain (Anthony and Heintz, 2008). Rodríguez-Moldes et al. (2017) established equivalences between catshark S27-S31 and mouse E10.5-14.5 stages, which suggests striking similarities in the temporal expression of BLBP between catshark and mouse. In *Xenopus* BLBP-positive cells line the telencephalic ventricles at stages 37/38 (Moreno and González, 2017). These *Xenopus* stages belong to a late embryonic period; however, as they show active neurogenesis it can be comparable to the catshark intermediate period. In the zebrafish forebrain, BLBP positive cells were described lining the ventricles in adults and 21 dpf juveniles (Grupp et al. 2010). We are not aware of studies of BLBP expression during zebrafish embryogenesis, precluding further comparisons.

A subpial layer of BLBP immunoreactive cells has been detected from S32-33 onwards. GFAP-immunoreactive subpial astrocytes have been described in the telencephalon of adult rays (Kálmán and Gould, 2001),

however, the morphology of the catshark BLBP-positive subpial cells resembles more to RGCs than to ordinary astrocytes, suggesting interspecific differences. In contrast with other vertebrates, the blood-brain barrier in cartilaginous fishes is not formed by endothelial cells but is formed by glial cell processes (Bundgaard and Cserr, 1981). Whether the submeningeal glial cells observed in cartilaginous fishes contribute to form the blood-brain barrier need to be further investigated.

In catshark embryos, numerous BLBP-positive cells line the telencephalic ventricles and their immunoreactive radial processes span the telencephalic walls from the ventricular zone to the pia. Scattered BLBP-immunoreactive cells outside the ventricular zone were also observed. However, in juveniles BLBP expression was restricted to ventricular glial cells and their subpial endfeet (present results). In mammals, BLBP is transiently expressed in the RGCs in the embryonic ventricular zone. It has been proposed that BLBP is involved in the establishment of the radial processes of RGC in the developing brain (Feng et al. 1994). Studies performed in zebrafish (März et al. 2010; Diotel et al. 2016) and *Xenopus* (D´Amico et al. 2011) have reported the presence of BLBP in RGCs of juvenile and adult brains, with a distribution pattern similar to that observed in the telencephalon of juvenile catshark. Likewise, BLBP expression in the catshark telencephalon might be related with the formation of the radial glia scaffold that persists in juveniles.

GS expression in the telencephalon of catshark appears in the later embryonic period previously to hatching, and the distribution pattern evidenced during embryonic development is similar to that of BLBP. In mammals GS is present in astrocytes and RGCs; in rat embryos, GS immunoreactivity was first observed in ventral ependymocytes of caudal neural tube at embryonic day 14 (E14) and radial glia shows GS immunoreactivity at E16 (Akimoto et al. 1993).

As in the rodent brain, in the catshark telencephalon GS is expressed in the later period of embryonic development. However, in teleosts and reptiles GS occurs earlier in development (Romero-Alemán et al. 2003; Grupp et al. 2010). The enzyme GS has a critical role in the metabolism of neurotransmitters released by neurons such as glutamate, which is uptaken by glia and converted by GS to glutamine to prevent neurotoxicity (Norenberg, 1979). GS has been reported as a marker of mature glial cells in the retina of this catshark species (Bejarano-Escobar, 2012; Sánchez-Farías and Candal, 2016). In juveniles GS was detected in ependymal cells (or tanycytes), which is in agreement with results observed in the adult human and mouse brain GS (Norenberg, 1979; Norenberg and Martinez-Hernandez, 1979; Bernstein et al. 2014); moreover, GS positive cells with morphology of RGCs were also found in the adult brain of amphibians (Kirkham et al. 2014) and fishes (teleosts: Grupp et al. 2010; Than-Trong and Bally-Cuif, 2015; chondrichthyes: Ari and Kálmán, 2008a; 2008b).

We can conclude that GFAP, BLBP and GS label cells with radial glia morphology during different developmental periods in the telencephalon of catshark. The onset expression timing of the three glial markers during catshark embryonic development shows many similarities with mammals. Present results reinforce the hypothesis that the basic developmental processes in this species (main molecular events, main morphogenetic processes) are quite similar to those of mammals (see also Rodríguez-Moldes et al. 2017 and references therein). However, in the catshark telencephalon RGCs are maintained after hatching, as in other species of fishes, which has been related with the high neurogenic potential of the glia in adult fishes (reviewed by Than-Trong and Bally-Cuif, 2015). Present results also indicate that the lesser spotted dogfish is an excellent model to study the development of the glial system in an evo-devo context.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

BLBP and GS adventricular positive cells may belong to the oligodendroglial lineage.

In embryonic and juvenile stages of catshark, scattered GS-positive cells are observed in the mantle zone of the medial and the caudal dorsal pallium (pars centromedialis). These cells also express BLBP in embryos, but not in juveniles (present results). They are characterized by their large size, the eccentric nucleus and a thick process, features that resemble to those of oligodendrocytes. Del Río Hortega (1928) made the first description of oligodendrocytes and classified them into four types attending to their size, shape, processes or even their interaction with axons (reviewed by Pérez-Cerdá et al. 2015). We found that GS-positive cells of the caudal dorsal pallium in juvenile fit well with type-3 oligodendrocytes, which are described by Del Rio Hortega as cells with a "bulky cell body" with one to four processes. In reptiles, it was shown that most of the cells positive for S-100 (also used as a marker of oligodendrocytes) also express GS during the embryonic period (Romero-Alemán et al. 2003). Interestingly, in adult humans and rodents, GS is expressed by subpopulations of oligodendrocytes (Bernstein et al. 2014). Ultrastructural studies focused on the mesencephalon of a lizard indicated that GS-positive cells may be oligodendrocytes (Monzón-Mayor et al. 1998). Oligodendrocytes with different size and shape were found in the brain of rainbow trout (Pérez et al. 1996) using NADPH diaphorase enzymohistochemistry, and these are morphologically similar to the GS-positive mantle cells of the catshark telencephalon. Differences in the size of oligodendrocytes has been related with the size of axons they ensheath (Díaz-Regueira and Anadón, 1998). Fibres in the catshark telencephalon are mostly unmyelinated, except in the compact bundles that run through the medial pallium and the centromedial dorsal pallium (Smeets et al. 1983). Curiously, we have also observed similar cells positive for GS in the rhombencephalon of this specie associated with myelinated tracts of

large diameter such as the medial longitudinal fascicle (results not shown). GS immunohistochemistry also revealed adventricularly disperse cells in the subpallium with a different morphology. These bipolar cells are orientated radially and horizontally in the subpallium and exhibit thin processes. They might correspond to either radial glial cells that have lost contact with the ventricular zone or to type-4 oligodendrocytes (or Schwannoid cells) of the classification of Del Rio Hortega, which use to show bipolar processes adhered to thick axons (for review see: Pérez-Cerdá et al. 2015). Numerous thick axons, some of them myelinated, run in the basal forebrain bundles through the catshark subpallium, including the area superficialis basalis (Smeets et al. 1983).

Since we have detected GS-positive cell type that morphologically resemble oligodendrocytes in regions that contain myelinated fibres we have carried out double immunofluorescence techniques using antibodies against GS and chondroitin sulfate proteoglycan NG2 (a marker of oligodendrocyte precursor cells, OPCs). Though GS-positive cell populations do not express NG2 (data not shown), we cannot exclude the possibility that these cells belong to the oligodendroglial lineage. Further investigations using markers of late progenitor pro-oligodendrocytes and mature oligodendrocytes like O4-antigen and myelin proteins (Ono and Ikenaka, 2013) might shed light about the nature of these glial cells.

Subsets of proliferating RGC are present in the catshark embryonic telencephalic ventricular zone.

In mammals, the development of the forebrain involves multiple types of progenitor cells, including NECs, RGCs, different pools of intermediate progenitor cells and basal radial glia cells. Apical progenitor cells (or RGCs) are primary progenitor cells generated from NECs; they undergo self-renewing division in the ventricular surface, producing neurons and generating different

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

pools of intermediate progenitors that themselves divide and give rise to differentiated neurons or different types of glial cells (astrocytes, oligodendrocytes and ependymal cells) at sequential stages in development. Although by the end of development most of the RGCs transform into astrocytes, in some brain areas RGCs persist after birth and function as primary progenitor cell (Götz et al. 1998; Marshall et al. 2003; Spassky et al. 2005; Kriegstein and Alvarez-Buylla, 2009; Taverna et al. 2014; Beattle and Hippenmeyer, 2017).

In mammals, the transition from NECs to apical RGCs involves the expression of several glial markers, including BLBP; several studies have shown that activation of the BLBP promoter in NECs promotes radial glia differentiation (for review see: Pinto and Götz, 2007). In rodents most of the precursor cells in the embryonic ventricular zone are RGCs (Noctor et al. 2002). We have detected that most of the ventricular cells express BLBP in S29-S30 catshark embryos, suggesting that these are RGC progenitors and that the transition from NECs (BLBP-negative) cells to RGCs occurs before these stages. Other feature of the apical RGCs of the mammalian dorsal telencephalon is the expression of the paired-box transcription factor 6 (Pax6) (Englund et al. 2005). Previous studies have found expression of Pax6 in the dorsal telencephalon of catshark at S29 (see Chapter 1). This data suggests that most of the BLBP-positive cells in the dorsal telencephalon also co-express Pax6.

On the other hand, our results indicate that the surface of the catshark telencephalic ventricles in later embryonic stages consists of subsets of RGCs; double immunofluorescence against GFAP, BLBP and GS reveals the presence of subpopulations of RGCs in the ventricular surface (see Fig. 7 and Suppl. Fig. 1). Due to the incompatibility among the antibodies used, double immunofluorescence between GFAP and BLBP was not carried out. However,

according to the distribution pattern we observed, a high degree of colocalization between GFAP and BLBP is expected. A molecular heterogeneity of RGCs is present in the developing telencephalon of mammals, with subsets of radial glial cells differing in the expression of the GLutamate ASpartate Transporter (GLAST), BLBP and the Radial Glial cell marker 2 (RC2), and whose proportions change throughout the neurogenic period (Hartfuss et al. 2001). In catshark embryos, we have not appreciated regional differences in the distribution of the subsets of radial glial although variations in thickness of the ventricular zone were observed.

Most of the RGCs expressing GFAP and BLBP are PCNA-positive, which indicates that they are proliferative (present results). Previous studies in this catshark have reported a high proliferative potential in S32-S34 embryos (Quintana-Urzainqui et al. 2015). Using fluorescently-tagged endogenous proliferating cell nuclear antigen (PCNA), Zerjatke et al. (2017) showed that differences in the intensity of the PCNA immunolabelling reflect different phases of the cell cycle. Based on this fact, GFAP- and BLBP-positive cells of the catshark telencephalon with intense PCNA are probably in S phase, while nuclei with a weak PCNA immunolabelling probably indicate cells that are in other phases of the cell cycle. In the mammalian telencephalon, nuclei undergoing the S phase form several layers at the basal surface of the ventricular zone. On the other hand, nuclei in M phase are close to the ventricle, and nuclei in G1 and G2 phases are in the mid region. This process is known as “interkinetic nuclear migration” (for review see: Kriegstein and Alvarez-Buylla, 2009). Such zonation of the ventricular zone can be observed on the basis of the intensity of PCNA immunoreactivity in the telencephalon of catshark.

On the other hand, the number of proliferating ventricular cells seems higher in the pallium than in the subpallium. Differences in the proliferative

potential between the dorsal and ventral telencephalon were also described in rodent embryos (Malatesta et al. 2003). Our results are in agreement with studies in other vertebrates showing proliferative RGCs. In mouse embryos, most of the proliferating ventricular cells have morphological and molecular characteristics of radial glia and express BLBP (Noctor et al. 2002; Anthony et al. 2004). Proliferating BLBP-positive cells are also present in the ventricular zone of *Xenopus* embryos (Moreno and González, 2017) and teleosts (Grupp et al. 2010). Also, in the developing dorsal telencephalon of reptiles ventricular radial glial cells undergo cell divisions (Clinton et al. 2014). Like in mammals (Hartfuss et al. 2001; Noctor et al. 2002), RGCs represent the majority of precursor cells in the catshark telencephalon. Our results also indicate that different cycling cells are present in the ventricular zone. Double GFAP/ GS and BLBP/GS immunolabelling clearly showed the presence of different RGCs subpopulations, and double immunofluorescence for PCNA and GFAP or BLBP revealed some proliferating cells that do not exhibit glial cell markers. Curiously, nuclei of most of these proliferating cells used to be strongly PCNA-immunoreactive, which means that cells are in S phase, and in consequence committed to undergo mitosis. Despite the actual nature of these cells is not known, they might correspond with some kind of intermediate progenitor cells.

Fate mapping experiments using Cre recombinase driven by RGCs promoters BLBP or GFAP in mammals indicate that most neurons throughout telencephalon originate from RGCs that express GFAP and BLBP and, interestingly, GFAP-positive cells also produce oligodendrocytes and astrocytes (Malatesta et al. 2003; Casper and McCarthy, 2006; Anthony and Heintz, 2008; Farkas and Huttner, 2008; Martynoga et al, 2012). Besides, in rodents RGCs give rise to most of the ependymal cells between E14 and E16, and these cells complete their maturation after birth (Spassky et al. 2005). If these results were extensive to basal vertebrates such as sharks, it would suggest that

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

BLBP/GFAP-positive proliferating cells may generate both neurons and different types of glial cell at the end of the embryonic period. At this point, further investigations are needed in order to elucidate the identity of the progeny originated by the proliferating RGC present in the catshark telencephalon.

Conclusion

This is the first study during embryogenesis focussed on the development of RGCs by using different glial markers in the telencephalon of the catshark. Radial glial markers such as GFAP, BLBP and GS are sequentially expressed along the development and its expression/distribution becomes overlapping and persistent in the postnatal telencephalon, evidencing a wide and organized RGCs pattern. We found that RGCs exhibit certain degree of molecular heterogeneity and the vast majority of these cells show proliferative capacity even in late periods of development. In addition, we found that some marker of RGCs also labelled cells with a presumptive oligodendroglial nature.

The high proliferative capacity of cartilaginous fishes and also the persistent presence of RGCs postnatally makes them excellent candidates for study, not only the ancestral condition of neurogenic and/or gliogenic processes, but also other possible roles of RGCs during development and postnatal stages. This work would lay the foundation for future studies that investigate the proliferative potential and progeny of RGCs in basal vertebrates.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

References

Akimoto J, Itoh H, Miwa T, Ikeda K (1993). Immunohistochemical study of glutamine synthetase expression in early glial development. *Brain Res Dev Brain Res* 72:9-14.

Alexandre P, Reugels AM, Barker D, Blanc E, Clarke JD (2010). Neurons derive from the more apical daughter in asymmetric divisions in the zebrafish neural tube. *Nat Neurosci* 13:673-9.

Alvarez-Buylla A, Theelen M, Nottebohm F (1990). Proliferation "Hot Spots" in adult avian ventricular zone reveal radial cell division. *Neuron* 5:101-109

Alvarez-Buylla A, García-Verdugo JM, Mateo AS, Merchant-Larios H (1998). Primary neural precursors and intermitotic nuclear migration in the ventricular zone of adult canaries. *J Neurosci* 18:1020-37.

Alvarez-Buylla A, Kriegstein, A (2013). Neural stem cells among glia. In: Rubenstein JLR, Rakic P, editors. Patterning and Cell Type Specification in the Developing CNS and PNS (Vol. 1). San Diego, CA, USA: Academic Press. p 685-705.

Anadón R, Molist P, Rodríguez-Moldes I, López JM, Quintela I, Cerviño MC, Barja P, González A (2000). Distribution of choline acetyltransferase immunoreactivity in the brain of an elasmobranch, the lesser spotted dogfish (*Scyliorhinus canicula*). *J Comp Neurol* 420:139-170.

Anthony TE, Klein C, Fishell G, Heintz N (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron* 41:881-90.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Anthony TE, Heintz N (2008). Genetic lineage tracing defines distinct neurogenic and gliogenic stages of ventral telencephalic radial glial development. *Neural Dev* 2008 3:30.

Ari C, Kálman M (2008a). Glial architecture of the ghost shark (*Callorhinchus milii*, Holocephali, Chondrichthyes) as revealed by different immunohistochemical markers. *J Exp Zool B Mol Dev Evol* 310:504-19.

Ari C, Kálmán M (2008b). Evolutionary changes of astroglia in Elasmobranchii comparing to amniotes: a study based on three immunohistochemical markers (GFAP, S-100, and glutamine synthetase). *Brain Behav Evol* 71:305-24.

Arochena M, Anadón R, Díaz-Regueira SM (2004). Development of vimentin and glial fibrillary acidic protein immunoreactivities in the brain of gray mullet (*Chelon labrosus*), an advanced teleost. *J Comp Neurol* 469:413-36.

Ballard WW, Mellinger J, Lechenault (1993). A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). *J Exp Zool* 267:318-336.

Beattie R, Hippenmeyer S (2017). Mechanisms of radial glia progenitor cell lineage progression. *FEBS* 591:3993-4008.

Bentivoglio M, Mazzarello P (1999). The history of radial glia. *Brain Res Bull* 49:305-315.

Bejarano-Escobar R, Blasco M, Durán AC, Rodríguez C, Martín-Partido G, Francisco-Morcillo J (2012). Retinal histogenesis and cell differentiation in an elasmobranch species, the small-spotted catshark *Scyliorhinus canicula*. *J Anat* 220:318-35.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Bernstein H, Bannier J, Meyer-Lotz G, Steiner J, Keilhoff G, Dobrowonly H, Walter M, Bogerts B (2014). Distribution of immunoreactive glutamine synthetase in the adult human and mouse brain. Qualitative and quantitative observations with special emphasis on extra-astroglial protein localization. *J Chem Neuroanat* 61-62:33-50.

Bundgaard M, Cserr HF (1981). A glial blood-brain barrier in elasmobranchs. *Brain Res* 226:61-73.

Casper KB, McCarthy KD (2006). GFAP-positive progenitor cells produce neurons and oligodendrocytes throughout the CNS. *Mol Cell Neurosci* 31:676-84.

Clinton BK, Cunningham CL, Kriegstein AR, Noctor SC Martínez-Cerdeño V (2014). Radial glia in the proliferative ventricular zone of the embryonic and adult turtle, *Trachemys scripta elegans*. *Neurogenesis*. DOI:10.4161/23262125.2014.970905.

Coolen M, Menuet A., Chassoux D, Compagnucci C, Henry S, Lévèque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodriguez- Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009). The dogfish Scyliorhinus canicula, a reference in jawed vertebrates. In: Behringer, R.R., Johnson, A.D., Krumlauf, R.E. (Eds.), Emerging Model Organisms, A Laboratory Manual, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 431–446.

D'Amico LA, Daniel Boujard D, Coumailleau P (2011). Proliferation, migration and differentiation in juvenile and adult *Xenopus laevis* brains. *Brain Research* 1405:31-48.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Del Río Hortega P (1928). Tercera aportación al conocimiento morfológico e interpretación funcional de la oligodendroglia. *Mem Real Soc Esp Hist Nat* 14:5-122

Díaz-Regueira S, Anadón R (1998). The macroglia of teleosts: characterization, distribution and development. In: Castellano B, González B, Nieto-Sampedro M, editors. Understanding Glial Cells. Dordrecht, The Netherlands: Kluwer Academic Publishers. p 19-46.

Diotel N, Vaillant C, Kah O, Pellegrini E (2016). Mapping of brain lipid binding protein (Blbp) in the brain of adult zebrafish, co-expression with aromatase B and links with proliferation. *Gene Expr Patterns* 20:42-54.

Englund C, Flink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci* 25:247-51.

Falk S, Götz M (2017). Glial control of neurogenesis. *Curr Opin Neurobiol* 47:188-195.

Farkas LM, Huttner WB (2008). The cell biology of neuronal stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. *Curr Opin Cell Biol* 20:707-15.

Feng L, Hatten ME, Heintz N (1994). Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. *Neuron* 12:895-908.

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012). Dynamic expression of Pax6 in the shark olfactory system: evidence for the presence of Pax6 cells along the olfactory nerve pathway. *J Exp Zool B Mol Dev Evol* 318:79-90.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Fox IJ, Paucar AA, Nakano I, Mottahedeh J, Dougherty JD, Kornblum HI (2004). Developmental expression of glial fibrillary acidic protein mRNA in mouse forebrain germinal zones--implications for stem cell biology. *Brain Res Dev Brain Res* 153:121-5.

González-Granero S, Lezameta M, García-Verdugo JM (2011). Adult neurogenesis in reptiles. In: Seki T, Sawamoto K, Parent JM, Alvarez-Buylla A (eds) Neurogenesis in the adult brain, vol. I. Springer, pp- 169-189.

Götz, M (2013). Radial glial cells. In: Kettenmann H, Ranson BR, editors. Neuroglia. New York: Oxford University Press. p 50-61.

Götz M, Huttner WB (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* 6:777-88.

Götz M, Stoykova A, Gruss P (1998). Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* 21:1031-44.

Grupp L, Wolburg H, Mack AF (2010). Astroglial structures in the zebrafish brain. J *Comp Neurol* 518:4277-87.

Hartfuss E, Galli R, Heins N, Götz M (2001). Characterization of CNS precursor subtypes and radial glia. *Dev Biol* 229:15-30.

Horstmann E (1954). Die Faserglia des Selachiergehirns. *Z Zellforsch Mikrosk Anat* 39:588-617.

Kálmán M (1998). Astroglial architecture of the carp (*Cyprinus carpio*) brain as revealed by immunohistochemical staining against glial fibrillary acidic protein (GFAP). *Anat Embryol (Berl)* 198:409-33.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Kálmán M, Gould RM (2001). GFAP-immunopositive structures in spiny dogfish, *Squalus acanthias*, and little skate, *Raia erinacea*, brains: differences have evolutionary implications. *Anat Embryol (Berl)* 204:59-80.

Kirkham M, Hameed LS, Berg DA, Wang H, Simon A (2014). Progenitor cell dynamics in the Newt Telencephalon during homeostasis and neuronal regeneration. *Stem Cell Reports* 2:507-19.

Kriegstein A, Alvarez-Buylla A (2009). The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci* 32:149-84.

Kriegstein AR, Götz M (2003). Radial glia diversity: A matter of cell fate. *Glia* 43:37-43.

Lazzari M, Franceschini V (2004). Glial fibrillary acidic protein and vimentin immunoreactivity of astroglial cells in the central nervous system of the African lungfish, *Protopterus annectens* (Dipnoi: Lepidosirenidae). *J Morphol* 262:741-9.

Lazzari M, Franceschini V (2006). Glial cytoarchitecture in the central nervous system of the soft-shell turtle, *Trionyx sinensis*, revealed by intermediate filament immunohistochemistry. *Anat Embryol* 211:497-506.

Li H, Jin G, Qin J, Yang W, Tian M, Tan X, Zhang X, Shi J, Zou L (2011). Identification of neonatal rat hippocampal radial glia cells in vitro. *Neurosci Lett* 490:209-14.

Lyons DA, Guy AT, Clarke JD (2003). Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. *Development* 130:3427-36.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Götz M (2003). Neuronal or glial progeny: Regional differences in radial glia fate. *Neuron* 37:751-764.

Malatesta P, Appolloni I, Calzolari F (2008). Radial glia and neural stem cells. *Cell Tissue Res* 331:165-78

Mamber C, Kamphuis W, Haring NL, Peprah N, Middeldorp J, et al. (2012). GFAPδ expression in glia of the developmental and adolescent mouse brain. *PLoS ONE* 7: e52659.

Marcus RC, Easter SS Jr (1995). Expression of glial fibrillary acidic protein and its relation to tract formation in embryonic zebrafish (*Danio rerio*). *J Comp Neurol* 359:365-81.

Martínez-Cerdeño V, Cunningham CL, Camacho J, Keiter JA, Ariza J, Lovern M and Noctor SC (2016). Evolutionary origin of Tbr2-expressing precursor cells and the subventricular zone in the developing cortex. *J Comp Neurol DOI*: 10.1002/cne.23879.

Marshall CA, Suzuki SO, Goldman JE (2003). Gliogenic and neurogenic progenitors of the subventricular zone: who are they, where did they come from, and where are they going? *Glia* 43:52-61.

Martynoga B, Drechsel D, Guillermot F (2012). Molecular control of neurogenesis: a view from the mammalian cerebral cortex. *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a008359.

März M, Chapouton P, Diotel N, Vaillant C, Hesl B, Takamiya M, Lam CS, Kah O, Bally-Cuif L, Strähle U (2010). Heterogeneity in progenitor cell subtypes in the ventricular zone of the zebrafish adult telencephalon. *Glia* 58:870-88.

Merrick SE, Pleasure SJ, Lurie DI, Pijak DS, Selzer ME, Lee VM (1995). Glial cells of the lamprey nervous system contain keratin-like proteins. *J Comp Neurol*. 355:199-210.

Messenger NJ, Warner AE (1989). The appearance of neural and glial cell markers during early development of the nervous system in the amphibian embryo. *Development* 107:43-54.

Monzón-Mayor M, Yanes C, De Barry J, Capdevilla-Carbonell C, Renau-Piqueras J, Tholey G, Gombos G (1998). Heterogeneous immunoreactivity of glial cells in the mesencephalon of a lizard: a double labeling immunohistochemical study. *J Morphol* 235:109-19.

Moreno N, González A (2017). Pattern of neurogenesis and identification of neuronal progenitor subtypes during pallial development in *Xenopus laevis*. *Front Neuroanat* DOI: 10.3389/fnana.2017.00024.

Noctor SC, Flint AC, Weissman TA, Wong WS, Clinton BK, Kriegstein AR (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J Neurosci* 22:3161-73.

Northcutt RG (1981) Evolution of the telencephalon in nonmammals. *Annu Rev Neurosci* 4:301–50

Norenberg MD (1979). Distribution of glutamine synthetase in the rat central nervous system. *J Histochem Cytochem* 27:756-62.

Norenberg MD, Martinez-Hernandez A (1979). Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res* 161:303-10.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Ono K, Ikenaka K (2013). Lineage and development: oligodendrocytes. In: Kettenmann H, Ranson BR, editors. Neuroglia, 3rd Ed. New York: Oxford University Press. p 148-158

Pérez SE, Adrio F, Rodríguez MA, Rodríguez-Moldes I, Anadon R (1996). NADPH-diaphorase histochemistry reveals oligodendrocytes in the rainbow trout (teleosts). *Neurosci Lett* 205:83-86.

Pérez-Cerdá F, Sánchez-Gómez MV, Matute C (2015). Pío del Río Hortega and the discovery of the oligodendrocytes. *Front Neuroanat* 9:92.

Pinto L, Götz M (2007). Radial glial cell heterogeneity--the source of diverse progeny in the CNS. *Prog Neurobiol* 83:2-23.

Podgornyi OV, Aleksandrova MA (2009). BLBP-immunoreactive cells in the primary culture of neural precursors from embryonic mouse brain. *Bull Exp Biol Med* 147125-31.

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E (2014). Developmental, tract-tracing and immunohistochemical study of the peripheral olfactory system in a basal vertebrate: insights on Pax6 neurons migrating along the olfactory nerve. *Brain Struct Funct* 219:85:104.

Quintana-Urzainqui I, Rodríguez-Moldes I, Mazan S, Candal E (2015). Tangential migratory pathways of subpallial origin in the embryonic telencephalon of sharks: evolutionary implications. *Brain Struct Funct* 220:2905-26.

Rakic P (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol* 145:61-83.

Rodríguez-Moldes I, Santos-Durán GN, Pose-Méndez S, Quintana-Urzainqui I, Candal E (2017). The brains of cartilaginous fishes. In: Kaas J (ed.), Evolution of Nervous Systems 2e. vol. 1, pp. 77–97. Oxford: Elsevier. ISBN: 9780128040423.

Romero-Alemán MdelM, Monzón-Mayor M, Yanes C, Arbelo-Galván JF, Lang D, Renau-Piqueras J, Negrín-Martínez C (2003). S100 immunoreactive glial cells in the forebrain and midbrain of the lizard *Gallotia galloti* during ontogeny. *J Neurobiol* 57:54-66.

Sánchez-Farías N, Candal E (2015). Doublecortin is widely expressed in the developing and adult retina of sharks. *Exp Eye Res* 134:90-100.

Sánchez-Farías N, Candal E (2016). Identification of radial glia progenitors in the developing and adult retina of sharks. *Front Neuroanat* 10:65.

Smeets WJAJ, Nieuwenhyus R, Roberts BL (1983). The central nervous system of cartilaginous fishes. Ed. Springer-Verlag.

Spassky N, Merkle FT, Flames N, Tramontin AD, García-Verdugo JM, Alvarez-Buylla A (2005). Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J Neurosci* 25:10-18.

Taverna E, Götz M, Huttner WB (2014). The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu Rev Cell Dev Biol* 30:465-502.

Than-Trong E, Bally-Cuif L (2015). Radial glía and neural progenitors in the adult zebrafish central nervous system. *Glia* 63:1406-28.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Thisse B, Pflumio S, Fürthauer M, Looping B, Heyer V, Degrave A, Woehl , Lux A, Steffan T, Charbonnier XQ, Thisse C (2001). Expression of zebrafish genome during embryogenesis. ZFIN direct Data 2001.

Turrero Garcia M, Harwell CC (2017). Radial glia in the ventral telencephalon. *FEBS Lett* 591:3942-3959.

Villar-Cheda B, Pérez-Costas E, Meléndez-Ferro M, Abalo XM, Rodríguez-Muñoz R, Anadón R, Rodicio MC (2006). Cell proliferation in the forebrain and midbrain of the sea lamprey. *J Comp Neurol* 494:986-1006.

Wasowicz M, Ward R, Repérant J (1999). An investigation of astroglial morphology in *Torpedo* and *Scyliorhinus*. *J Neurocytol* 28:639-53.

Zerjatke T, Gak IA, Kirova D, Fuhrmann M, Daniel K, Gonciarz M, Müller D, Glauche I, Mansfeld J (2017). Quantitative cell cycle analysis based on an endogenous all-in-one reporter for cell tracking and classification. *Cell Rep* 19:1953-1966.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Abbreviation list

BLBP: brain lipid binding protein

BSA: basal superficial area.

ChP: choroid plexus

DMP: dorsomedial pallium

DP: dorsal pallium

Dpcm: dorsal pallium centromedial pars

GFAP: glial fibrillary acidic protein

GS: glutamine synthase

Hab: habenula

LGE-h: lateral ganglionic eminence homologue

MGE-h: medial ganglionic eminence homologue

MP: medial pallium

OB: olfactory bulb

P: pallium

PCNA: proliferating cell nuclear antigen

pLP: presumptive lateral pallium

pMGE-h: presumptive medial ganglionic eminence homologue

pMP: presumptive medial pallium

PSB: pallial-subpallial boundary

pVLP: presumptive ventrolateral pallium

pVP: presumptive ventral pallium

RGCs: radial glial cells

SP: subpallium

Spt: septum

VLP: ventrolateral pallium

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 1. Primary and secondary antibodies used in this study.

Table 1. Primary and secondary antibodies used.

Primary Antibody	Source	Working dilution	Secondary Antibody	Source	Working dilution
GFAP	Polyclonal rabbit anti-GFAP Dako Cat. Nº. Z033429	1:500	**Goat anti-rabbit HRP coupled**	Dako, Glostrup, Denmark	1:200
GS	Monoclonal mouse anti-GS Millipore Cat. Nº. MAB302	1:500	**Goat anti-mouse HRP coupled**	Dako, Glostrup, Denmark	1:200
BLBP	Polyclonal rabbit anti-BLBP Millipore Cat. Nº. ABN14	1:300	**488-cojugated donkey anti-mouse**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
PCNA	Monoclonal mouse anti-PCNA Sigma Cat. Nº. P8825	1:500	**546-conjugated donkey anti-rabbit**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
PH3	Polyclonal rabbit anti-PH3 Millipore 06-570	1:300			

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Figure 1. Immunoblots of SDS-polyacrylamide gel of *S. canicula* adult brain protein extracts stained by anti-GFAP (lane 2), anti-BLBP (lane 3), and anti-GS (lane 4) antibodies. GFAP lane showed single a band of around 49 kDa. BLBP lane showed a single band of around 13 kDa. GS lane showed a single band between 38-49 kDa.

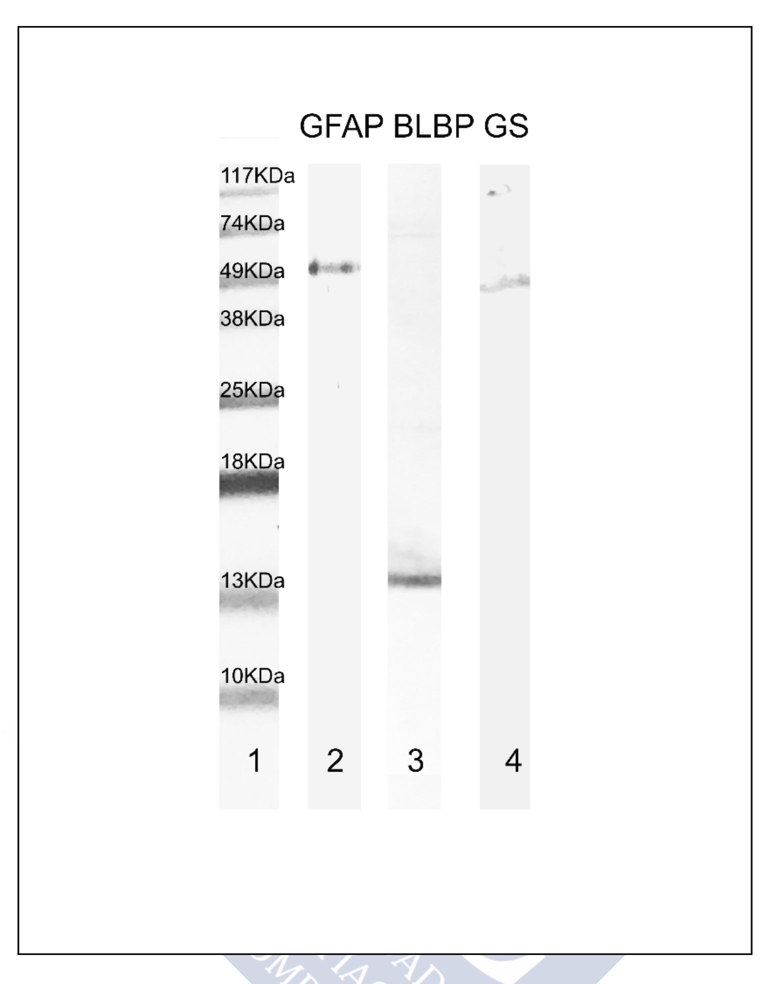


FIGURE 1

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Figure 2. Schema summarizing the expression pattern of GFAP (green color), BLBP (orange color), GS (red color) from S29 to S32. The choroid plexus has not been represented in this schema. Scale bars: 200 µm.

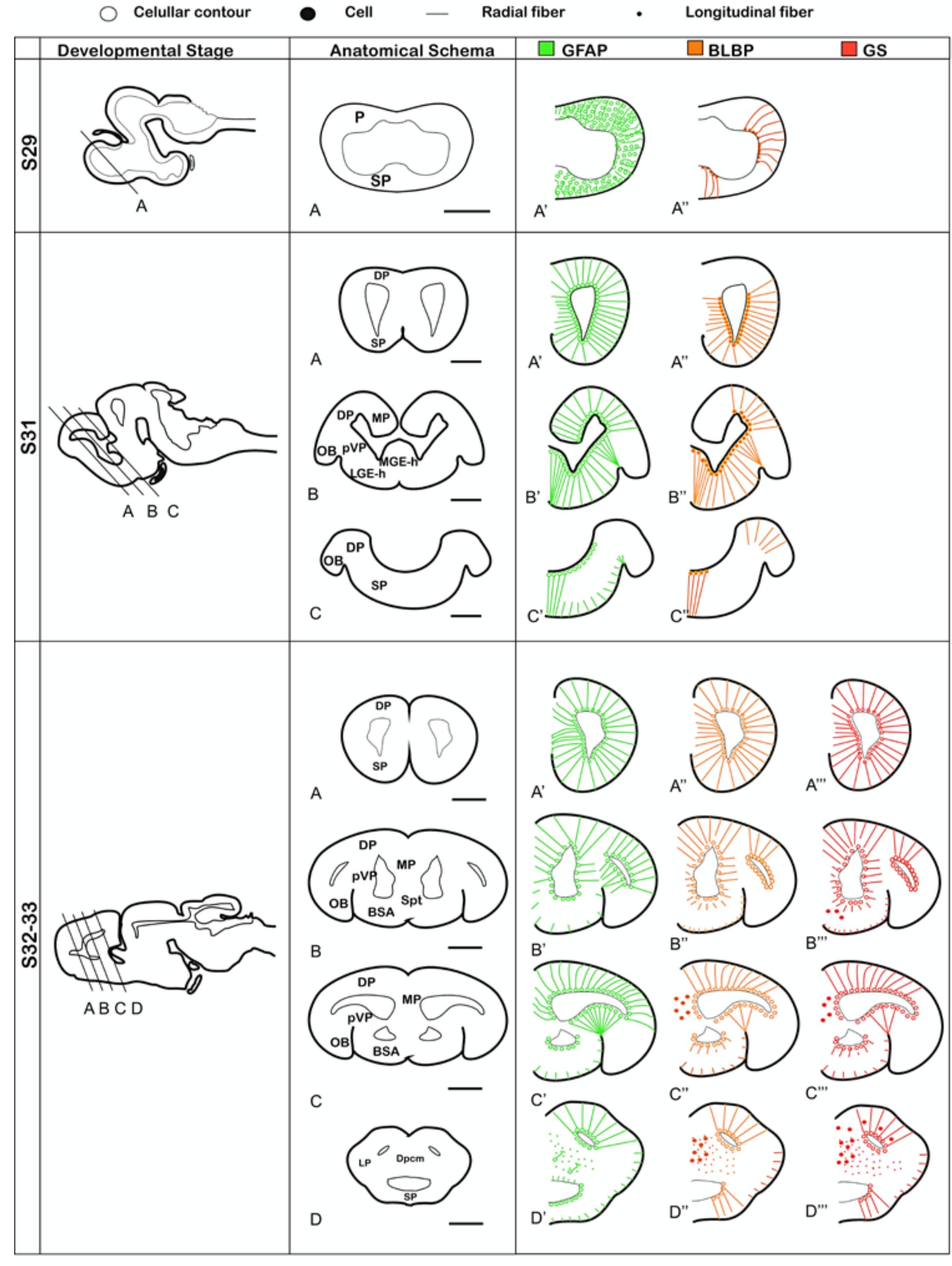


FIGURE 2

Figure 3. Schema summarizing the expression pattern of GFAP (green color), BLBP (orange color), GS (red color) in posthatching juveniles. The choroid plexus has not been represented in this schema. Scale bars: 500 µm.

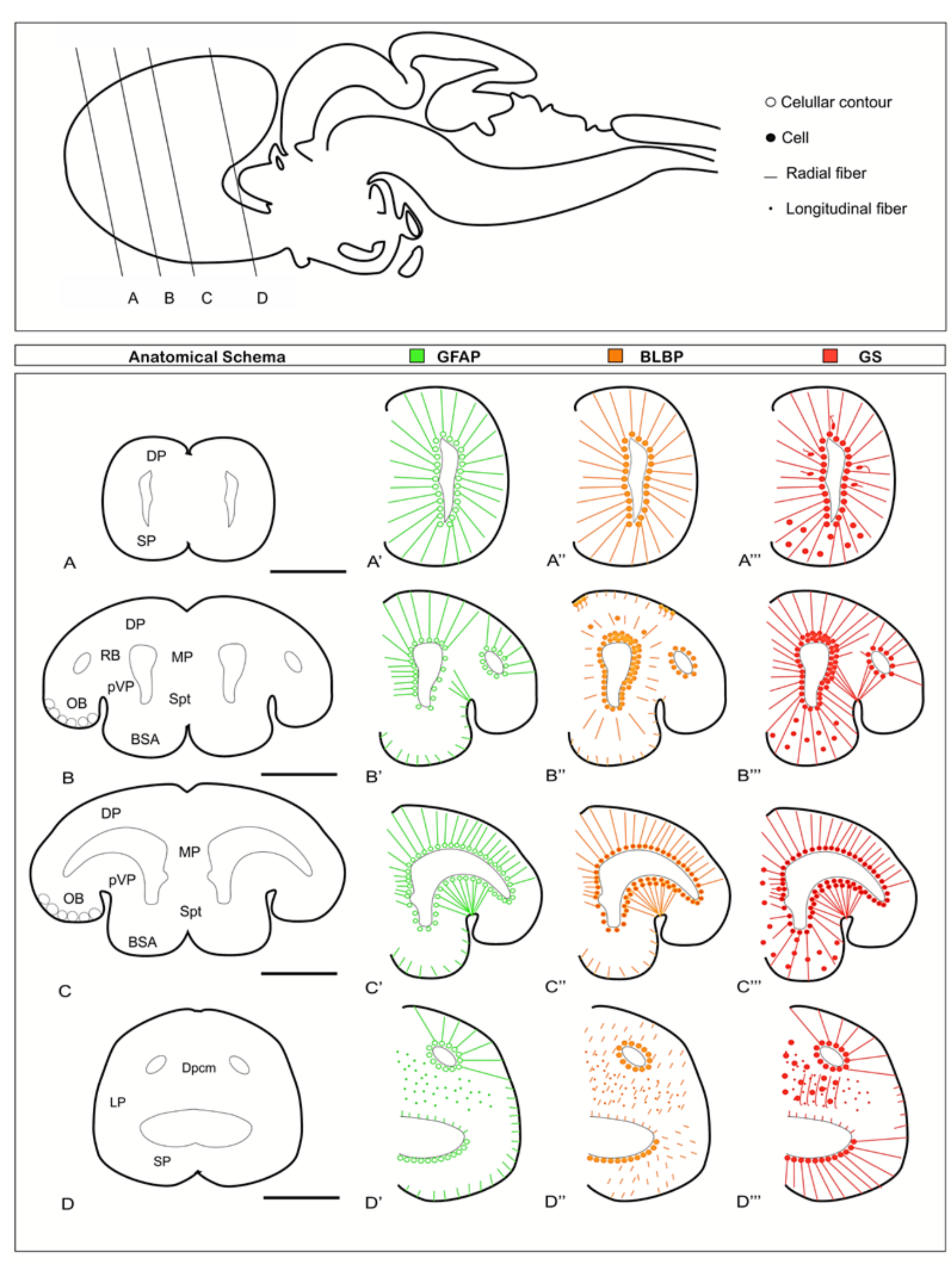


FIGURE 3

Figure 4. Photomicrographs of transverse sections of the telencephalon of *S. canicula* showing the expression pattern of GFAP in embryos (A-K) and juveniles (L-Q). **(A)** Section of the telencephalon of a S25 embryo showing GFAP immunoreactivity in numerous endfeet in the pial surface of pallium (arrows). **(B-F)** Photomicrographs of a S29 embryo. **(B)** Photomicrograph at lower magnification showing GFAP immunoreactivity in radial processes extending from the ventricular surface to the pia in the pallium (arrows) and subpallium (arrowheads). **(C-E)** Photomicrographs at higher magnification showing an intense GFAP immunolabelling in the midline of the telencephalon (arrows) **(C, D)**, in contrast to the rest of the telencephalon which shows weak immunoreactivity (arrows) **(E)**. **(F)** Photomicrograph at higher magnification of the choroid plexus which show an intense GFAP immunoreactivity. **(G-H)** Photomicrographs from a S31 embryo at different magnifications. **(G)** Transverse section of the telencephalon showing radial processes (arrows), endfeet (arrowheads) and numerous positive cells lining the ventricular zone (empty arrowheads) positive for GFAP. Note the intense immunoreactivity in the choroid plexus (asterisk). **(G')** Photomicrograph at higher magnification of the ventricular surface showing the basal portion of cells close to the ventricle (arrowheads) immunoreactive to GFAP. **(H)** Transverse section of the subpallial midline showing intense immunoreactivity for GFAP at the ventricular and pial zone and numerous radial processes positive for GFAP (arrows); cells GFAP positive are intermingled between radial processes (arrowheads). **(I-K)** Photomicrographs from S32 embryos. **(I)** Section of the rostral telencephalon showing intense convergent processes (arrows) in the midline of subpallium. **(J-J')** Photomicrographs at different magnifications of the dorsal pallium showing curvy radial processes (arrows) and numerous cells in the ventricular zone showing GFAP immunoreactivity in the periphery of the cell body (arrowheads in J'). **(K)** Photomicrograph at high magnification of the medial pallium showing intense GFAP positive radial processes converging in the pallial midline (arrows). **(L-Q)** Transverse sections of GFAP immunoreactivity in the telencephalon of juveniles. **(L)** Panoramic view of the ventricular zone of the telencephalon showing differences in the expression of GFAP between medial pallium, presumptive ventral pallium and subpallium. Note strong immunoreactivity in the choroid plexus (asterisk). **(M-O)** Magnifications of the ventricular zone, pial surface and blood vessels respectively showing ventricular positive cells (arrows in M), endfeet in the pial surface (arrows in N) and endfeet around blood vessels (arrows in O). **(P-Q)** Details of the caudal telencephalon showing the presence of small cells close to blood vessels (empty arrowheads) and endfeet positive for GFAP surrounding blood vessels (arrowheads in **P),** as well as the presence of numerous endfeet in the roof of the caudal ventricle (arrows in **Q).** Scale bars: 200µm (O), 100µm (B, G, I, J, P), 50µm (A, H, J', M, N, Q), 20 µm (C, D, E, F, K), 10 µm (G', L).

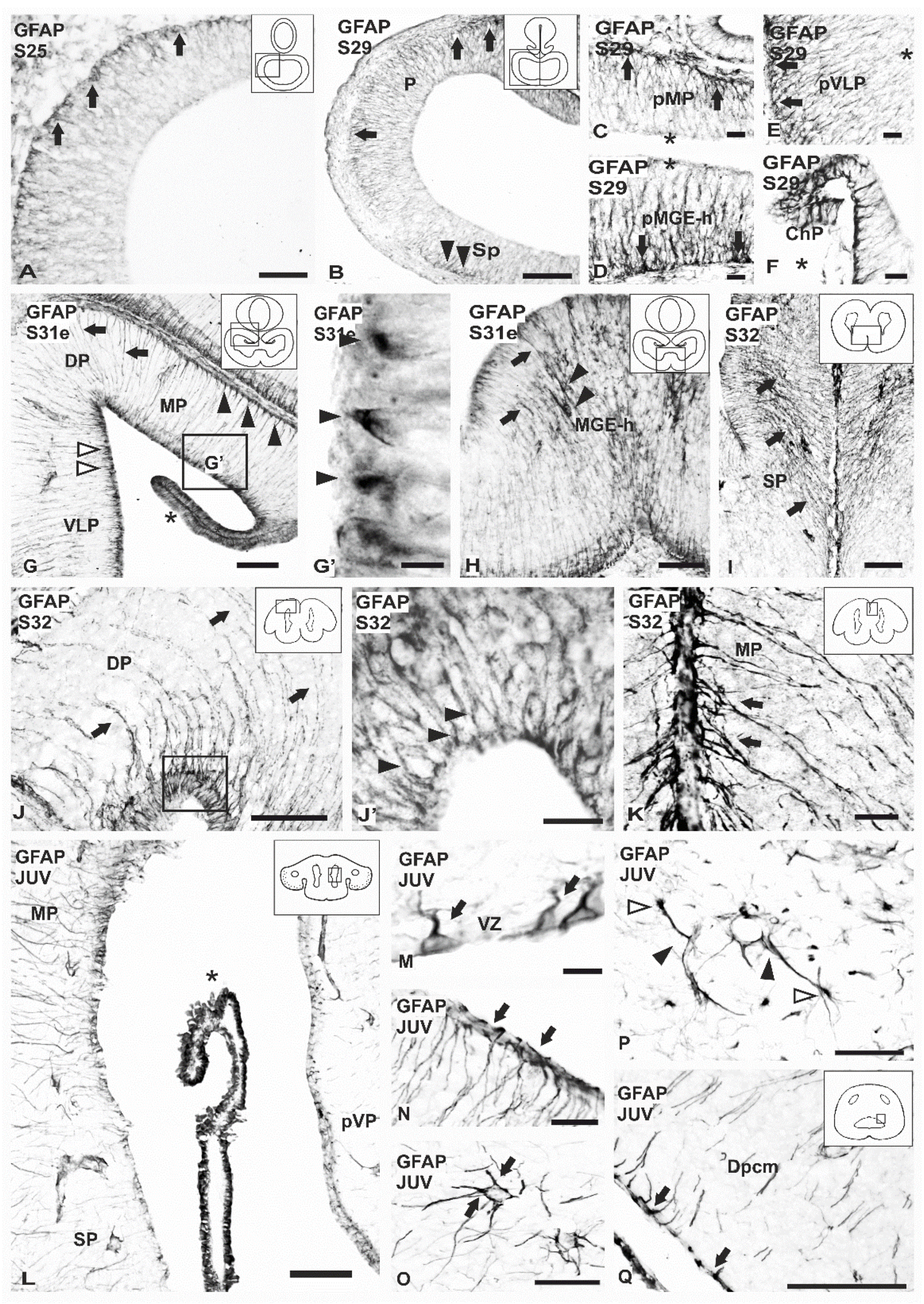


FIGURE 4

Figure 5. Photomicrographs of different sections of the telencephalon of *S. canicula* showing the expression pattern of BLBP during development **(A-F)** and juveniles **(G-I'')**. **(A-B')** Sagittal sections of the telencephalon of a S29-30 embryo showing BLBP immunoreactivity restricted to lateral portions of the pallium **(A)** and to medial portions of the subpallium **(B).** Note the intense immunolabeling in the choroid plexus (arrow)**. (B')** Detail of the ventricular surface of the pMGE-h showing ventricular contacting positive cells (arrows). **(C-F)** Transverse sections of the telencephalon of a S32 showing BLBP immunolabeling. BLBP positive cells are mainly restricted to the ventricular zone **(C),** both in pallium **(C')** and subpallium **(C'').** Note that immunoreactivity is concentrated in the basal (arrows) and apical portion of the cells (arrowheads). Dilatations are pointed with empty arrowheads. (**D**) Photomicrograph of the caudal telencephalon showing intense immunolabeling in the ventricular zone of the subpallium. **(E)** Magnification showing BLBP positive cells located far away from the ventricular zone in the centromedial portion of the caudal dorsal pallium (arrows). **(F)** Transverse section showing the pial surface where radial processes (arrows) and endfeet structures (arrowheads) positive for BLBP are observed. **(G-I'')** Photomicrographs at different magnifications showing BLBP expression pattern in juveniles of *S. canicula*. **(G)** Transverse section of the pallium showing immunoreactivity for BLBP in ventricular cells (arrows) and in long radial prolongations (arrowheads). **(G'-G'')** Details of the ventral and dorsal pallial ventricle respectively showing BLBP positive cells in the ventricular zone (arrows) and a few in subventricular positions (arrowheads). **(H)** Detail of a group of BLBP positive cells at the pial surface of the pallium (arrows). **(I)** Panoramic view of the caudal telencephalon showing magnifications of BLBP positive cells in the subpallial ventricular zone (arrows in **I')** and endfeet in the ventricular roof (arrows in **I'').** Scale bars: 200 µm (A, B, C, D, G, I), 100 µm (C', C'', F), 50 µm (B', E, G', H', I', I''), 20 µm (G'').

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

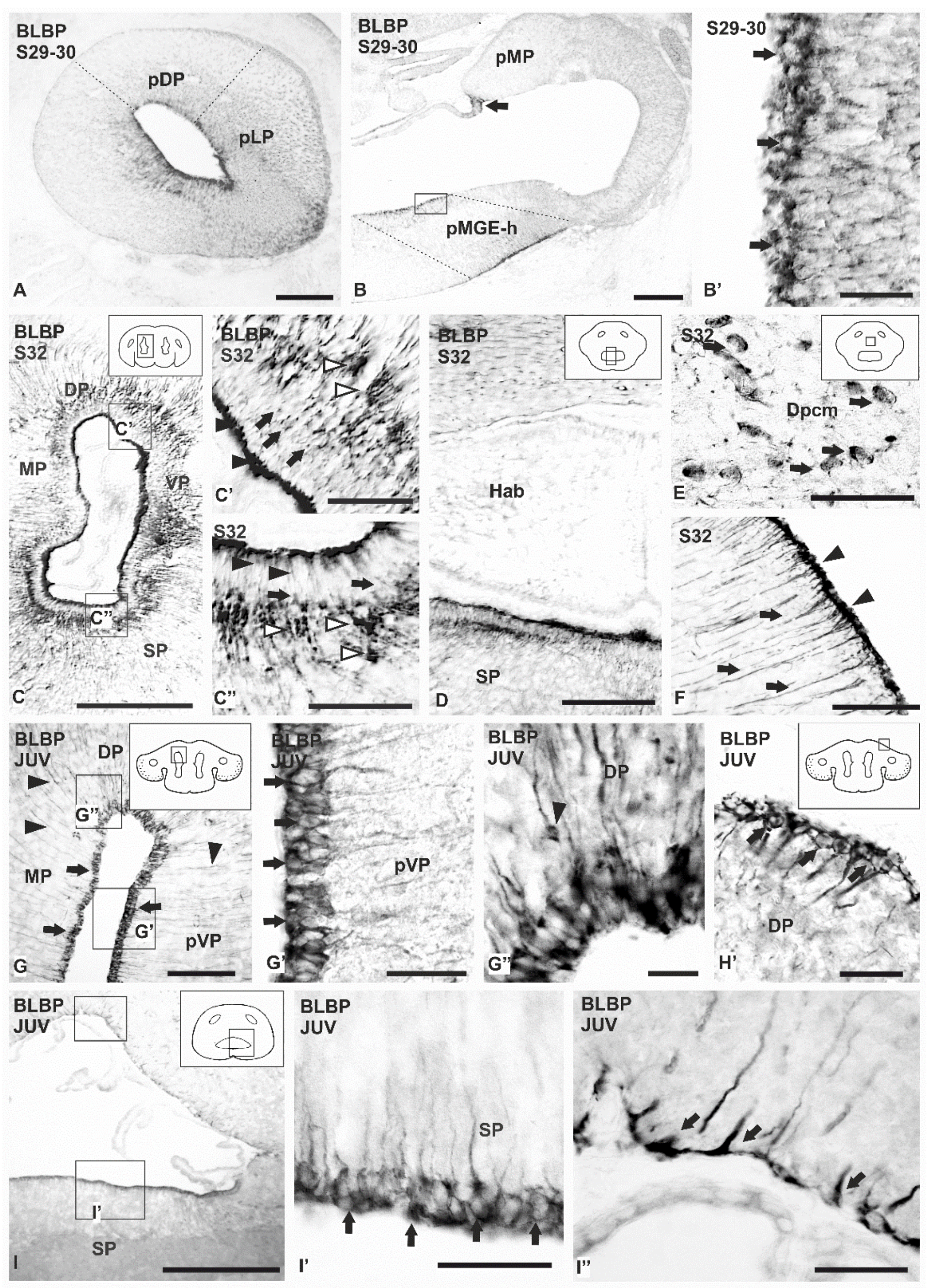


FIGURE 5

Figure 6. Photomicrographs of different sections of the telencephalon of *Scyliorhinus canicula* showing the expression pattern of GS during development **(A-D)** and juveniles **(E-N).** (A) Panoramic view of a transverse section of the telencephalon of a S32 of development showing GS immunolabeling. GS positive cells are mainly restricted to the ventricular zone, both in pallium (arrows) **(A')** and subpallium (empty arrowheads) **(A'').** Dilatations are pointed with arrowheads in A'-A''. **(B)** Disperse GS positive cells are present in the subpallium near to the ventricular zone (arrows) and large cells are observed in the caudal dorsal pallium pars centromedialis (arrowheads) **(C). (D)** Photomicrograph showing radial processes (arrows) projecting to the pial surface, where they ramify and finish in endfeet (arrowheads). **(E-N)** Transverse sections of the telencephalon of catshark showing immunoreactivity for GS in the ventricular cells in pallium **(E)** and subpallium **(F)**. **(G-I).** Details of the ventricular zone of the telencephalon at different levels showing GS positive cells. **(J)** Transverse section of the pallial ventricle showing paraventricular radial cells with a long basal prolongation (arrows)**. (K-L)** Photomicrographs of disperse cells in the subpallium (arrows) and in the caudal telencephalon (arrowheads), respectively, immunoreactive for GS**. (M-N)** Transverse sections of the telencephalon showing differences in the amount of immunoreactive processes between pallium **(M)** and subpallium **(N).** Scale bars: 500 µm (A), 200 µm (E, F), 100 µm (B), 50 µm (D, J, K, L, M, N), 20 µm (A', A'', C), 10 µm (G, H, I).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

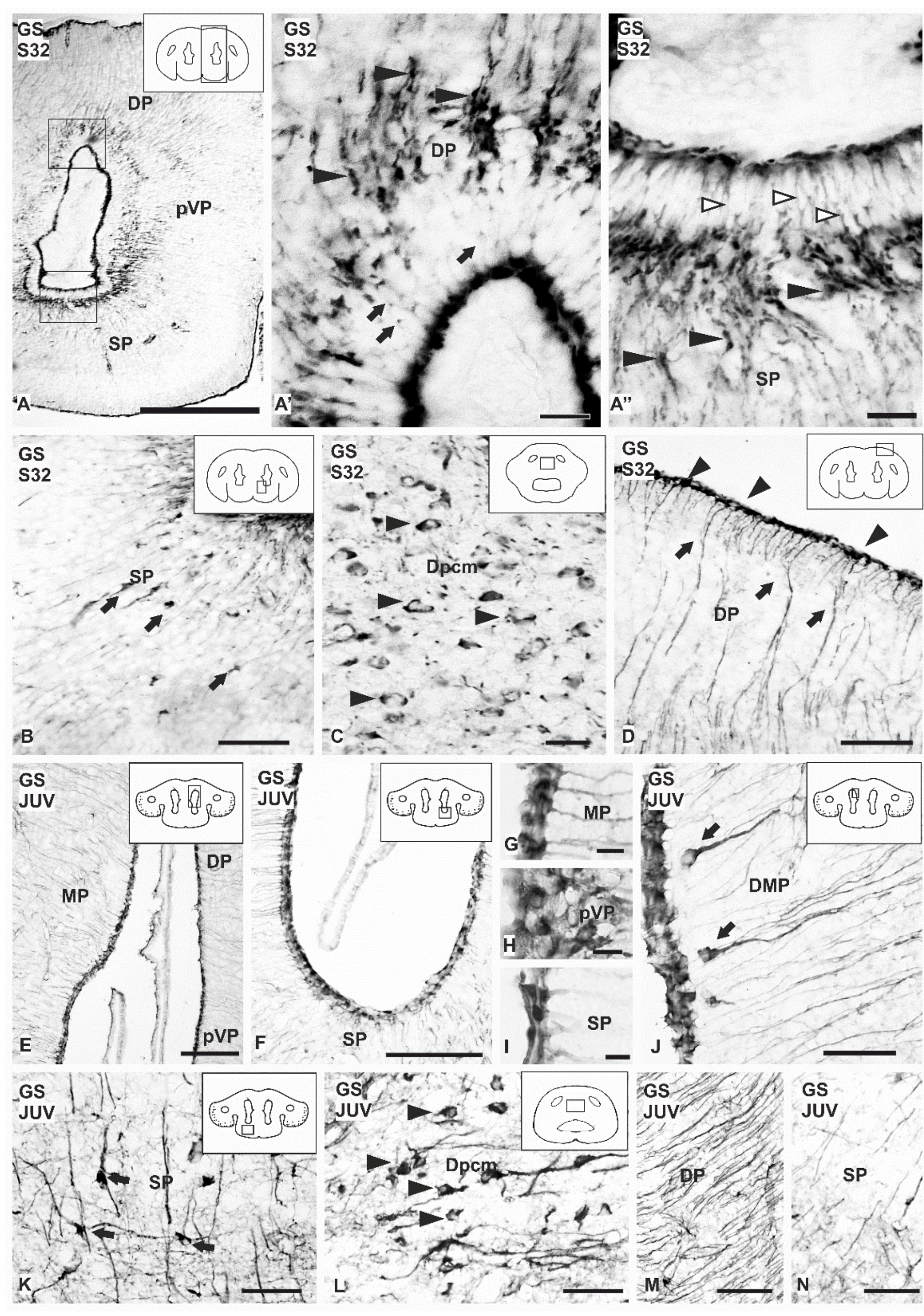


FIGURE 6

Figure 7. Photomicrographs of the telencephalic ventricle during development showing double immunolabeling for GFAP and GS **(A-A'')**, BLBP and GS **(D-D'')** and cell counting **(B, C, E, F)**. **(A-A'')** Details of the ventricular zone of a S33 of development showing double-immunolabelled cells (white stars) and a few cells where both glial markers do not colocalize (yellow stars). **(B-C)** Bar representations of percentage of GFAP/GS single and double-labelled cells in the dorso-medial pallium and presumptive ventral pallium, respectively. (**D-D'')** Details of the ventricular zone of a late S32 of development showing double-immunolabelled cells (white stars) and a few cells where both glial markers do not colocalize (yellow stars). **(E-F)** Bar representations of percentage of GFAP/GS single and double-labelled cells in the dorso-medial pallium and presumptive ventral pallium, respectively. Scale bars: 25 µm.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

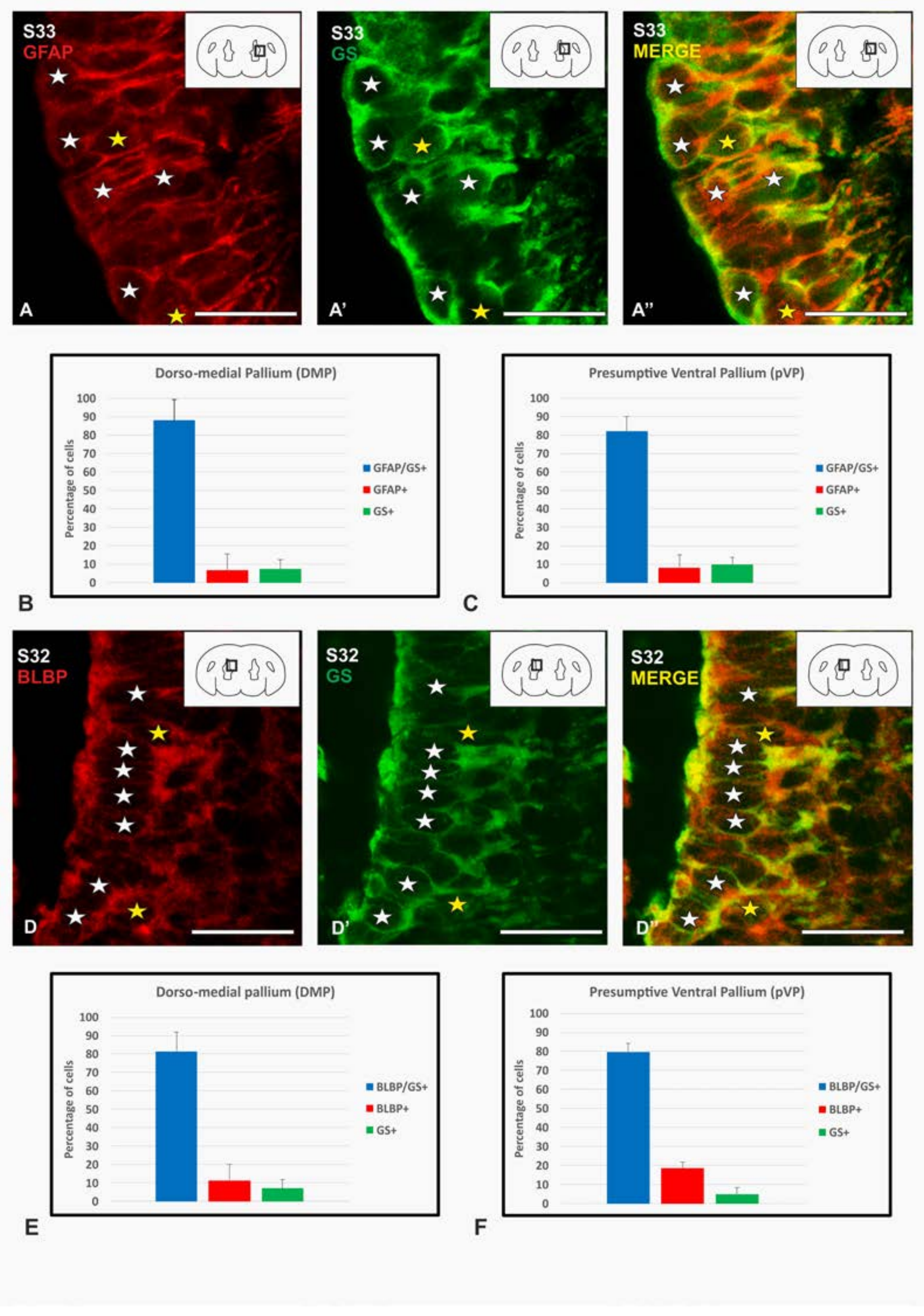


FIGURE 7

Figure 8. Photomicrographs of different sections of the telencephalon of *S. canicula* in S33 embryos showing double immunofluorescence for PCNA and GFAP or BLBP. **(A)** Panoramic view of the telencephalic ventricle showing double immunofluorescence for PCNA and GFAP. **(B-C'')** Details of the ventricular zone of dorsal pallium and presumptive ventral pallium showing numerous double-labelled cells (white stars) and some BLBP- PCNA+ cells (yellow stars). **(D)** Panoramic view of the telencephalic ventricle showing double immunofluorescence for PCNA and BLBP. **(E-F'')** Details of the ventricular zone of dorsal pallium and presumptive ventral pallium showing numerous double-labelled cells (white stars) and some BLBP- PCNA+ cells (yellow stars). In higher magnification details the ventricle is on the left side of the photomicrographs. Scale bars: 200 µm (A, D), 25 µm (B, B', B'', C, C', C'', E, E', E'', F, F', F'').

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

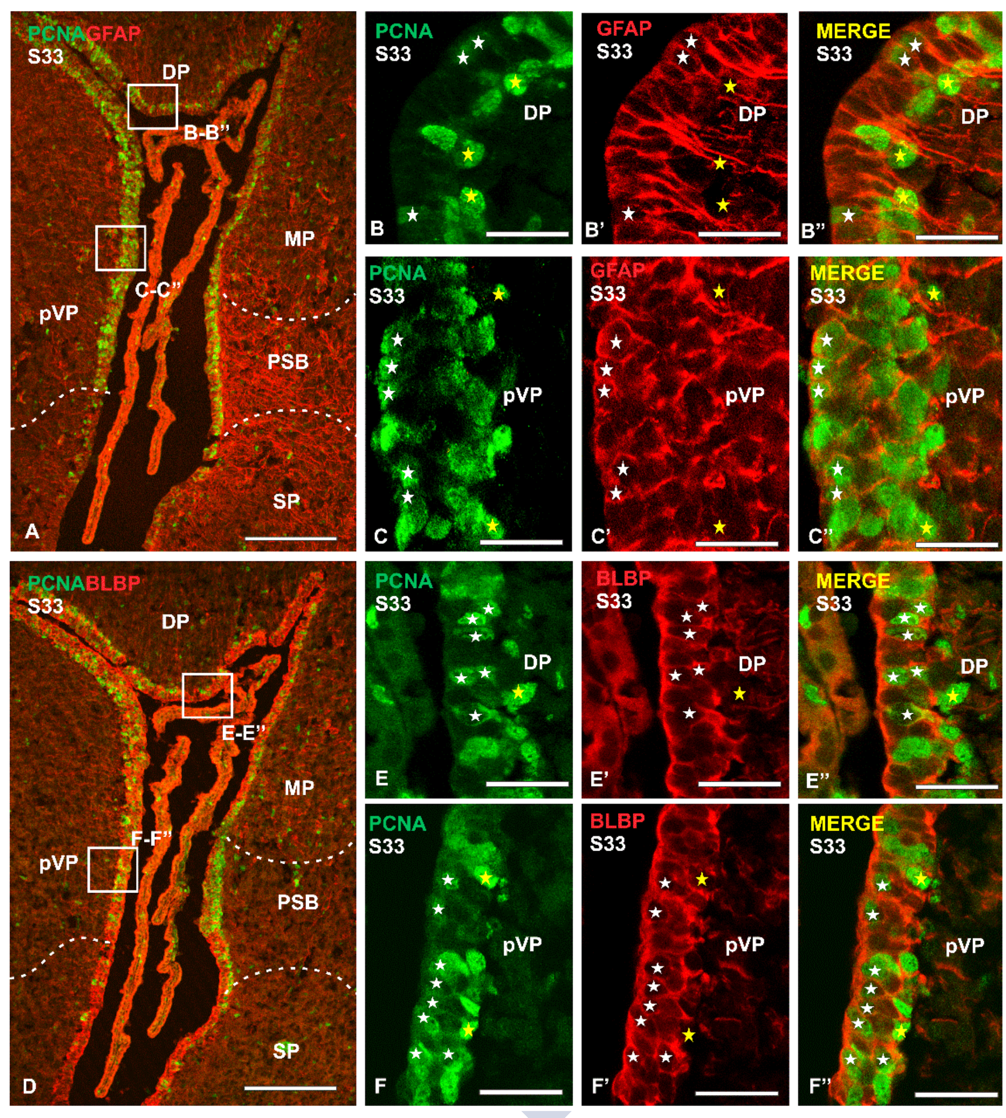


FIGURE 8

Figure 9. Transverse sections of the pallium of a S34 of development (prehatching) at different magnifications showing double immunofluorescence for the three glial markers BLBP, GFAP, GS and the proliferating markers PCNA, PH3. **(A)** Panoramic view of the telencephalic ventricle showing double immunofluorescence for BLBP and PCNA. **(B)** Panoramic view of the telencephalic ventricle showing double immunofluorescence for GFAP and PCNA. **(C)** Panoramic view of the telencephalic ventricle showing double immunofluorescence for GS and PH3. **(D-D'')** Details of the ventricular zone of a S34 of development showing double-immunolabeled cells for BLBP and PCNA (white stars). **(E-E'')** Details of the ventricular zone of a S34 of development showing double-immunolabeled cells for GFAP and PCNA (white stars). **(F-F'')** Details of the ventricular zone of a S34 of development showing double-immunolabeled cells for GS and PH3 (white stars). In higher magnification details the ventricle is on the right side of the photomicrographs. Scale bars: 100 µm (A, B, C) 25 µm (D-F'').

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

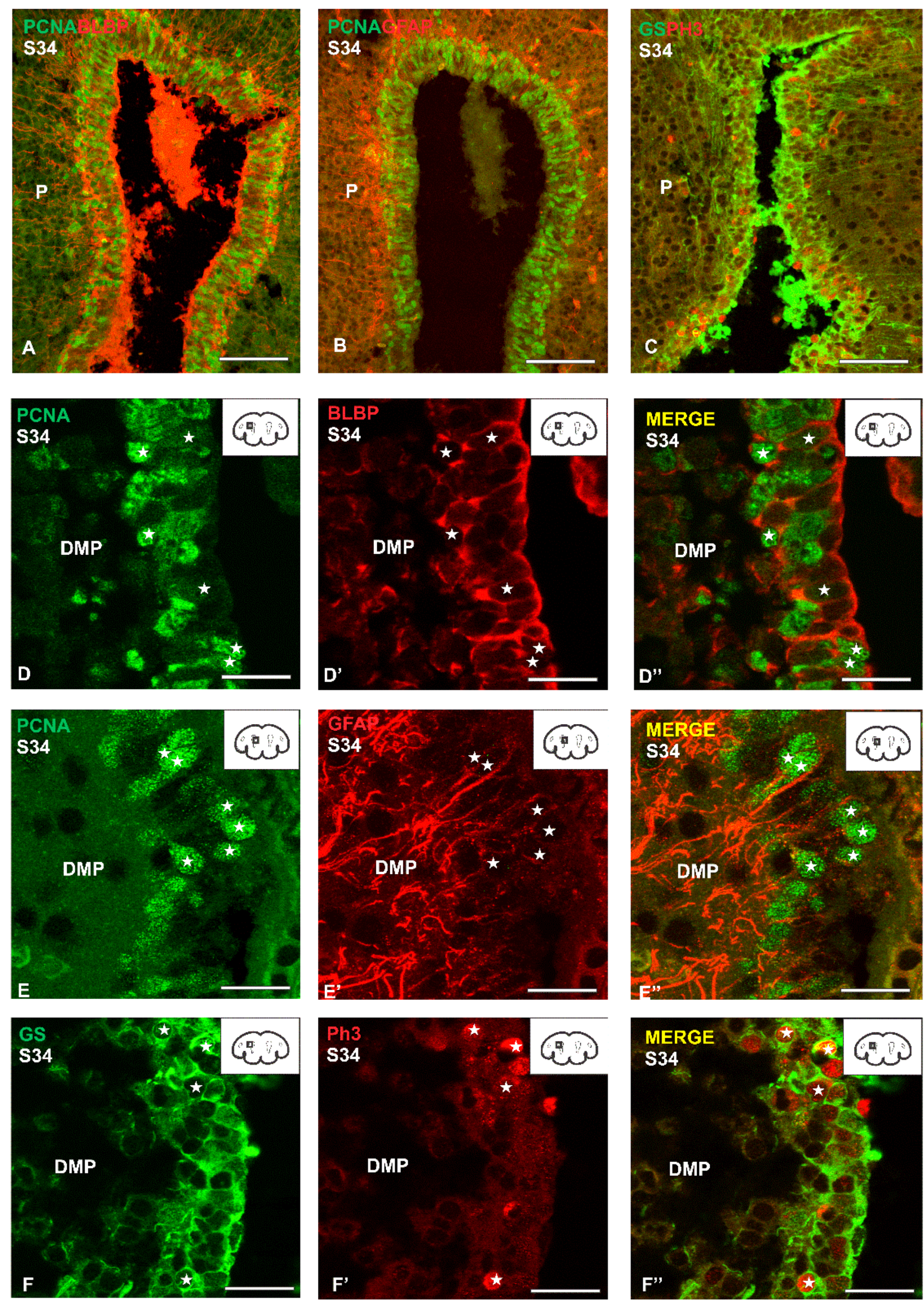
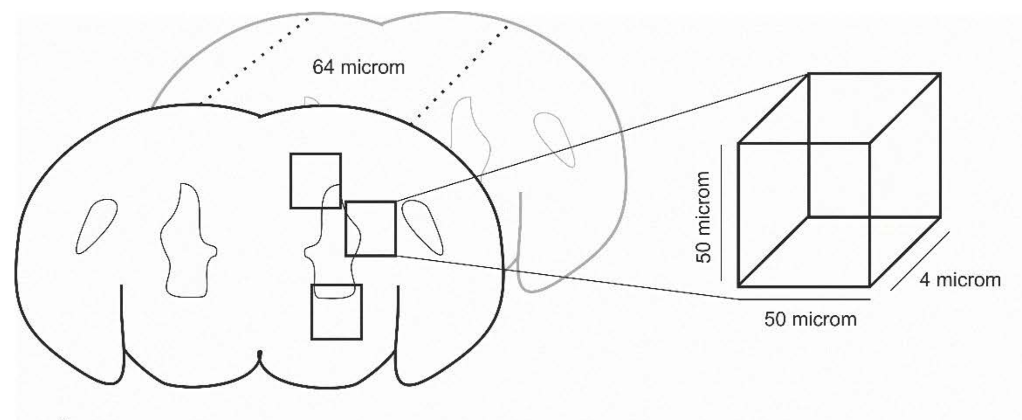


FIGURE 9

Supplementary Figure 1. (A) Schema of the areas selected for cell counting, box area and distance between sections selected. **(B)** Table showing averages and standard deviations included in Figs. 7B, C, E, F. (**C, D).** Bar representations showing percentage of cells grouped by marker and area.



A

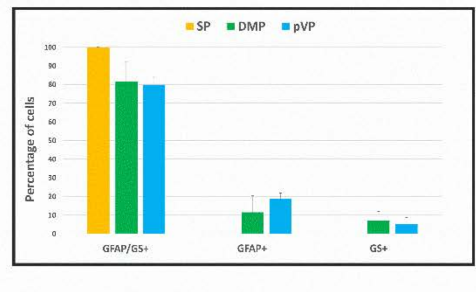
CELL NUMBERS CORRESPONDING TO FIGURE 7 (B, C)

	BLBP+GS+	BLBP+ only	GS+ only
SP	100 ± 0	0 ± 0	0 ± 0
DMP	81,47 ± 10,57	11,43 ± 8,88	7,083 ± 4,78
PVP	79,82 ± 4,45	18,61 ± 3,08	5,13 ± 3,44

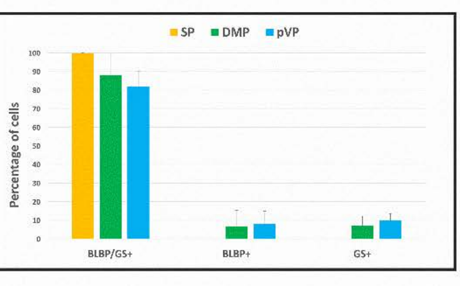
CELL NUMBERS CORRESPONDING TO FIGURE 7 (E, F)

	GFAP+GS+	GFAP+ only	GS+ only
SP	100 ± 0	0 ± 0	0 ± 0
DMP	88,13 ± 11,34	6,81 ± 8,70	7,32 ± 4,97
PVP	82,02 ± 8,05	8,17 ± 6,8	9,79 ±3,76

B



C



D

SUPPL. FIGURE 1

CHAPTER 3

CHARACTERIZATION OF NEUROGENIC NICHES IN THE TELENCPEHALON OF JUVENILE AND ADULT SHARKS

Introduction

Neurogenesis can be defined as a series of developmental events leading to a new neuron (Kemperman, 2002). This definition involves the existence of a number of progenitor cells that progressively restricts its proliferative potential and number of possible fates to become a fully functional neuron (Hevner et al. 2006). Despite it was believed that the neurogenic process was only restricted to the developmental period, in the past 60 years it has been discovered that adult individuals also present constitutive neurogenesis. In the mammalian brain, adult neurogenesis takes place mainly in two regions of the telencephalon: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (first described by Altman, 1963) and the subventricular zone (SVZ) of the lateral walls of telencephalic ventricles (first described by Altman 1969). Despite adult neurogenesis in the rodent brain has been largely proved, in primates this issue remains, to date, controversial (Boldrini et al. 2018; Sorrells et al. 2018; reviewed by Kempermann et al. 2018).

In the adult telencephalic niche of mammals, new-born neurons are generated from adult neural stem cells (aNSCs) also called, in a general way, adult progenitors (Ming and Song, 2011). Adult progenitors in mouse have been extensively characterised. Two models about the identity of these precursors were initially postulated (Ming and Song, 2011): (1) progenitors are radial glial cells that express both astroglial markers and stem cell markers (Álvarez-Buylla and Lim, 2004; Ma et al. 2009); and (2) progenitors do not express glial markers but they express stem cell markers such as *Sex determining region Y-box 2* (Sox2; Suh et al. 2007). Later, it has been discovered that both models were not mutually exclusive but rather complementary, revealing the wide diversity of adult progenitor cells (Bonaguidi et al. 2012), and the necessity of deepen in the characterization of progenitor cells in the adult brain.

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Nowadays, it is accepted that adult progenitor cells in mammals can be subdivided in radial and non-radial progenitors. Radial progenitors are cells that have the capacity of self-renewal, show long-time maintenance of the immature state and can generate different kind of neurons (Bonaguidi et al. 2016). Radial progenitors occasionally divide and generate non-radial progenitors (Bonaguidi et al. 2012). However, they normally display a relatively quiescent state. These type of cells are known as B cells in the SVZ and as Type-1 cells in the SGZ (Doetsch et al. 1997, 1999; Seri et al. 2004; Ming and Song, 2011; Bond et al. 2015; Bonaguidi et al. 2016; Lim and Alvarez-Buylla, 2016). These progenitors express the glial fibrillary acidic protein (GFAP), the brain lipid binding protein (BLBP) and Sox2, among others. On the other hand, non-radial progenitors are intermediate progenitor cells (IPCs). IPCs are actively dividing cells that lack radial prolongations and express proliferating and neuronal lineage markers that depend on their future phenotype (Suh et al. 2007; Lugert et al. 2010): GABAergic progenitors express the distal less marker Dlx2 and glutamatergic progenitors express the T-box transcription factor Tbr2 (Hodge et al. 2012). These cells are known as C cells in the SVZ and as Type-2 cells in the SGZ (Doetsch et al. 1997, 1999; Seri et al. 2004; Steiner et al. 2006; Ming and Song, 2011; Bond et al. 2015; Bonaguidi et al. 2016; Lim and Alvarez-Buylla, 2016). IPCs undergo mitosis generating more IPCs or two migratory neuroblasts. These neuroblasts exit the neurogenic niche and migrate to their final destinations in the brain. In the case of the SVZ, these neuroblasts are called A cells and migrate following a particular tangential pathway to the olfactory bulb called rostral migratory stream (RMS) (reviewed by Lim and Alvarez-Buylla, 2016). In the SGZ, these cells are called Type-3 cells and migrate locally to their final destination on the hippocampus. Both neuroblasts of the SVZ and SGZ express the same lineage markers than the IPCs. In the SVZ, Dlx2- (GABAergic phenotype) or Tbr2- (glutamatergic phenotype) expressing neuroblasts have

been found (Lledo et al. 2008; Hodge et al. 2012). However, in the SGZ, only glutamatergic Tbr2-expressing neuroblasts have been detected (Hodge et al. 2012). The expression of neuronal lineage markers as the cytoskeletal proteins Doublecortin (DCX) and the polysialylated-neural cell adhesion molecule (PSA-NCAM) are usually used as markers of migratory neuroblasts. The absence of proliferation markers allows to differentiate postmitotic neuroblasts (immature neurons) from progenitor cells (Hodge et al. 2012).

Adult neurogenesis has also been studied in the telencephalon of other non-mammalian species such as birds (Goldman and Nottebohm 1983; Alvarez-Buylla et al. 1990, 1992, 1998; Mazengenya et al. 2018), reptiles (Perez-Cañellas and García-Verdugo, 1996; Font et al. 2001), amphibians (Simons et al. 2008; Kirham et al. 2014) and teleost fishes (Adolf et al. 2006; Grandel et al. 2006; Zupanc 2006; März et al. 2010; reviewed by Ganz and Brand, 2016). High similarities between mammals and these groups have been found regarding the main type of cells and the molecular markers they express. However, the organization of different cell types within the niche differs among vertebrates. In mammals the distinction between progenitor radial glia and differentiated glia is clear. However, in fishes, but also sometimes in amphibians or even reptiles, the term radial glia has been used to refers both radial glial progenitors and radial glial differentiated populations including, in the telencephalon, ependymal cells (ependymoglia) or tanycites (reviewed by Cuoghi and Mola, 2009). Although progenitor radial glial cells, ependymoglial cells and tanycytes are indeed different types of cells, in anamniotes these cell types share most part of molecular markers (Kirkham et al. 2014; Than-Trong and Bally-Cuif, 2015).

Comparative studies have evidenced that the neurogenic capacity becomes more restricted to anterior parts of the brain during the course of evolution. Besides, fishes are the group of vertebrates with the highest

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

neurogenic potential (Ganz and Brand, 2016). However, most studies in fishes have been performed in modern teleost as zebrafish, and almost none were performed in cartilaginous fishes (Quintana-Urzainqui et al. 2015).

The telencephalon of cartilaginous fishes is a big non-laminated structure that represents the 50% of the total cerebral mass (Yopak et al. 2015). Contrary to the everted telencephalon of teleosts, the telencephalon of cartilaginous fishes develops by evagination as in all other jawed vertebrates (Bulter and Hodos, 2005; Nieuwenhuys, 2009), which eases comparative studies. Besides, its phylogenetic position as a sister group of gnathostomes with a bony skeleton that gave rise to land vertebrates makes it essential in assessing the ancestral condition of adult neurogenesis in the brain of jawed vertebrates (Rodríguez-Moldes et al. 2011). Developmental studies in cartilaginous fishes have evidenced high similarities to mammals concerning proliferating patterns and migratory routes in the developing telencephalon (Carrera et al. 2008; Quintana-Urzainqui et al. 2015). Concerning adult neurogenesis, recent studies have evidenced the existence of adult progenitors in the telencephalon of cartilaginous fishes (Quintana-Urzainqui et al. 2015). This study proposed the existence of a RMS homologue in sharks. However, a deep molecular characterization of juvenile progenitor cells and new-born neurons in the telencephalon is lacking.

With the aim of extending the knowledge on the evolution of adult neurogenesis, we have performed a detailed analysis of the proliferating niches in the telencephalon of juvenile and adult specimens of the lesser spotted dogfish *Scyliorhinus canicula*, by using antibodies against the proliferating cell nuclear antigen (PCNA). Then we have characterized different types of cells located in the neurogenic niches of the telencephalon. We have investigated the expression pattern of the stem cell factor Sox2 and the astroglial markers GFAP

(glial fibrillary acidic protein), BLBP (brain lipid binding protein) and GS (glutamine synthase), markers typically used for detecting progenitor radial glial cells in vertebrates; and we have also examined the expression of neuronal lineage markers *ScDlx2*, *ScTbr2* and doublecortin (DCX) in order to determinate the possible existence of IPCs and neuroblasts.

Materials and Methods

Experimental animals

In the present study we have analysed 10 juveniles of *S. canicula* from 10 to 25 cm long (early and late juveniles) and one adult (50 cm long). Individuals were kindly provided by the aquarium of O Grove (Galicia, Spain). Sharks were raised in seawater tanks under standard conditions of temperature (15-16 ºC), pH (7.5-8.5) and salinity (35 g/L); suitable measures were taken to minimize animal pain and discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by Spanish Royal Decree 53/2013 for animal experimentation and were approved by the Ethics Committee of the University of Santiago de Compostela.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Tissue processing

Juveniles were deeply anesthetized with 0.5% tricaine methane sulfonate (MS- 222; Sigma, St. Louis, MO) in seawater and then perfused intracardially with elasmobranch Ringer´s solution (see Ferreiro-Galve et al. 2012) followed by 4 % PFA in Elasmobranch Phosphate Buffer (EPB). Brains were removed and postfixed in the same fixative for 24-48 h at 4 ºC. Subsequently, they were rinsed in PB saline (PBS), cryoprotected with 30 % sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (18-20 µm thick) were obtained in

transverse planes on a cryostat and mounted on to Superfrost Plus (Menzel-Glasser, Madison, WI, USA) slides.

In situ hybridization

We applied in situ hybridization (ISH) for *S. canicula Sox2, Tbr2/Eomes* and *Dlx2 (ScSox2, ScTbr2, and ScDlx2)* probes. These genes were selected from a collection of *S. canicula* embryonic cDNA library (mixed stages S9 to 22) and submitted to high throughput EST sequencing (coordinated by Dr. Sylvie Mazan). Sense and antisense digoxigenin-UTP-labeled *ScSox2, ScTbr2* and *ScDlx2* were synthesized directly by transcription *in vitro*. ISH was performed on cryostat sections of juveniles following standard protocols (Coolen et al. 2009). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65º C and incubated with the alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Roche Applied Science, Manheim, Germany) overnight at 4º C. The colour reaction was performed in the presence of BM-Purple (Roche). Finally, sections were dehydrated and coverslipped. Control sense probes did not produce any detectable signal.

Immunohistochemistry

Sections were pre-treated with 0.01 M citrate buffer pH 6.0 for 30 min at 90 ºC for heat-induced epitope retrieval and allowed to cool for 20 min at room temperature (RT). Sections were rinsed in 0.05 M Tris-buffered saline (TBS) pH 7.4 for 5 min and treated with 10% H_2O_2 in TBS for 30 min at RT to block endogenous peroxidase activity. Sections were rinsed in 0.05 M TBS pH 7.4 for 5 min, and incubated approximately for 15 h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate secondary antibody (see Table 1) for 1

hour at RT. All dilutions were made with TBS containing 15% normal goat serum (Millipore, Billerica, MA), 0.2% Triton X-100 (Sigma) and 2% bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Then, sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each. The immunoreaction was developed with 0.25 mg/ml diaminobenzidine (DAB) tetrahydrochloride (Sigma) in TBS pH 7.4 and 0.00075 % H_2O_2, or with SIGMAFAST™ 3.3-DAB tablets as indicated by the manufacturers. For immunohistochemistry against PCNA in adult individuals 2.5mg/mL of nickel ammonium sulphate was added. Finally, the sections were dehydrated, and coverslipped. Information about the primary and secondary antibodies is included in Table 1.

Double in situ hybridization-immunohistochemistry

We applied double ISH-immunohistochemistry for *ScSox2, ScTbr2 and ScDlx2* probes and PCNA antibody. In this procedure, ISH has been performed first, following the procedure described above. Colour reaction was stopped by rinsing twice in phosphate buffer solution (PBS) for 10 min each and then in PFA 4% for 45 min. Then, immunohistochemistry was performed as described above.

Double-immunofluorescence

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 90 ºC and allowed to cool for 20 min at RT. Sections were rinsed in 0.05 M TBS pH 7.4 for 5 min and incubated approximately for 15 h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate combination of fluorescent dye-labelled secondary antibodies (see Table 1) for 1 hour at RT. All dilutions were made with TBS containing 15

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

% normal donkey serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % BSA (Sigma). All incubations were carried out in a humid chamber. Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each and in distilled water for 30 min too. Sections were then allowed to dry for 30 min at 37 ºC, and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany). For double immunofluorescence using antibodies of the same species, microwaving protocols defined by Tornehave et al. (2000) where applied. Information about the primary and secondary antibodies is included in Table 1.

Control and specificity of antibodies

The PCNA is present in proliferating cells, and its expression is stronger during the S phase (Zerjatke et al. 2017). However, its expression is persistent along the entire cell cycle. The PCNA antibody has been previously used to label progenitor cells in the brain and retina of dogfish (i.e. Quintana-Urzainqui et al. 2015; Sanchez-Farías and Candal, 2015). The specificity of the antibodies against the glial markers GFAP, BLBP and GS has been tested by western blot (data not shown). The specificity of the anti-DCX antibody has been also tested by western blot by Pose-Mendez et al (2014).

Imaging

Fluorescent sections were photographed with the Leica TCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Confocal images were acquired separately for each laser channel with steps of 1 µm along the z-axis, and collapsed images were obtained with the LITE software (Leica). On the other hand, light field images were obtained with an Olympus BX51 microscope equipped with an Olympus DP71 colour digital camera. Both fluorescent and light field photographs were adjusted for contrast,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

brightness and intensity using Corel Draw X3. Plates also were prepared using the same software.

Results

The telencephalon of *S. canicula* has been classically subdivided in three parts: the olfactory bulbs, the telencephalic hemispheres (pair ventricles, divided in pallium and subpallium) and the impar telencephalon (enclosing an unpair ventricle, from the anterior commissure to the caudal part of the optic chiasm; Smeets et al. 1983). In this classic view, the telencephalic hemispheres are located topographically rostral to the impar telencephalon that, in turn, is located rostral to the hypothalamic subdivisions (See Figure 1A-C). However, modern neuromorphology evidences that this view does not reflect the topologically invariant organization of the telencephalon. According to the prosomeric model, the prosencephalon is divided in two transverse segments termed prosomeres (Fig. 1D, E). The caudal one includes the telencephalic hemispheres (divided in pallium and subpallium). The rostral one includes the preoptic area, a subpallial compartment located between the anterior commissure and the hypothalamus. (for more information about the prosomeric model in the catshark, see Santos-Durán et al. 2015 and Rodríguez-Moldes et al. 2017). Despite developmental and genoarchitecture studies in *S. canicula* support the prosomeric model, descriptions in what follows are made according to rostro-caudal topographical axes that, for most species and according to literature, is the prevailing way to describe neuroanatomical subdivisions in the adult.

In the present study we have investigated the expression pattern of the proliferating cell nuclear antigen (PCNA) in other to determine the presence of proliferating areas in the telencephalic hemispheres and preoptic area of juveniles of *S. canicula.* Then we aimed at characterizing proliferating cells

within the ventricular layer by analyzing the distribution pattern of the stem cell marker *ScSox2*, the glial markers GFAP, BLBP and GS and the IPC and neuronal lineage markers *ScDlx2, ScTbr2* and DCX.

PCNA expression pattern

In juveniles PCNA-ir cells are mainly located in the ventricular zone (VZ) of the olfactory bulb, lateral ventricles of the telencephalic hemispheres and also in the VZ of the preoptic area. Rostrally, in the telencephalic hemispheres PCNA-ir cells were distributed homogeneously through the VZ of the pallial and subpallial subdivisions (not shown). In intermediate levels of the telencephalon, numerous PCNA-ir cells were appreciated in the VZ, but remarkable differences among the VZ of different telencephalic regions were observed. First, PCNA positive cells were considerably more abundant in the VZ of the pallium than in the VZ of the subpallium (Fig. 2A, A'). In addition, differences in the number of PCNA-ir cells were observed along the pallial VZ: dorsolateral and ventral portions of the pallial ventricle exhibit considerably more PCNA-ir cells than the VZ of the medial pallial subdivision (Fig. 2A, A''). Also, fusiform PCNA positive cells can be detected emerging from the dorsal pallial ventricle (arrows in Fig. 2B). Olfactory ventricles have also numerous PCNA-ir cells (Fig. 2C). In the preoptic region (Fig. 2D), numerous PCNA-ir cells were observed in the lateral portions of ventricle, but the medial VZ is devoid of PCNA immunoreactivity (Fig. 2D, D'). Disperse adventricular PCNA-immunoreactive (-ir) cells were detected both in the pallium and subpallium, as well as in the olfactory bulb (Fig. 2A-C), and their amount decreased in the preoptic region (Fig. 2D-D'). In addition, immunoreactive cells can be appreciated in the choroid plexus, specially concentrated in the region that makes contact with the VZ (arrow in Fig. 2E). A similar pattern can be

observed in adult specimens (50 cm long Fig. 2F-H), though the relative number of PCNA-ir cells appears to decrease with respect to juveniles.

Sox2 expression pattern

In the adult mammalian brain, a subpopulation of progenitor cells retains the expression of the stem cell marker Sox2 (Bonaguidi et al. 2016). In addition, previous studies in adult zebrafish have reported that, in the VZ of the telencephalic hemispheres, most ventricular cells express Sox2 (März et al. 2010). In order to investigate whether Sox2 is expressed in the whole ventricular layer or it defines subpopulations of proliferating cells as in mammals, we studied the expression pattern of *ScSox2* by *in situ* hybridization in the telencephalon of *S canicula*.

The whole VZ of the telencephalic hemispheres (Figs. 3A, A') and olfactory bulbs (not shown) were positive for *ScSox2*. Also, the impar ventricle exhibited *ScSox2* expressing cells in the ventricular surface (Fig. 3B). *ScSox2* expressing cells were absent from the choroid plexus (arrow in Fig. 3B').

In order to determinate if *ScSox2* positive cells exhibit proliferative characteristics we performed double ISH-immunohistochemistry for *ScSox2* and PCNA. We detected numerous double-labeled cells for *ScSox2* and PCNA in the VZ of all pallial areas studied, including the dorsolateral pallium (Figs. 3C-C''), the medial pallium (Figs. 3D, D') and the presumptive ventral pallium (Figs. 3D, D'') and in the olfactory ventricle. A few double labeled cells were also observed in the subpallium (not shown).

Double immunofluorescence against glial markers

The brain of teleost fishes possesses a considerable quantity of radial glial cells/ependymal cells in the ventricular layer that have been shown to be immunoreactive for the astroglial markers GFAP, BLBP and GS (Rubio et al.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

1992; Kálmán, 1998; Kálmán and Ari, 2002; Arochena et al. 2004; Lazzari and Franceschini, 2004; Grupp et al. 2010; Diotel et al. 2016). The brain of cartilaginous fishes also exhibits ependymoglial cells (or tanycytes, Horstmann, 1954) in the ventricular layer that have been labeled with same antibodies than in teleosts (Wasowicz et al. 1999; Kálmán and Gould, 2001; Ari and Kálmán, 2008a, b). As studies in zebrafish have established that most of proliferating cells in the ventricular layer exhibit glial nature (reviewed by Than-Trong and Bally-Cif, 2015), we have performed double immunofluorescence for PCNA and the glial markers GFAP, BLBP and GS. We have mainly focused our analysis in the medial pallium and in the presumptive ventral pallium as representative areas containing low and high densities of proliferating cells, respectively.

Double immunofluorescence against GFAP and PCNA revealed abundant double-labelled cells in the VZ of the telencephalon. In the rostral telencephalon, where no differences regarding PCNA immunoreactivity were observed along the VZ, some double-labeled cells have been observed (stars in Figs. 4A, A', A''). However, in medial levels of the telencephalon, where the VZ of different telencephalic regions exhibits different levels of proliferation, the number of double-labelled cells increases considerably, especially in the presumptive ventral pallium, where the VZ seems to be wider than in other regions of the telencephalic ventricle (Figs. 4B, B', B'').

Concerning BLBP, double labeled cells can be appreciated in rostral levels, where the VZ is homogenous. However, at intermediate levels, both in the medial and presumptive ventral pallium numerous double-labeled cells have been found (stars in Figs. 4C, C', C'' and 3D, D', D'' respectively).

Finally, double immunolabeling for GS and PCNA has also been performed. Surprisingly, despite the abundancy of GS positive cells in the VZ,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

only a few double labeled cells have been found, even in the presumptive ventral pallial subdivision, where both GS-ir and PCNA-ir cells are more abundant than in other regions of the telencephalon (Figs. 4E, E', E'', E''', E'''').

Expression of molecular markers of IPCs and neuroblasts: Dlx2, Tbr2 and DCX

During adult neurogenesis, in mammals, the neurogenic niches generate GABAergic and glutamatergic new neurons (Hevner et al. 2006; Lledo et al. 2008; Mind and Song, 2011; Hodge et al. 2012; Bond et al. 2015; Lim and Alvarez-Buylla, 2016). These cell lineages are generated by intermediate progenitors (IPC) in the niche, which express Dlx2 and Tbr2 respectively. Here we have investigated whether PCNA-ir cells express markers of IPCs and what cell lineage they generate by studying the expression pattern of *ScTbr2* and *ScDlx2* by *in situ* hybridization. *ScTbr2* expressing cells can be observed in the OB, dorsolateral pallium and in the presumptive ventral pallial subdivision (Fig. 5A), but only in the presumptive ventral pallium they were located close to the VZ. However, no double *ScTbr2*-expressing and PCNA-ir cells have been observed in the telencephalic VZ (Fig. 5A'). As *ScTbr2* did not show any signal in the VZ of the telencephalic hemispheres, we focused on *ScDlx2* expression pattern. We have found *ScDlx2* expression in the pallial VZ of the telencephalic hemispheres, coincident with PCNA positive territories, and also in a periventricular band in the dorsal and medial pallial territories (Figs. 5B, B'). Double-labeled cells were clearly observed in the VZ of the dorsal (Figs. 5C, C') and medial pallium (Fig. 5D).

As part of the neurogenic niche, a population of migratory neuroblasts have been found in mammals and other species of vertebrates (i.e Alvarez-Buylla et al. 1998; März et al. 2010; Ming and Song, 2011; Kirham et al. 2014; Bond et al. 2015; Macedo-Lima et al. 2016). For that, we have also investigated

the distribution pattern of the microtubule associated protein DCX, a marker of migratory neuroblasts (Gleeson et al. 1999). DCX-immunoreactive cells were observed in both the VZ of the olfactory and pair ventricles (Figs. 6A’, A’’). DCX-immunoreactive cells have been also observed subventricularly. No immunoreactivity for DCX has been found in the VZ of the impair ventricle (Fig. 6B). In order to further characterize DCX positive cells, we have performed double immunofluorescence for DCX and PCNA. We have observed abundant double-labelled cells (yellow stars in Figs. 6C, C’, C’’). In addition, DCX/PCNA-ir cells are also present even in subventricular positions (yellow stars in Fig. 6D, D’, D’’).

Discussion

The study of adult neurogenesis has been a subject of interest during the past decades, not only in mammals, but also in many different species of birds (reviewed by Barnea and Pravosudov, 2011), reptiles (reviewed by González-Granero, 2011), amphibians (Simons et al. 2008; Kirkham et al. 2014) and teleost fishes (reviewed by Ganz and Brand, 2016). Previous reports from our group have shown that the telencephalon of sharks contains proliferative cells in the VZ of the adult telencephalon (Quintana-Urzainqui et al. 2015). With the aim of extending the knowledge about adult neurogenesis in the telencephalon of sharks we have analysed the proliferating VZs of the telencephalon searching for different types of progenitors and studying the neuronal commitment of these progenitors. Then we have compared the characteristics of the neurogenic niche of sharks with other vertebrates. A summary of the main cell types located in the neurogenic niches across vertebrates is provided in Figure 7.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Telencephalic proliferation across evolution: the origin of adult neurogenesis.

One of the most interesting facts of neurogenic process is that, as we descend in the evolutive scale, the number of neurogenic niches and proliferating cells increases considerably (Ganz and Brand, 2016). In mammals and birds, neurogenesis in mainly restricted to some parts of the telencephalon such as the SVZ of the lateral ventricles and the SGZ of the dentate gyrus of the hippocampus in case of mammals (reviewed by Bond et al. 2015), and the VZ of the high vocal centre nucleus (HVC) (Goldman and Nottebohm, 1983) or the hippocampus in birds (Barnea and Nottebohm, 1994). In reptiles (mainly lizards) and amphibians (frog and salamander), it seems that the VZ of the telencephalon, regardless of the telencephalic subdomain, exhibits proliferating cells (reptiles: D'amico et al. 2011; reviewed by González-Granero, 2011; amphibians: Simons et al. 2008; Kirkham et al. 2014). Despite proliferating cells have been found in all the pallial subdivisions and in the subpallium, these cells are not abundant. However, in amphibians (an also in birds), there are several points of the VZ where proliferation is especially higher, which have been called "hot spots" (Álvarez-Buylla, et al. 1990; Kirkham et al. 2014). In teleost fishes, adult proliferation in the telencephalon has been extensively studied in several species like zebrafish, medaka or killyfish, among others (Ekström et al. 2001; Zupanc et al. 2005; Grandel et al. 2006, Kuroyanagi et al. 2010; Tozzini et al. 2012; reviewed by Ganz and Brand, 2016). In this group of fishes, the proliferation pattern is drastically higher compared to other vertebrates and can be observed more like a continuous band of proliferating cells rather than a few disperse ventricular cells. In the present study, we have investigated proliferation in the mature telencephalon of *S. canicula* by studying the expression pattern of PCNA, which labels proliferating cells (reviewed in Zerjatke et al. 2017). As in teleost fishes, in *S. canicula* the VZ of both the

pallium and the subpallium exhibit a rather continuous band of proliferating cells, though the subpallium exhibits a considerable less number of proliferating cells than the pallium. Despite we cannot conclude the existence of separate neurogenic niches, we have observed differences in the density of PCNA-ir cells in the VZ of different pallial regions, with a hot spot of proliferation in the ventral pallium. Interestingly, in zebrafish, the pallium presents smaller numbers of proliferating cells than the supallium, where cells are arranged in a more compact way (Grandel et al. 2006). However, it remains unexplored if this difference in the proliferation pattern results in different allometric relationships of different telencephalic domains to overall telencephalon size. In addition, as in other fishes (Zupanc et al. 2006), we have found many disperse cells throughout the telencephalon of *S. canicula* in adventricular positions, which suggest that migratory neuroblasts maintain their progenitor capacity long after they leave the ventricular zone.

The fact that basal vertebrates as sharks exhibit high levels of proliferation in the telencephalon in juveniles and adults (present results) confirms that the number of neurogenic niches and proliferating cells increases as we descend in the evolutive scale (Ganz and Brand, 2016). However, in contrast to what it could be expected, studies in lampreys (the most basal vertebrates) have demonstrated that PCNA immunoreactivity disappears at the end of the developmental period (Villar-Cheda et al. 2006). Therefore, the presence of a high number of proliferating cells in the telencephalon of the lesser spotted dogfish and the absence of proliferation in adult lampreys point to an evolutionary origin of adult neurogenesis in the transition from agnathans to jawed vertebrates.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

aNSCs in the telencephalon on shark express radial glia markers and show proliferative activity.

In the adult neurogenic niches of mammals, many cellular types coexist. One of these cells are radial glial cells (B cells in the SVZ and Type-1 in the SGZ). These cells have a glial nature and act as progenitor cells in the first steps of the neurogenic process, though they are relatively quiescent (see Introduction). They express glial markers such as GFAP, BLBP, GS, and stem markers such as Sox2, among others (Doetsch et al. 1997, 1999; Seri et al. 2004; Ming and Song, 2011; Götz et al. 2013; Bond et al. 2015; Bonaguidi et al. 2016; Lim and Alvarez-Buylla, 2016). In birds, studies using electron microscopy and pulse-chase labelling have identified radial glial cells in the VZ of the telencephalon with stem cell and neurogenic capacity (Álvarez-Buylla et al. 1990, 1998). In reptiles, similar studies with BrdU and tritiated thymidine have identified proliferating cells in the VZ of the telencephalon of lizards that were pointed to be proliferating radial glial cells (García-Verdugo et al. 1981; Pérez-Cañellas and García-Verdugo, 1996; Font et al. 2001; Grandel and Brand, 2013). Besides, studies in turtles allowed to find persistent radial glial cells in the VZ of the telencephalon (Clinton et al. 2014). However, these studies do not mention the proliferative capacity of these cells. In amphibians, studies in newts have evidenced that the entire VZ is comprised of radial glial cells labelled with GFAP or GS that additionally coexpressed Sox2. These cells also express proliferating markers in the previous mentioned hot spots, and they have neurogenic potential (Kirkham et al. 2014). However, outside the hot spots, they mainly rest in a quiescent state.

In teleost fishes, a detailed study in the telencephalon of zebrafish has identified numerous radial glial cells that express abundant glial markers such as BLBP or GFAP, and Sox2 (März et al. 2010). Many of these cells incorporate

BrdU at slow rates, which means they have proliferative capacity but they are mostly quiescent cells, as it was reported in the mammalian B cells. In the telencephalon of the catshark we have identified a dense VZ with radial cells immunoreactive to GFAP, BLBP, GS and *ScSox2*. GFAP, BLBP or GS positive cells with morphology of radial glia have already been reported in this species (Wasovicz et al. 1999; Kálmán and Ari, 2002), but its proliferative potential has never been investigated in juvenile specimens. Here, very few GS-ir cells were shown to be proliferative (PCNA-ir). In contrast, numerous BLBP-ir and GFAP-ir in the VZ were additionally PCNA-ir. However, we also found GFAP-ir, BLBP-ir and GS-ir cells that do not show proliferative activity. Besides, double ISH-IHC for PCNA-*ScSox2* has evidenced the presence of numerous double labelled cells. This scenario is quite similar to that reported in teleosts where quiescent and proliferating subtypes of radial glia have been defined (März et al. 2010), clearly supporting an ancient origin of radial glial cells as progenitor cells in the postnatal neurogenic process. Lampreys also exhibit radial glial-like cells in the adult telencephalon. These radial cells extend processes form the VZ to the pia (as in amphibians and fishes), but they express cytokeratins instead of glial markers such as GFAP (Merrick et al. 1995). However, no PCNA positive cells have been found in the telencephalic VZ of this species (Villar-Cheda et al. 2006), suggesting that adult radial glial-like cells in lampreys do not have proliferative potential. Of note, studies about proliferation in larvae of lampreys have shown that seasonal changes or lesions lead to a reactivation of proliferation in cytokeratin positive cells in the rombencephalon or spinal cord (Zhang et al. 2014), so it cannot be discarded that radial glial-like cells in the telencephalon of adult lampreys might be quiescent cells waiting for exogenous signals to be reactivated.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Intermediate progenitor cells and neuronal commitment

Once radial glial cells reactivate their cellular cycle and undergo mitosis, they generate fast cycling progenitors that subsequently generate neuroblasts. Fast cycling progenitors, also called IPCs or non-radial progenitors, exhibit neuronal commitment markers such as Dlx2 (GABAergic lineage marker) or Tbr2 (glutamatergic lineage markers) (Doetsch et al. 1997, Ming and Song, 2011; Hodge et al. 2012; Bond et al. 2015; Lim and Alvarez-Buylla, 2016). These IPCs are located close to the radial glial progenitors and do not exit the neurogenic niche. In contrast, migratory neuroblasts express DCX or PSA-NCAM and are able to exit the neurogenic niche to reach their final destination in the telencephalon (Doetsch et al. 1997). IPCs have been found in the brain of birds by using electron microscopy and tritiated thymidine incorporations (Álvarez-Buylla et al. 1998). However, their future neuronal phenotype has not been investigated, but the presence and increase of DCX immunoreactivity in the adult brain of birds with favourable neurogenic conditions clearly suggest a neuronal fate (Barnea and Pravasudov, 2011; Mazengenya et al. 2018). In reptiles, DCX positive cells have been detected in the adult brain of crocodiles (Ngwenya et al, 2017) and turtles (Macedo-Lima et al. 2016), but the existence of adult IPCs has not been proved. On the contrary, in amphibians, drug treatment with AraC (an IPC killer) and BrdU incorporations have clearly evidenced their existence (Kirkham et al. 2014). As in birds, there is no clue about the future phenotype of these cells, but the high levels of PSA-NCAM surrounding the proliferative ventricle clearly support their neuronal fate (Kirkham et al. 2014). In teleosts, experiments with BrdU incorporations have evidenced the existence of fast dividing cells, that express low levels of glial markers and start to express neuronal lineage markers such as PSA-NCAM, which match the definition of IPCs. (März et al. 2010). Close to this cell type, PSA-NCAM positive cells have been found, which have typical morphology

and markers of neuroblasts (März et al. 2010). The phenotype of these cells has not been investigated. However, BrdU pulse-chase studies have pointed the possibility that telencephalic progenitors contribute with dopaminergic cells to the olfactory bulb in fishes (Grandel et al. 2006). In addition, studies in the adult telencephalon have found Tbr2 and Prox1 positive cells (both markers of glutamatergic cells) in the VZ of different portions of the pallium of zebrafish (Ganz et al. 2015), suggesting the possibility of adult glutamatergic neurogenesis. In the present work we have investigated the existence of IPCs and migratory neuroblasts by using double IHQ against PCNA and ISH for *ScTbr2* (glutamatergic IPCs), *ScDlx2* (GABAergic IPCs) and DCX (neuronal commitment). We did not find *ScTbr2* expression neither in the VZ nor in the SVZ of the telencephalic ventricles, but rather in the intermediate zone, being especially abundant in the ventral pallium. Indeed, Tbr2 expression has been previously used to identify the ventral pallial subdomain in amphibians (Brox et al. 2004). The absence of *ScTbr2* in the VZ and SVZ suggests that IPCs (which are located in/close the ventricle) does not express *ScTbr2* and that the expression of this transcription factor is rather upregulated in migratory neuroblasts, which evidences the need of specific markers for glutamatergic IPCs. Further studies using cell-tracking are needed to clarify this point. In contrast we found *ScDlx2* positive cells ventricularly and subventricularly, many of them also positive for PCNA. Whether they are IPCs is not clear. BrdU or IPC inhibition treatments would be necessary to clarify this matter. However, the abundancy of double *ScDlx2-PCNA* positive cells suggests that some of them might be IPCs. As well, we found DCX positive cells in the ventricular and in subventricular positions, some of them also immunoreactive to PCNA. This clearly suggests that proliferating cells in the mature brain of sharks give rise to new neurons. Previous studies of our group have detected tyrosine hydroxylase positive cells in the VZ of the telencephalic walls, which have been

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

interpreted as newborn dopaminergic cells that would contribute to OB neurogenesis via a RMS-like (Quintana-Urzainqui et al. 2015). These dopaminergic cells in mammals are also GABAergic (Lledo et al. 2008), so we cannot discard that some of our S*cDlx2*-PCNA positive cells are also TH-positive.

Conclusions

This work represents the first study in cartilaginous fishes that has investigated adult neurogenesis by characterizing the telencephalic neurogenic niche. We have shown that the telencephalic VZ of the catshark exhibits high levels of proliferation, as in teleost fishes, but in contrast to lampreys, pointing to an evolutionary origin of adult neurogenesis in the transition from agnates to gnatostomes. We also have shown that the VZ exhibits high numbers of radial glial cells with proliferative potential, which are the main type of progenitors in fishes. Besides, we have pointed to the existence of IPCs of GABAergic, but not glutamatergic nature. Finally, some DCX-immunoreactive neuroblasts were found to be proliferative (immunoreactive to PCNA), which evidenced the neurogenic process in the telencephalon. In summary, we can conclude that basal vertebrates as sharks represent the ancestral condition of adult neurogenesis and that the main type of cells was present at the beginning of the scale of vertebrates and have persisted along the evolution.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

References

Adolf B, Chapouton P, Lam CS, Topp S, Tannhäuser B, Strähle U, Götz M, Bally-Cuif L (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev Biol* 295:278-93.

Altman J (1963). Autoradiographic investigation of cell proliferation in the brains of rats and cats. *Anat Rec* 145:573-591.

Altman J (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol* 137:433-457.

Álvarez-Buylla A, Theelen M, Nottebohm F (1990). Proliferation "Hot Spots" in adult avian ventricular zone reveal radial cell division. *Neuron* 5:101-109

Álvarez-Buylla A, Ling CY, Nottebohm F (1992). High vocal center growth and its relation to neurogenesis, neuronal replacement and song acquisition in juvenile canaries. *J Neurobiol*.23:396-406.

Álvarez-Buylla A, García-Verdugo JM, Mateo AS, Merchant-Larios H (1998). Primary neural precursors and intermitotic nuclear migration in the ventricular zone of adult canaries. *J Neurosci* 18:1020-37.

Álvarez-Buylla A, Lim DA (2004). For the long run: maintaining germinal niches in the adult brain. Neuron 41:683-6.

Ari C, Kálmán M (2008a). Glial architecture of the ghost shark (Callorhinchus milii, Holocephali, Chondrichthyes) as revealed by different immunohistochemical markers. *J Exp Zool B Mol Dev Evol* 310:504-19.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Ari C, Kálmán M (2008b). Evolutionary changes of astroglia in Elasmobranchii comparing to amniotes: a study based on three immunohistochemical markers (GFAP, S-100, and glutamine synthetase). *Brain Behav Evol* 71:305-24.

Arochena M, Anadón R, Díaz-Regueira SM (2004). Development of vimentin and glial fibrillary acidic protein immunoreactivities in the brain of gray mullet (Chelon labrosus), an advanced teleost. *J Comp Neurol* 469:413-36

Barnea A, Nottebohm F (1994). Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. *Proc Natl Acad Sci U S A*. 91:11217-21.

Barnea A, Pravosudov V (2011) Birds as a model to study adult neurogenesis: bridging evolutionary, comparative and neuroethological approaches. *Eur J Neurosci* 34:884-907.

Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, Rosoklija GB, Stankov A, Arango V, Dwork AJ, Hen R, Mann JJ (2018). Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell* 22:589-599.

Bonaguidi MA, Song J, Ming GL, Song H (2012). A unifying hypothesis on mammalian neural stem cell properties in the adult hippocampus. *Curr Opin Neurobiol* 22:754-61.

Bonaguidi MA, Stadel RP, Berg DA, Sun J, Ming GL, Song H (2016). Diversity of Neural Precursors in the Adult Mammalian Brain. *Cold Spring Harb Perspect Biol* 8:a018838

Bond AM, Ming GL, Song H (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell* 17:385-95.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Butler AB, Hodos W (2005). Comparative vertebrate neuroanatomy. Evolution and adaptation. 2nd ed. Wiley-Liss, Hoboken, NJ.

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R and Rodríguez-Moldes I (2008). Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. *Brain Res. Bull* 75(2-4):405-9.

Clinton BK, Cunningham CL, Kriegstein AR, Noctor SC, Martínez-Cerdeño V (2014). Radial glia in the proliferative ventricular zone of the embryonic and adult turtle, *Trachemys scripta elegans. Neurogenesis (Austin*) doi: 10.4161/23262125.2014.970905

Coolen M, Menuet A, Chassoux D, Compagnucci C, Henry S, Leveque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodriguez- Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009) The dogfish Scyliorhinus canicula, a reference in jawed vertebrates. Emerging model organisms. A laboratory manual. *Cold Spring Harbor, New York*, pp 431–446.

Cuoghi B, Mola L (2009). Macroglial cells of the teleost central nervous system: a survey of the main types. *Cell Tissue Res* 338:319-32.

D'Amico LA, Boujard D, Coumailleau P (2011). Proliferation, migration and differentiation in juvenile and adult *Xenopus laevis* brains. *Brain Res* 1405:31-48.

Diotel N, Vaillant C, Kah O, Pellegrini E (2016). Mapping of brain lipid binding protein (Blbp) in the brain of adult zebrafish, co-expression with aromatase B and links with proliferation. *Gene Expr Patterns* 20:42-54.

Doetsch F, García-Verdugo JM, Alvarez-Buylla A (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 17:5046-61.

Doetsch F, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97:703-16.

Ekström P, Johnsson CM, Ohlin LM (2001). Ventricular proliferation zones in the brain of an adult teleost fish and their relation to neuromeres and migration (secondary matrix) zones. *J Comp Neurol* 436:92-110.

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012). Dynamic expression of Pax6 in the shark olfactory system: evidence for the presence of Pax6 cells along the olfactory nerve pathway. *J Exp Zool B Mol Dev Evol* 318:79-90.

Font E, Defilis E, Pérez-Cañellas MM, García-Verdugo JM (2001). Neurogenesis and neuronal regeneration in the adult reptilian brain. *Brain Behav Evol* 58:276-295.

Ganz J, Kroehne V, Freudenreich D, Machate A, Geffarth M, Braasch I, Kaslin J and Brand M (2015). Subdivisions of the adult zebrafish pallium based on molecular marker analysis *F1000Res* 3:308.

Ganz J, Brand M (2016). Adult Neurogenesis in Fish. *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a019018.

García Verdugo JM, Berbel PJ, López García C (1981). [Golgi and electron microscopy study of cerebral ependymocytes of the lizard Lacerta galloti]. *Trab Inst Cajal* 72:269-78

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Gleeson JG, Lin PT, Flanaga LA and Walsh CA (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* 23:257-71.

Goldman SA, Nottebohm F (1983). Neuronal production, migration and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci USA* 80:2390-2394

González-Granero S, Lezameta M, García-Verdugo JM (2011). Adult neurogenesis in reptiles. In: Seki T, Sawamoto K, Parent JM, Alvarez-Buylla A (eds) Neurogenesis in the adult brain, vol. I. Springer, pp- 169-189.

Götz, M (2013). Radial glial cells. In: Kettenmann H, Ranson BR, editors. Neuroglia. New York: Oxford University Press. p 50-61

Götz M, Sirko S, Beckers J, Irmler M (2015). Reactive astrocytes as neural stem or progenitor cells: In vivo lineage, In vitro potential, and Genome-wide expression analysis. *Glia* 63:1452-68.

Grandel H, Kaslin J, Ganz J, Wenzel I, Brand M (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Dev Biol* 295:263-77.

Grandel H, Brand M (2013). Comparative aspects of adult neural stem cell activity in vertebrates. *Dev Genes Evol* 223:131-47.

Grupp L, Wolburg H, Mack AF (2010). Astroglial structures in the zebrafish brain. J *Comp Neurol* 518:4277-87

Hevner RF, Hodge RD, Daza RA, Englund C (2006). Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci Res* 55:223-33.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Hodge RD, Kahoud RJ, Hevner RF (2012). Transcriptional control of glutamatergic differentiation during adult neurogenesis. *Cell Mol Life Sci* 69:2125-34.

Horstmann E (1954). Die Faserglia des Selachiergehirns. *Z Zellforsch Mikrosk Anat* 39:588-617.

Kálman M (1998). Astroglial architecture of the carp (Cyprinus carpio) brain as revealed by immunohistochemical staining against glial fibrillary acidic protein (GFAP). *Anat Embryol (Berl)* 198:409-33.

Kálman M, Gould RM (2001). GFAP-immunopositive structures in spiny dogfish, Squalus acanthias, and little skate, Raia erinacea, brains: differences have evolutionary implications. *Anat Embryol (Berl)* 204:59-80.

Kálman M, Ari C (2002). Distribution of GFAP immunoreactive structures in the rhombencephalon of the sterlet (Acipenser ruthenus) and its evolutionary implication. *J Exp Zool* 293:395-406.

Kempermann G (2002). Regulation of adult hippocampal neurogenesis - implications for novel theories of major depression. *Bipolar Disord* 4: 17-33.

Kempermann G, Gage FH, Aigner L, Song H, Curtis MA, Thuret S, Kuhn HG, Jessberger S, Frankland PW, Cameron HA, Gould E, Hen R, Abrous DN, Toni N, Schinder AF, Zhao X, Lucassen PJ, Frisén J (2018). Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell*. doi: 10.1016/j.stem.2018.04.004.

Kirkham M, Hameed LS, Berg DA, Wang H, Simon A (2014). Progenitor cell dynamics in the Newt Telencephalon during homeostasis and neuronal regeneration. *Stem Cell Reports* 2:507-19.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Kuroyanagi Y, Okuyama T, Suehiro Y, Imada H, Shimada A, Naruse K, Takeda H, Kubo T, Takeuchi H (2010). Proliferation zones in adult medaka (*Oryzias latipes*) brain. *Brain Res* 1323:33-40.

Lazzari M, Franceschini V (2004). Glial fibrillary acidic protein and vimentin immunoreactivity of astroglial cells in the central nervous system of the African lungfish, Protopterus annectens (Dipnoi: Lepidosirenidae). *J Morphol* 262:741-9

Lledo PM, Merkle FT, Alvarez-Buylla A (2008). Origin and function of olfactory bulb interneuron diversity. *Trends Neurosci* 3:392-400.

Lim DA, Alvarez-Buylla A (2016). The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. Cold Spring Harb Perspect Biol doi: 10.1101/cshperspect.a018820.

Lugert S, Basak O, Knuckles P, Haussler U, Fabel K, Götz M, Haas CA, Kempermann G, Taylor V, Giachino C (2010). Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell* 6:445-56.

Ma DK, Bonaguidi MA, Ming GL, Song H (2009). Adult neural stem cells in the mammalian central nervous system. *Cell Res* 19:672-82

Macedo-Lima M, Freire MA, de Carvalho Pimentel H, Rodrigues Ferreira Lins LC, Amador de Lucena Medeiros KA, Viola GG, Dos Santos JR, Marchioro M (2016). Characterization of NADPH Diaphorase- and Doublecortin-Positive Neurons in the Lizard Hippocampal Formation. *Brain Behav Evol* 88:222-234

März M, Chapouton P, Diotel N, Vaillant C, Hesl B, Takamiya M, Lam CS, Kah O, Bally-Cuif L, Strähle U (2010). Heterogeneity in progenitor cell

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

subtypes in the ventricular zone of the zebrafish adult telencephalon. *Glia* 58:870-88.

Mazengenya P, Bhagwandin A, Manger PR, Ihunwo AO (2018). Putative adult neurogenesis in old world parrots: the congo African grey parrot (*Psittacus erithacus*) and timneh grey parrot (*Psittacus timneh*). *Front Neuroanat* 12:7.

Merrick SE, Pleasure SJ, Lurie DI, Pijak DS, Selzer ME, Lee VM (1995). Glial cells of the lamprey nervous system contain keratin-like proteins. *J Comp Neurol*. 355:199-210.

Ming GL, Song H (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70:687-702.

Ngwenya A, Patzke N, Herculano-Houzel S, Manger PR (2017). Potential adult neurogenesis in the telencephalon and cerebellar cortex of the Nile crocodile revealed with doublecortin immunohistochemistry. *Anat Rec (Hoboken)* doi: 10.1002/ar.23738.

Nieuwenhuys R (2009). The forebrain of actinopterygians revisited. *Brain Behav Evol*.73:229-52.

Pérez-Cañellas MM, García-Verdugo JM (1996). Adult neurogenesis in the telencephalon of a lizard: a [^{3}H]thymidine autoradiographic and bromodeoxyuridine immunocytochemical study. *Brain Res Dev Brain Res* 93:49-61.

Pose-Méndez S, Candal E, Adrio F, Rodríguez-Moldes I (2014). Development of the cerebellar afferent system in the shark Scyliorhinus canicula: insights into the basal organization of precerebellar nuclei in gnathostomes. *J Comp Neurol* 522:131-68

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Quintana-Urzainqui I, Rodríguez-Moldes I, Mazan S, Candal E (2015). Tangential migratory pathways of subpallial origin in the embryonic telencephalon of sharks: evolutionary implications. *Brain Struct Funct* 220:2905-26.

Rodríguez-Moldes I, Carrera I, Pose-Méndez S, Quintana-Urzainqui I, Candal E, Anadón R, Mazan S, Ferreiro-Galve S (2011). Regionalization of the shark hindbrain: a survey of an ancestral organization. *Front. Neuroanat* 5:16.

Rodríguez-Moldes I, Santos-Durán GN, Pose-Méndez S, Quintana-Urzainqui I, Candal E (2017). The Brains of Cartilaginous Fishes. In: Kaas J (ed.), Evolution of Nervous Systems 2e. vol. 1, pp. 77–97. Oxford: Elsevier. ISBN: 9780128040423.

Rubio M, Suarez I, Bodega G, Fernandez B (1992). Glial fibrillary acidic protein and vimentin immunohistochemistry in the posterior rhombencephalon of the Iberian barb (*Barbus comiza*). *Neurosci Lett* 134:203-6.

Sánchez-Farías N, Candal E (2015). Doublecortin is widely expressed in the developing and adult retina of sharks. *Exp Eye Res* 134:90-100.

Santos-Durán GN, Menuet A, Lagadec R, Mayeur H, Ferreiro-Galve S, Mazan S, Rodríguez-Moldes I, Candal E (2015). Prosomeric organization of the hypothalamus in an elasmobranch, the catshark Scyliorhinus canicula. *Front Neuroanat* 9:37.

Seri B, García-Verdugo JM, Collado-Morente L, McEwen BS, Alvarez-Buylla A (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *J Comp Neurol* 478:359-78.

Simmons AM, Horowitz SS, Brown RA (2008). Cell proliferation in the forebrain and midbrain of the adult bullfrog, *Rana catesbeiana. Brain Behav Evol* 71:41-53.

Smeets WJAJ, Nieuwenhyus R, Roberts BL (1983). The central nervous system of cartilaginous fishes. Ed. Springer-Verlag.

Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, James D, Mayer S, Chang J, Auguste KI, Chang EF, Gutierrez AJ, Kriegstein AR, Mathern GW, Oldham MC, Huang EJ, Garcia-Verdugo JM, Yang Z, Alvarez-Buylla A (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555:377-381

Steiner B, Klempin F, Wang L, Kott M, Kettenmann H, Kempermann G (2006). Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. *Glia* 54:805-14.

Suh H, Consigulio A, Ray J, Sawai T, D'Amour KA, Gage FH (2007). In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell Stem cell* 1:515-28.

Than-Trong E, Bally-Cuif L (2015). Radial glia and neural progenitors in the adult zebrafish central nervous system. *Glia* 63:1406-28.

Tornehave D, Hougaard DM, Larsson L (2000). Microwaving for double indirect immunofluorescence with primary antibodies from the same species and for staining of mouse tissues with mouse monoclonal antibodies. *Histochem Cell Biol* 113:19-23.

Tozzini ET, Baumgart M, Battistoni G, Cellerino A (2012). Adult neurogenesis in the short-lived teleost Nothobranchius furzeri: localization of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

neurogenic niches, molecular characterization and effects of aging. *Aging Cell* 11:241-51.

Villar-Cheda B, Pérez-Costas E, Meléndez-Ferro M, Abalo XM, Rodríguez-Muñoz R, Anadón R, Rodicio MC (2006). Cell proliferation in the forebrain and midbrain of the sea lamprey. *J Comp Neurol* 494:986-1006.

Wasowicz M, Ward R, Repérant J (1999). An investigation of astroglial morphology in torpedo and scyliorhinus. *J Neurocytol* 28:639-53.

Yopak KE, Lisney TJ, Collin SP (2015). Not all sharks are "swimming noses": variation in olfactory bulb size in cartilaginous fishes. *Brain Struct Funct* 220:1127-43.

Zhang, G, Vidal Pizarro I, Swain GP, Kang AH, Selzer ME (2014). Neurogenesis in the lamprey central nervous system following spinal cord transection. *J Comp Neurol* 522:1316–1332.

Zerjatke T, Gak IA, Kirova D, Fuhrmann M, Daniel K, Gonciarz M, Müller D, Glauche I, Mansfeld J (2017). Quantitative Cell Cycle Analysis Based on an Endogenous All-in-One Reporter for Cell Tracking and Classification. *Cell Rep.* 19:1953-1966.

Zupanc GK, Hinsch K, Gage FH (2005). Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. *J Comp Neurol* 488:290-319.

Zupanc GK (2006). Neurogenesis and neuronal regeneration in the adult fish brain. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 192:649-70.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

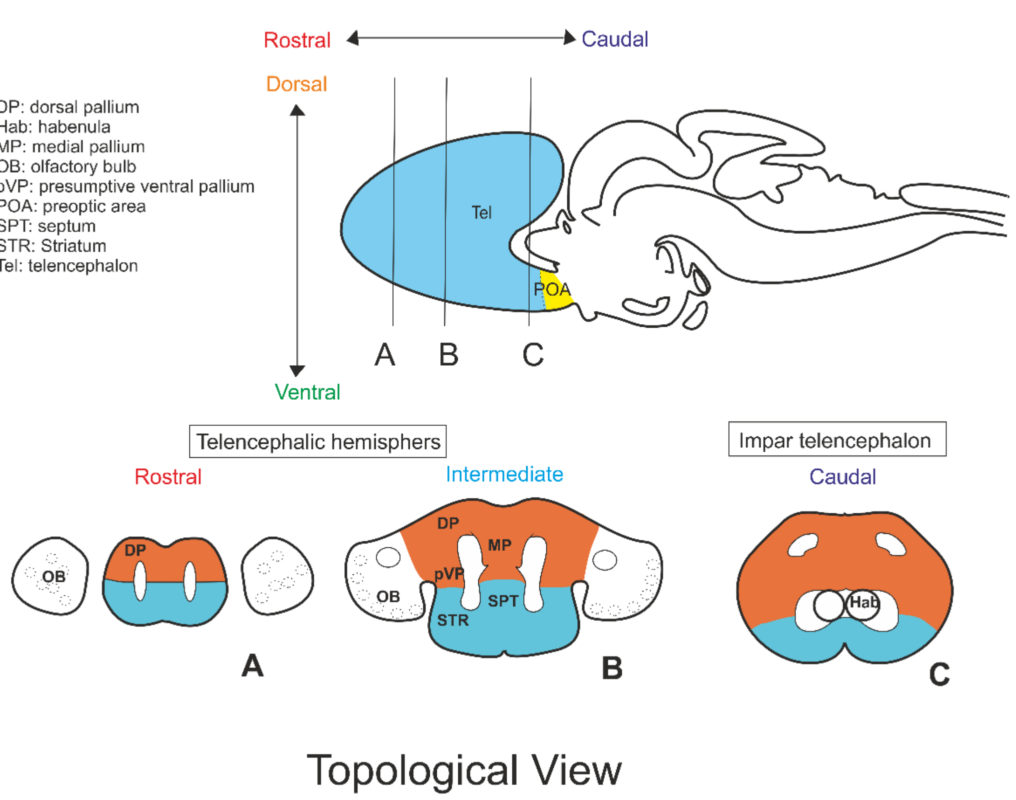
Table 1. Primary and secondary antibodies used.

Table 1. Primary and secondary antibodies used.					
Primary Antibody	**Source**	**Working dilution**	**Secondary Antibody**	**Source**	**Working dilution**
PCNA	Monoclonal mouse anti-PCNA Sigma (Cat. nº P8825)	1:500	Goat anti-mouse HRP coupled	Dako, Glostrup, Denmark	1:200
GFAP	Policlonal rabbit anti-GFAP Dako (Cat nº. Z0334)	1:500	488-cojugated donkey anti-mouse	Alexa Fluor Molecular Probes, Eugene, OR	1:200
GS	Monoclonal mouse anti-GS Millipore (Cat nº. MAB302)	1:500	546-conjugated donkey anti-rabbit	Alexa Fluor Molecular Probes, Eugene, OR	1:200
BLBP	Policlonal rabbit anti-BLBP Millipore (Cat nº. ABN14)	1:300	546-conjugated donkey anti-mouse	Alexa Fluor Molecular Probes, Eugene, OR	1:200
DCX	Policlonal rabbit anti-DCX Cell Signalling (Cat nº. 4604)	1:300			

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Figure 1. Schema showing the anatomy of the brain of the catshark, making emphasis on the telencephalon, showing the topographic and topologic view of the anatomy of the telencephalon. Note the changes in how dorso-ventral and rostro-caudal axis are considered in the topographical and topological view. Pallium and subpallium are represented in red (pallium) and blue (subpallium) in transverse sections. Schemas based on Smeets et al. 1983 and Santos-Durán et al. 2015.

Topographical View



Topological View

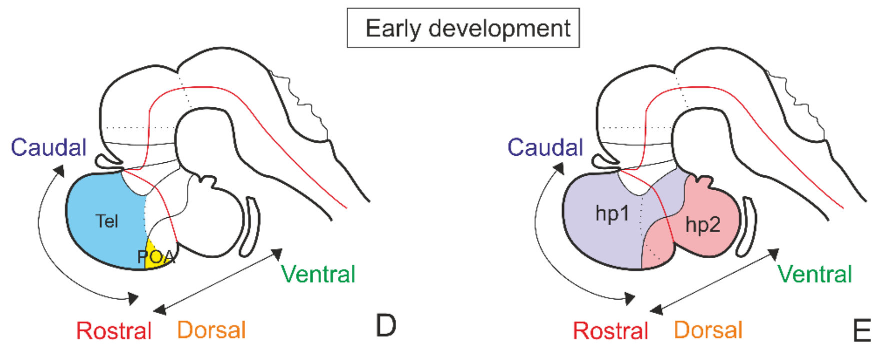


FIGURE 1

Figure 2. Photomicrographs at different magnifications showing the expression pattern of PCNA in juveniles **(A-E)** and adults **(F-H)** of *S. canicula*. **(A-A'')** Photomicrographs at different magnifications of the VZ of the telencephalon showing the different density of PCNA immunoreactive cells between the subpallium **(A')** and different subdivisions of the pallium **(A'').** **(B)** Detail of the dorsal pallium showing PCNA immunoreactive cells out of the VZ (arrows). **(C)** Detail of the VZ of the olfactory bulb showing PCNA immunoreactive cells. **(D-E)** Photomicrographs at different magnifications of the caudal telencephalon showing PCNA immunoreactivity restricted to a few subzones of the subpallial VZ. Note also the presence of some immunoreactive cells in the choroid plexus (arrow in **E**). **(F-H)** Photomicrographs at different magnifications of the telencephalic hemispheres of an adult specimen showing PCNA immunoreactivity. Dotted lines represent pallial-subpallial boundary. Scale bars: 200 µm (A, D, F), 100 µm (A', A'', C, G), 50 µm (B, D', E, H).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

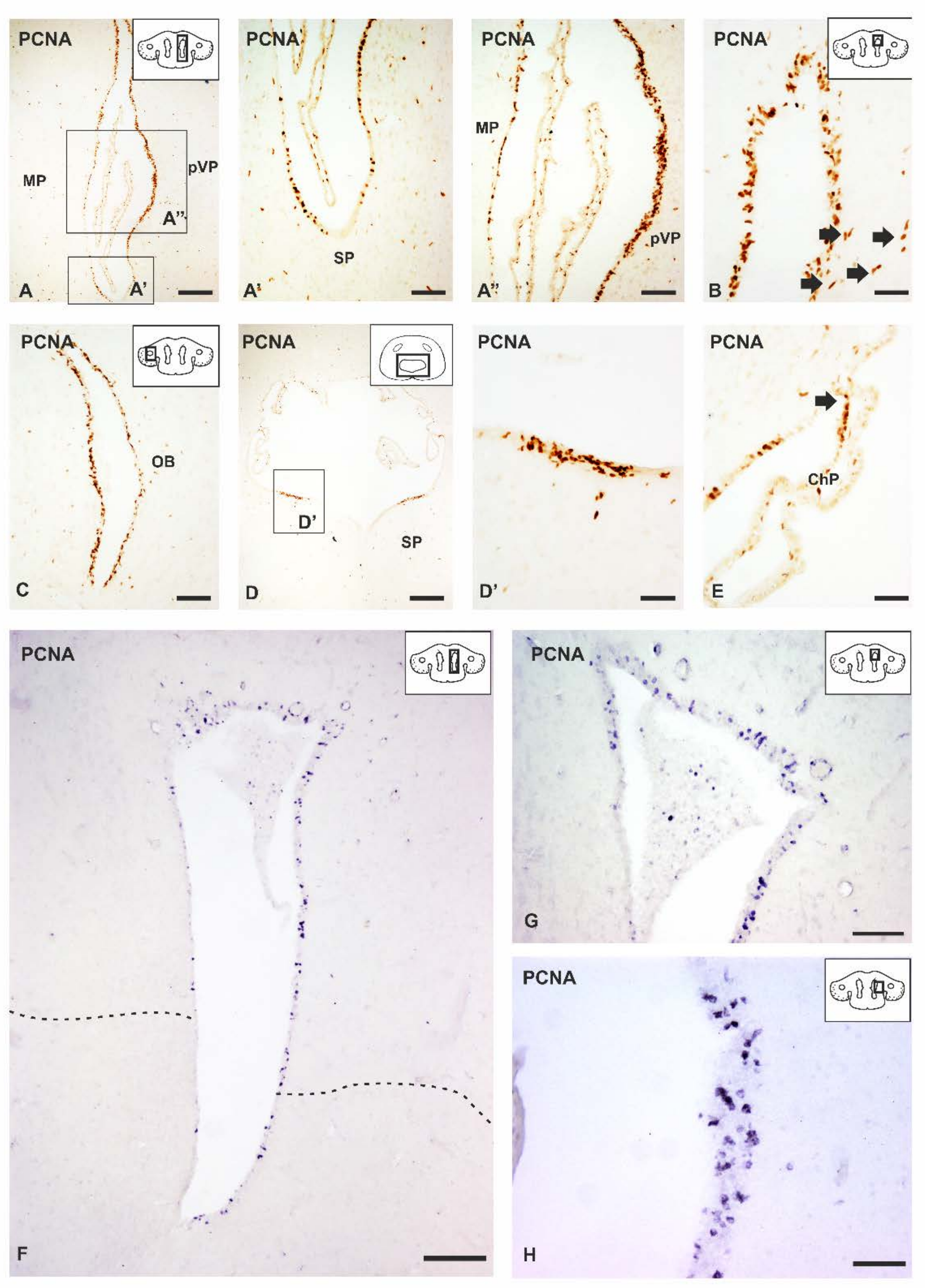


FIGURE 2

Figure 3. Photomicrographs at different magnifications showing the expression pattern of *ScSox2* and *ScSox2*-PCNA in the telencephalon of catshark juveniles. **(A-B')** Photomicrographs at different magnifications showing the expression pattern of *ScSox2* in the VZ of the telencephalic hemispheres (**A, A'**) and in the VZ of the impair telencephalon (**B, B'**). Note that the expression of *ScSox2* in the impair telencephalon abuts the choroid plexus, which is negative for ScSox2 (**arrow in B'**). **(C-D'')** Photomicrographs at different magnifications showing double ISH-IHC between *ScSox2*-PCNA in the dorsal **(C-C''),** medial **(D')** and presumptive ventral pallium **(D'').** Note differences between the medial and the presumptive ventral pallium regarding the organization of the VZ and coexpression of *ScSox2* and PCNA **(D-D'').** Scale bars: 200 µm (B, D), 100 µm (A, C), 50 µm (A', B', C', D', D''), 10 µm (C'').

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

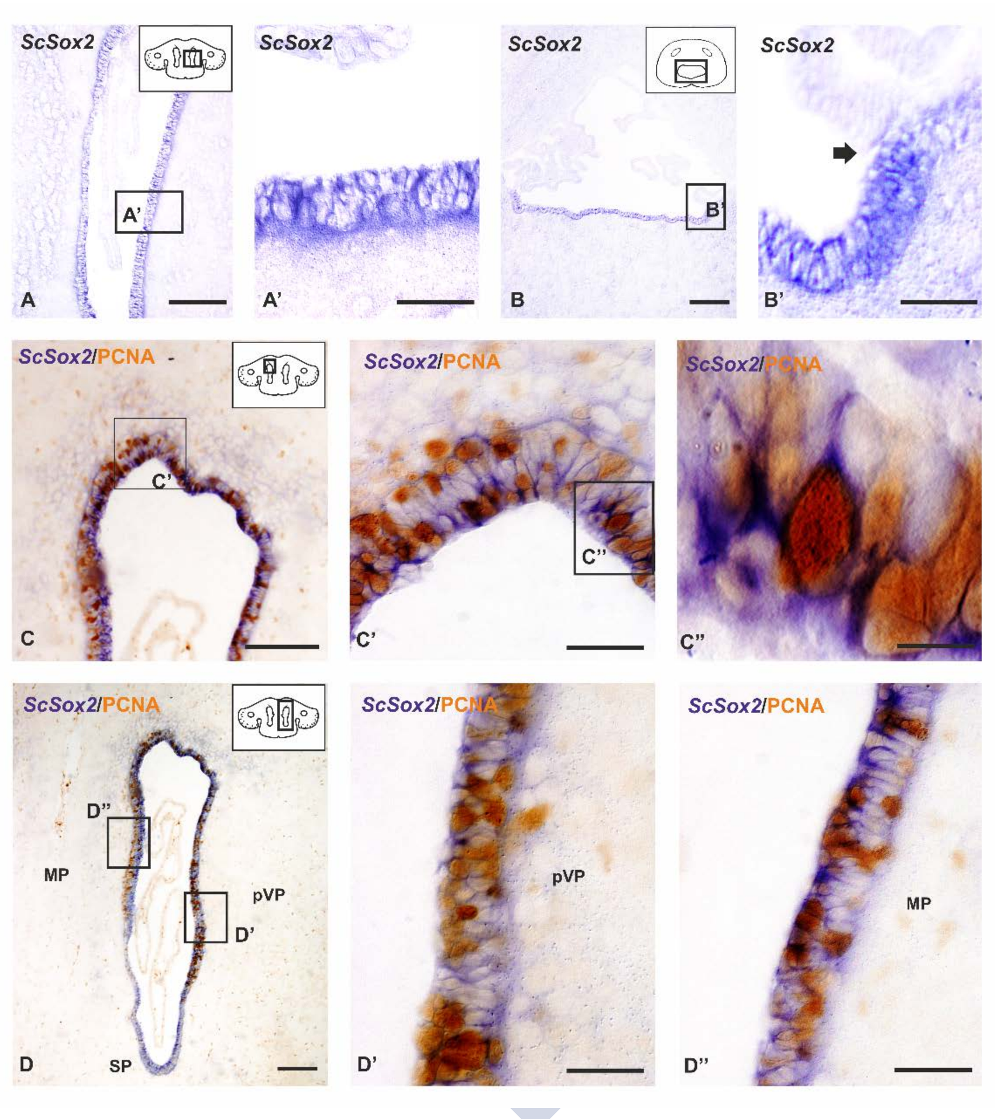


FIGURE 3

Figure 4. Details of the VZ of the pallium of catshark juveniles showing double labelled cells (**yellow stars**) for PCNA and different radial glial markers. Detail of the VZ of rostral pallium **(A-A'')** and presumptive ventral pallium **(B-B'')** showing double-immunofluorescence against GFAP and PCNA. Detail of the VZ of dorsomedial pallium **(C-C'')** and presumptive ventral pallium **(D-D'')** showing double-immunofluorescence against BLBP and PCNA. **(E-E'''')** Photomicrograph of the presumptive ventral pallium showing double-immunofluorescence against GS and PCNA**.** Scale bars: 25 µm.

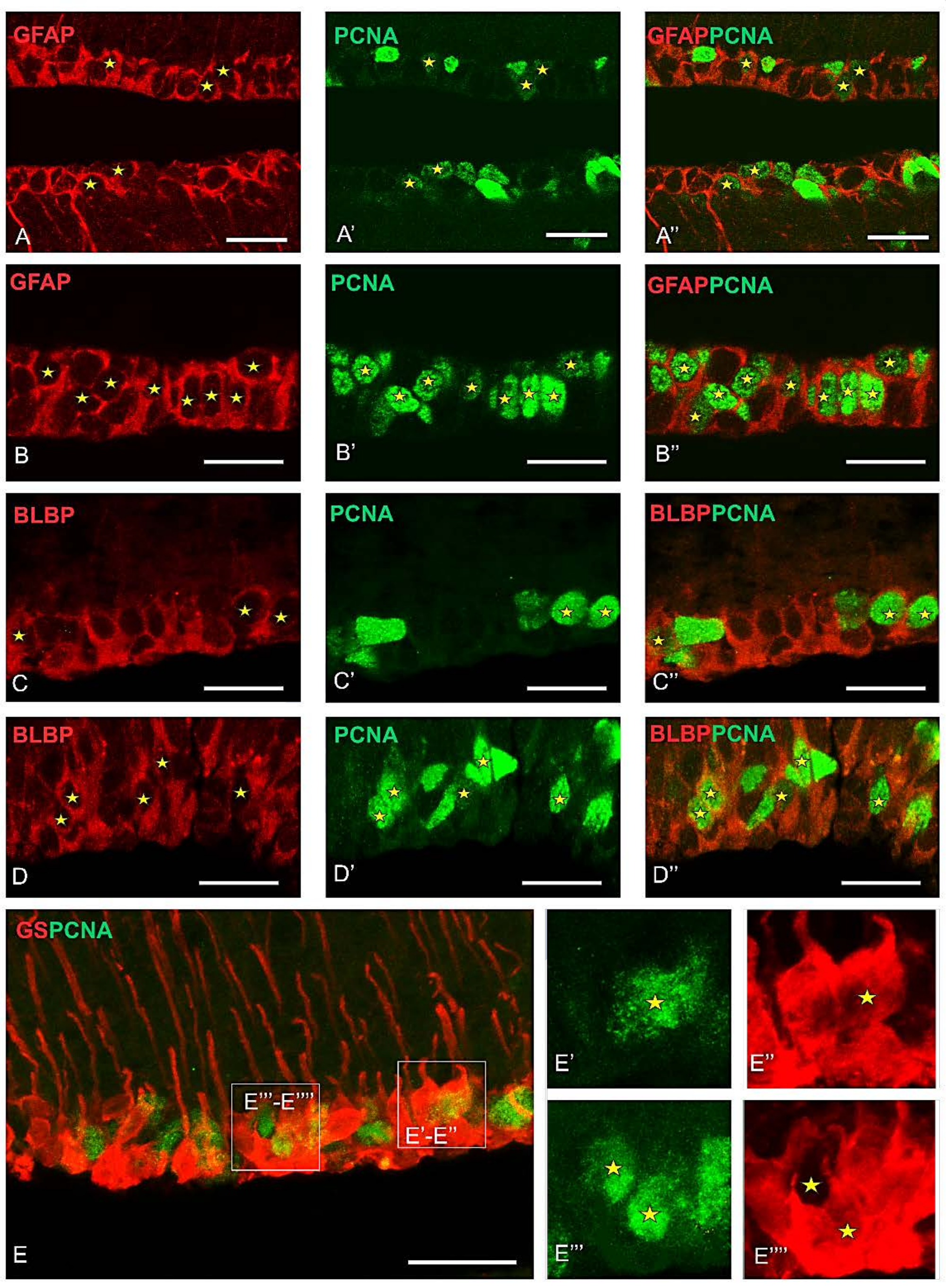


FIGURE 4

Figure 5. Photomicrographs at different magnifications showing the expression pattern of *ScTbr2*-PCNA and *ScDlx2*-PCNA in juveniles of catshark. **(A-A')** Panoramic and detail of the telencephalon showing the expression pattern of *ScTbr2*-PCNA. Note that *ScTbr2* is not expressed in PCNA immunoreactive populations in the VZ. **(B-D)** Photomicrographs at different magnifications showing the expression pattern of *ScDlx2*-PCNA in the VZ of the pallium. Note that many *ScDlx2* positive cells coexpress PCNA. Scale bars: 500 µm (A), 100 µm (B), 50 µm (A', B', C), 10 µm (C', D).

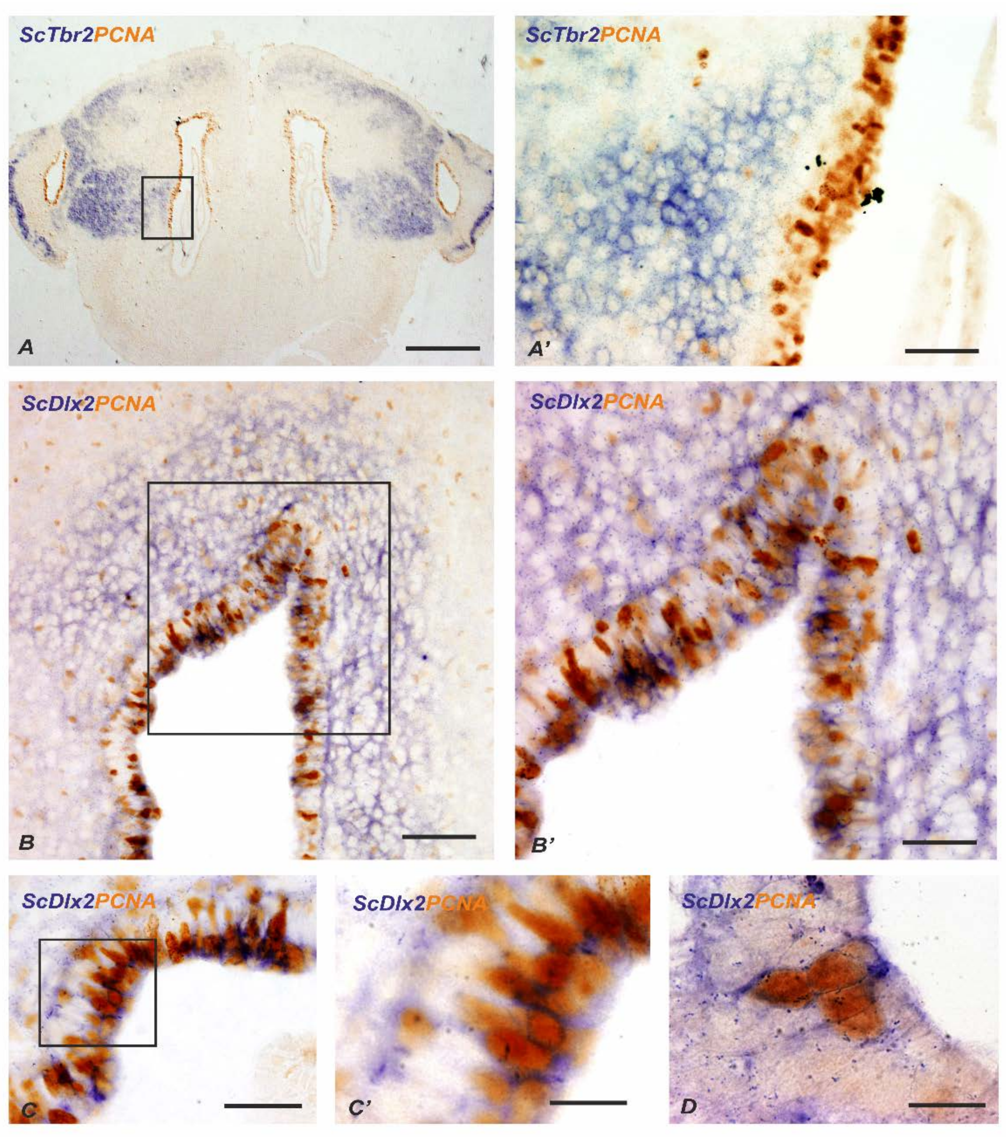


FIGURE 5

Figure 6. Photomicrographs at different magnifications showing the expression pattern of DCX and coexpression of DCX-PCNA in juveniles of catshark. **(A-B)** Schema and panoramic views of the telencephalic ventricle **(A')**, olfactory bulb **(A'')** and VZ of the impair telencephalon **(B)** showing the expression pattern of DCX. **(C-D'')** Details of the VZ **(C-C'')** and subventricular portions **(D-D'')** of the telencephalon showing DCX-PCNA double-labelled cells **(yellow stars)**. Scale bars: 200 µm (A', A''), 100 µm (B) 25 µm (C-D'').

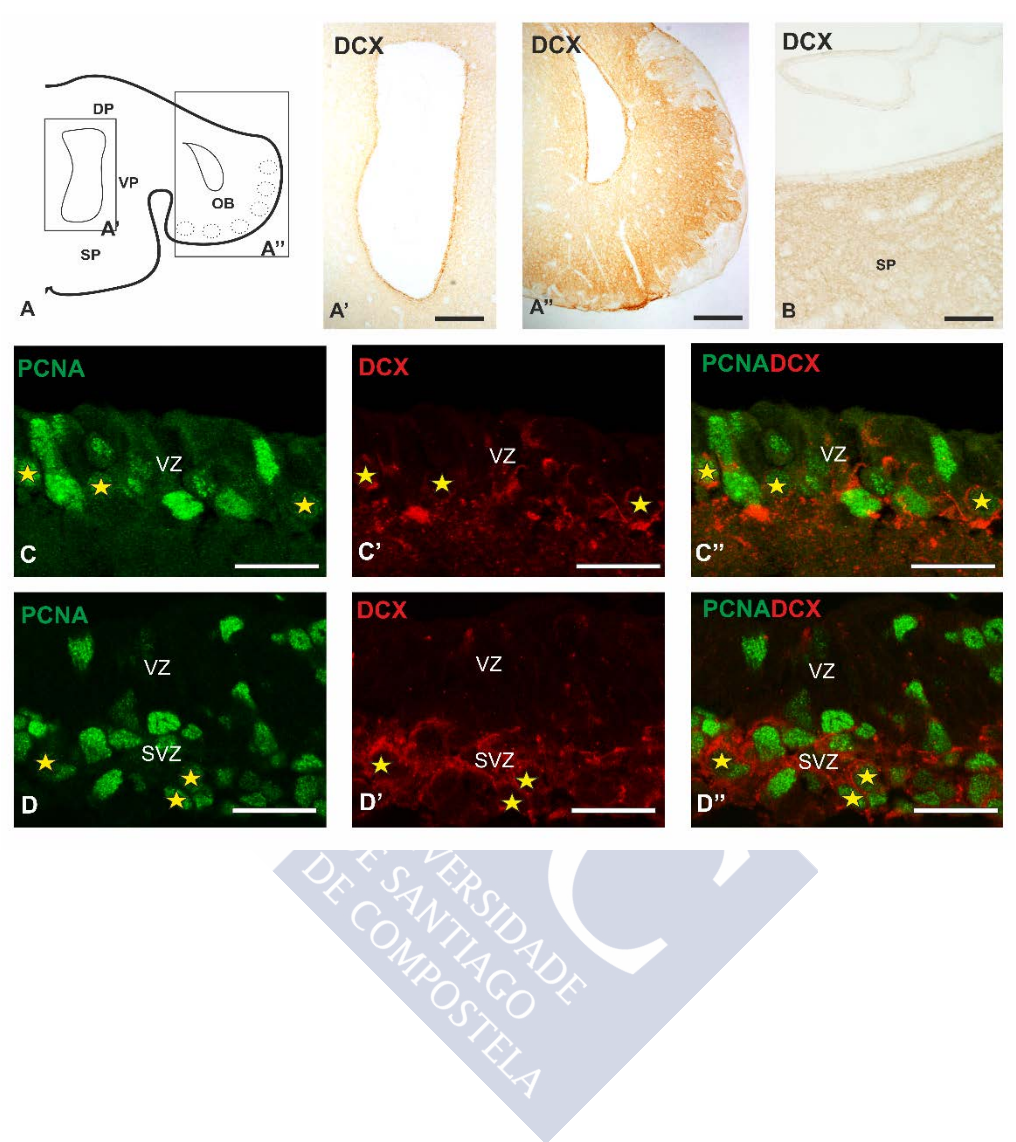


FIGURE 6

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Figure 7. Summary of the main cell types in adult neurogenesis across evolution.

COMPOSITION OF THE ADULT NEUROGENIC NICHE ACROSS VERTEBRATES

	RGC	IPC	Neuroblast	Main References
Mammals	BLBP/GFAP+ Sox2+ PCNA+	Dlx2/Tbr2 PCNA+	Dcx+ PSA-NCAM+	Doetch et al., 1997 Rev. Hodge et al., 2012 Rev. Bond et al., 2015
Birds	Electron micorscopy ^{3}HT Methylen blue	Electron microscopy ^{3}HT	Electron microscopy DCX+	Álvarez-Buylla et al., 1990 Álvarez-Buylla et al., 1998 Rev. Barnea and Pravasudov, 2011
Reptiles	BrdU ^{3}HT Methylen blue Golgy	ND	DCX+	García-Verdugo et al., 1981 Rev. González-Granero., 2011 Macedo-Lima et al., 2016
Amphibians	Glial Marker+ Sox2+ PCNA+	BrdU AraC sensitive	PSA-NCAM+	Simons et al., 2008 Kirkham et al., 2014
Teleosts	Glial Marker+ Sox2+ BrdU+	BrdU+ ?Glial marker+ ?Sox2	PSA-NCAM+ ?TH+	Grandel et al., 2006 März et al., 2010 Rev. Ganz and Brand., 2016
Chondrictyans	Glial Marker+ Sox2+ PCNA+	Dlx2+ PCNA+	DCX+ Dlx2+ Tbr2+	Quintana-Urzainqui et al., 2015 Present results
Cyclostomes	Citokeratins+ PCNA-	---	---	Merrick et al., 1995 Villar-Cheda et al., 2006

FIGURE 7

CHAPTER 4

COEXISTENCE OF NEUROGENIC PALLIAL MARKERS AND RADIAL GLIA MARKERS IN THE DEVELOPING OLFACTORY BULB OF CATSHARK (*Scyliorhinus canicula*)

Introduction

The sense of smell is essential for a variety of behaviors in vertebrates like mating, feeding, fear, and aggression. The organization of the olfactory system is well conserved throughout all vertebrates not only in terms of function, but also in connectivity and also concerning the developmental origin of different structures within the peripheral and central olfactory system. Numerous investigations in mammals indicate that the olfactory system constitutes an excellent model to study various developmental aspects of the nervous system such as neurogenesis, neuronal migration, axon guidance or interactions between glial cells and neurons (Bailey et al. 1999; Blanchart et al. 2011; Díaz-Guerra et al. 2013; Lim and Alvarez-Buylla, 2016).

In rodents, the OB becomes evident macroscopically around the day 12 of embryonic development (E12) when the projecting axons of the olfactory receptor neurons (ORNs, located in the olfactory epithelium) reach the telencephalic vesicle and induce the growth of the olfactory primordium (OP) (Gong and Shipley, 1995). For the proper growth of olfactory axons is necessary to establish the olfactory nerve pathway, which requires a class of peripheral glial cells exclusive of the olfactory system, termed olfactory ensheathing cells (OECs). In mammals, OECs produce neurotrophic factors, express cell adhesion molecules and several glial markers as brain lipid binding protein (BLBP), glial fibrillary acid protein (GFAP) and S100β (Valverde et al. 1992; Miller et al. 2010; Huilgol and Tole, 2016). However, information about OECs is more limited as we descend into the evolutionary scale of vertebrates (amphibians: Huang et al., 2005; Lazzari et al., 2016; teleost fishes (Lazzari et al., 2013, 2014), being very scarce in cartilaginous fishes (Quintana-Urzainqui et al., 2012).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

During morphogenesis of the mouse OB, projection neurons (mitral cells) are born first from pallial progenitor cells around E12, and then interneurons (granule and periglomerular cells) born in the subpallium migrate tangentially toward their destination (Blanchart et al. 2006; Huilgol and Tole, 2016). The transcriptional program involved in the specification and differentiation of mitral cells of the OB is well known in mammals, and curiously is the same that operates on the specification/differentiation of pallial glutamatergic neurons (for review see; Bulfone et al. 1998; Díaz-Guerra et al, 2013; Imamura and Greer, 2013; Kahoud et al. 2014; Roybon et al. 2015). In Chapter 1, we show that pallial glutamatergic markers are present in the developing pallium of catshark. However, information about this issue in the OB is lacking.

In rodents, mitral cells arise from paired-box-gene (Pax6) positive progenitor radial glia located in the ventricular zone of the rostral pallium (Winpenny et al. 2011; Imamura et al., 2011; Imamura and Greer, 2013). Different experimental approaches carried out in mammals have shown that Pax6, together with Sox2 (a transcription factor expressed in stem cells), are involved in self-renewal and differentiation of specific neural cell types (Devollade et al. 1998; Nomura et al. 2004; Kohwi et al. 2005; Gómez-López et al. 2011; Curto et al. 2014). Pax6 regulates the expression of two T-box genes (Tbr2 and Tbr1) (Bulfone et al. 1995; Mendez-Gómez et al. 2011; Mizuguchi et al. 2012; Imamura and Greer, 2013), and both transcription factors are expressed in mitral cells (glutamatergic) in the embryonic OB in rodents (Bulfone et al. 1999; Brill et al. 2009; Winpenny et al. 2011; Mizuguchi et al. 2012; Roybon et al. 2015), amphibians (Brox et al. 2004) and zebrafish (Mione et al. 2001; Mueller et al. 2008; Mueller and Wullimann, 2016). Mutant mice, where expression of Pax6, Tbr1 and Tbr2 is altered, show a disrupted process of OB morphogenesis (Bulfone et al. 1998; Nomura and Osumi, 2004; Kahoud et al.

2014). The basic-helix-loop-helix transcription factor NeuroD is also implicated in the terminal differentiation of mitral cells (Boutin et al. 2009; Osorio et al. 2010; Roybon et al. 2015). The expression of these transcription factors occurs before the formation/maturation of glomeruli in rodents and also before the mature organization of the OB is achieved. Interactions glia-neuron seems to play an important role in diverse aspects of the development of the OB, including formation of the glomeruli (Bailey et al. 1999; Okere and Kaba, 2000). Using antibodies against GFAP, the glial citoarchitecture of the OB was analyzed and a wide morphological diversity of glial cells was found in rodents, which contribute to the correct organization of the OB (Bailey and Shipley, 1993; Chiu and Greer, 1996; Bailey et al. 1999; Olude et al. 2014, 2015).

Despite most of the studies about the olfactory system are focused on mammals, nowadays other animal models are necessary to understand the molecular events that take place during development of the olfactory system. Because of its key phylogenetic position, the catshark *Scyliorhinus canicula* is a very good model to understand evolution and development of the olfactory system across vertebrates. Moreover, cartilaginous fishes possess a well-developed sense of smell that is important for survival, localizing preys, avoiding predators, and chemosensory communication (for review see: Yopak et al. 2015). Using optical and electron microscopy the organization of the adult OB and olfactory epithelium have been extensively studied in elasmobranchs (for review see: Ferreiro-Galve et al., 2012; Quintana-Urzainqui et al., 2012); in contrast, developmental studies are scarce, and mainly focused on peripheral olfactory system and olfactory placode (Fishelson and Baranes, 1997; Ferrando et al., 2007; Ferreiro-Galve et al., 2012; Quintana-Urzainqui et al., 2012). Using tract-tracing and immunohistochemical techniques the maturation of the peripheral olfactory system of the catshark *Scyliorhinus canicula* have been described according to key developmental events (Quintana-Urzainqui et al.,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2012). Some information concerning neurogenic events related with interneurons formation exists in the OB of sharks (Quintana-Urzainqui et al., 2015), but information about projection neurons is lacking. As far as we concern, the only transcription factor studied in relation to mitral cell neurogenesis in the OB of the catshark is Pax6 (Ferreiro-Galve et al. 2012; Quintana-Urzainqui et al., 2015). Numerous Pax6 positive cells are present in ventricular zone of the lateral/dorsal pallium of embryos before and after the appearance of the primordial OB (Ferreiro-Galve et al. 2012; Quintana-Urzainqui et al., 2015), however, the molecular phenotype of these cells is unknown. In addition, although GFAP expression pattern has also been studied in the adult brain of cartilaginous fishes (Wasovicz et al. 1999; Kálmán and Gould, 2001; Ari and Kálmán, 2008a, b), descriptions about the glial system in the OB are not available. Therefore, data about the glial system in the olfactory system during embryonic and postnatal development is lacking in cartilaginous fishes.

With the purpose of shedding light into the development of the central olfactory system in an evo-devo context, the aims of the present work are: (1) to characterize the Pax6 immunoreactive cells present in the ventricular zone of the dorsal/lateral pallium embryos of catshark; to achieve this goal we have combined markers for Pax6, stem cells (Sox2), radial glia cells (GFAP, BLBP) and proliferating cells (PCNA, proliferative cell nuclear antigen); (2) to study the expression pattern of transcription factors related to differentiation of glutamatergic cells (such as Tbr2, NeuroD and Tbr1) in late embryos of catshark (defined by Quintana-Urzainqui et al. 2015) using immunocytochemistry and *in situ* hybridization techniques; and (3) to study the expression pattern of several radial glia markers during embryonic development and in postnatal individuals using antibodies against GFAP, brain lipid binding protein (BLBP), glutamine

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

synthase (GS). We have also evaluated the proliferating capacity of radial glial cells by using antibodies against the proliferating cell nuclear antigen (PCNA).

Materials and Methods

Experimental animals

In the present study we have analysed 15 embryos of *S. canicula* from stages 30 (S30) to 34 (S34) of development and 3 juveniles. Embryos were provided by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France) and the Oceanographic Observatory of Banyuls sur Mer (France). Juveniles were kindly provided by the aquarium of O Grove (Galicia, Spain). Embryos were staged by their external features according to Ballard et al. (1993). Sharks were raised in seawater tanks under standard conditions of temperature (15-16 ºC), pH (7.5-8.5) and salinity (35 g/L) and suitable measures were taken to minimize animal pain and discomfort. All procedures were made according to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by Spanish Royal Decree 53/2013 for animal experimentation and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5 % tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4 % paraformaldehyde (PFA) in elasmobranch's phosphate buffer [EPB: 0.1 M phosphate buffer (PB) containing 1.75 % of urea, pH 7.4] for 48-72 h depending on the stage of development. Juveniles were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer´s solution (see Ferreiro-Galve et al. 2012) followed by 4 % PFA in EPB. Brains of perfused juveniles were removed and postfixed in the

same fixative for 24-48 h at 4 ºC. Subsequently, they were rinsed in PB saline (PBS), cryoprotected with 30 % sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (16-18 μm thick mostly) were obtained in transverse planes on a cryostat and mounted on to Superfrost Plus (Menzel-Glasser, Madison, WI, USA) slides.

In situ hybridization

We applied *in situ* hybridization (ISH) for *S. canicula Tbr2/Eomes, NeuroD1, vGlut1 and Sox2 (ScSox2, ScTbr2, ScNeuroD1, ScvGlut1)* genes. These probes were selected from a collection of *S. canicula* embryonic cDNA library (mixed stages, S9 to S22), submitted to high throughput EST sequencing (coordinated by Dr. Sylvie Mazan). Sense and antisense digoxigenin-UTP-labeled *ScTbr2, ScNeuroD1, ScvGlut1 and ScSox2* were synthesized directly by transcription *in vitro*. *In situ* hybridization was performed on cryostat sections of S30, S31 and S32 embryos following standard protocols (Coolen et al. 2009). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65 ºC and incubated with the alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Roche Applied Science, Manheim, Germany) overnight at 4 ºC. The color reaction was performed in the presence of BM-Purple (Roche). Color reaction was stopped by rinsing in PFA 4% for 45 min. Finally, sections were dehydrated and coverslipped. Control sense probes did not produce any detectable signal.

Immunohistochemistry

Sections were pre-treated with 0.01 M citrate buffer pH 6.0 for 30 min at 90 ºC for heat-induced epitope retrieval and allowed to cool for 20 min at room temperature (RT). Sections were rinsed in 0.05 M Tris-buffered saline (TBS) pH 7.4 for 5 min and treated with 10 % H_2O_2 in TBS for 30 min at RT to

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

block endogenous peroxidase activity. Sections were rinsed in 0.05 M TBS pH 7.4 for 5 min and incubated approximately for 15 h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate HRP coupled secondary antibody (see Table 1) for 1 h at RT. All dilutions were made with TBS containing 15 % normal goat serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Then, sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each. The immunoreaction was developed with 0.25 mg/ml diaminobenzidine (DAB) tetrahydrochloride (Sigma) in TBS pH 7.4 and 0.00075 % H_2O_2, or with SIGMAFAST™ 3.3-DAB tablets as indicated by the manufacturers. In some procedures, 2.5 mg/ml nickel ammonium sulphate was added. Finally, the sections were dehydrated, and coverslipped.

Double in situ hybridization- immunohistochemistry

We applied double *in situ* hybridization-immunohistochemistry for *ScTbr2* and *ScNeuroD* probes and the Tbr1 antibody. After colorimetric detection of probes, sections were rinsed three times in 1 M PBS for 10 min each, rinsed in PFA 4 % for 45 min and immunohistochemistry was performed as described above.

Double-immunofluorescence

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 90 ºC and allowed to cool for 20 min at RT. Sections were rinsed in 0.05 M TBS (pH 7.4) for 5 min and incubated approximately for 15 h at RT with primary antibodies (see Table 1). Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each, and incubated in the appropriate combination of fluorescent dye-labeled secondary antibodies (see Table 1) for 1 h at RT. All dilutions were made with TBS containing 15 %

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

normal donkey serum (Millipore, Billerica, MA) 0.2% Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Sections were rinsed three times in 0.05 M TBS pH 7.4 for 10 min each and in distilled water for 30 min. Sections were then allowed to dry for 30 min at 37 ºC, and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany). Information about the primary and secondary antibodies is included in Table 1.

Control and specificity of antibodies

The PCNA antibody has been previously used to label progenitor cells in the brain, retina and olfactory system of *S. canicula* (i.e Ferreiro-Galve et al., 2010; Quintana-Urzainqui et al. 2012; Quintana-Urzainqui et al. 2015; Sánchez-Farías and Candal, 2015). In addition, the specificity of the antibody against Pax6 has been tested by preabsortion test in *S. canicula* (Ferreiro-Galve et al. 2012) and the specificity of the antibodies against glial markers (GFAP, GS and BLBP) have also been tested by western blot (see Chapter 2).

Imaging

Fluorescent sections were photographed with the Leica TCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Confocal images were acquired separately for each laser channel with steps of 2 µm along the z-axis, and collapsed images were obtained with the LITE software (Leica). Some sections were photographed with an Olympus AX70 epifluorescence photomicroscope fitted with an Olympus DP70 color digital camera. Light field images were obtained with an Olympus BX51 microscope equipped with an Olympus DP71 color digital camera. Images were adjusted for contrast, brightness and intensity using Corel Draw X7.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Results

In the catshark, the OP emerges in lateral portions of the telencephalic hemispheres in the transition from S30 to S31 of development. In S31 the OP is observed as a well-defined protrusion. While no cell layering can be appreciated at this stage, incipient glomeruli began to appear, called protoglomeruli, in the distal portion of the OP. As the development proceeds to S32, protoglomeruli are more evident and the basic layering of the OB can be appreciated, which are, from the outside to inside, the olfactory nerve layer, glomerular layer (protoglomeruli) and granular layer (see General Introduction). Mitral cells, which in the mammalian brain and in other vertebrates constitute a proper layer, do not form a defined layer in the catshark, but rather they are diffusely distributed between the glomerular and granular layers. Form S32 onwards the OB grows and the glomerular layer and granular layer are properly recognizable. For further information about the OB development in the catshark see Quintana-Urzainqui et al. 2012, 2015.

Expression of Pax6 and progenitor cell markers in the ventrolateral pallium at stage 30/31.

At S30 of development, before the appearance of the OP, the telencephalon is constituted by a large ventricle surrounded by the telencephalic walls. These walls are constituted by a proliferating ventricular zone and an intermediate zone comprised of neuroblasts (see Chapter 1). Pallium and subpallium are recognizable because of their differential expression of GAD (Carrera et al., 2008; Quintana-Urzainqui et al., 2015) but their main subdivisions are not yet stablished (Fig. 1A). As OB progenitor cells should be present in the pallial proliferating ventricular zone before the appearance of the OP, we have studied the expression pattern of Pax6 (pallial progenitor marker), *ScSox2* (stem cell marker), BLBP-PCNA and GFAP-PCNA (radial glial

progenitor cell markers) in the ventricular zone of the ventrolateral portions of the pallium (the presumptive area where the OP is going to emerge) of a S30 embryo (square in Fig. 1A) and then we tracked Pax6 expression to a S31.

Pax6 expression was restricted to the pallial ventricular zone in the telencephalon of a S30 of development, including its ventrolateral portions (Fig. 1B). *ScSox2* expression was also present in the pallial ventricular zone, where its expression coincides with that of Pax6 (compare Fig. 1B and C). In the same region where numerous Sox2 and Pax6 positive cells were present, numerous BLBP and GFAP positive cells were observed; immunoreactivity for both radial glia markers was present in the periphery of the cell bodies and in their basal and apical processes. Double immunofluorescence BLBP/PCNA and GFAP/PCNA showed that all BLBP and GFAP expressing cells also coexpress PCNA (Fig. 1D, and stars in Figs. 1D', D''; Fig. 1E, and stars in Figs. 1E', E'').

In S31, Pax6 immunoreactive cells can be observed in the ventricular zone of the ventrolateral portions of the telencephalon (Fig. 1F). Interestingly, stream of Pax6 positive cells can be appreciated running from the ventricular zone to the OP (Fig. 1F'). Curiously, cells that invade the OP show a weaker Pax6 immunoreactivity.

Expression pattern of transcription factors (Tbr2, Tbr1 and NeuroD) in late embryos of catshark.

We have analyzed the expression pattern of *ScTbr2*, *ScNeuroD*, and Tbr1 at stages S31 and S32. At S31 (Figs. 2A-C), i*n situ* hybridizations for *ScTbr2* and *ScNeuroD* revealed a band of cells adjacent to the most distal region of the OP, where the first protoglomeruli appear, close to the ON entry (Figs. 2A, B), while a faint labelling was observed in proximal regions of the OP. In contrast, Tbr1 immunohistochemistry revealed immunoreactive cells in all regions of the OP, except in the protoglomerular region (Fig. 2C).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

At S32, the olfactory nerve layer begins to form bordering the protoglomerular layer. The expression pattern of the three transcription factors was restricted to a dense band of cells in the region adjacent to the protoglomeruli (Figs. 2D, E, F). As in S31, Tbr1 positive cells were also located in proximal regions of the prospective OB (Fig. 2F). As the expression pattern of *ScTbr2* and *ScNeuroD* was highly coincident but that of Tbr1 was not, we decided to perform double *in situ* hybridization-immunohistochemistry for *ScTbr2* and Tbr1 and for *ScNeuroD* and Tbr1 at S32 (Figs. 2G-H'). We found that most of the *ScTbr2* expressing cells were also positive for Tbr1 (Fig. 2G-G'). However, some cells expressed only *ScTbr2* or Tbr1. Curiously, most of the cells that only expressed Tbr1 were located in lateral regions of the distal OB (Fig. 2G'), in contrast to the ventral region, where most of the cells were positive for *Tbr2* only (Fig. 2G''). Concerning *ScNeuroD* and Tbr1 cell populations, we have observed a high coexpression of both factors in the same cells (Fig. H-H') and no differences between the lateral and ventral regions were appreciated.

In mammals and other vertebrate groups these transcription factors are part of a molecular cascade related with the differentiation of glutamatergic neurons (reviewed by Hevner et al. 2006). However, there is no information about the relationship between these factors and the glutamatergic system in sharks. We have studied the glutamatergic marker *ScvGlut1* (vesicular glutamatergic transporter) in S32 embryos by *in situ* hybridization. We found numerous *ScvGlut1* positive cells adjacent to the protoglomerular region (Fig. 2I), in the region occupied by *ScTbr2*/Tbr1/*ScNeuroD* expressing cells.

Expression pattern of radial glia markers (GFAP, BLBP and GS) in late embryos and juveniles of catshark.

The expression of radial glia markers (GFAP, BLBP and GS) was firstly detected in the transition from S31 to S32 in the OB of catshark. In all the stages of development studied (even in juveniles), all glial markers were found either in cells with bipolar morphology with their soma located mainly in the olfactory ventricular zone, or in adventricular cells showing different morphologies.

GFAP immunoreactivity has been detected in the olfactory nerve layer of a S32 embryo and some scattered processes can be seen in other layers of the OB. Numerous GFAP immunoreactive processes course along the olfactory nerve. Although, the amount of GFAP immunoreactive processes increases in S33 embryos, GFAP positive somas were not observed in the OB until S34 (Fig. 3A). At S34, numerous GFAP expressing cells with bipolar morphology were observed in the ventricular zone of the OB (ependymal cells and/or tanycytes; Horstmann, 1954). The glomerular layer showed the highest levels of GFAP immunolabeling, where cells with a bipolar morphology were observed surrounding glomeruli, forming a structure like a glomerular shell; some of the positive cells showed a thin and a thick branched process; numerous GFAP-ir processes were visualized within the glomeruli (Fig. 3B). In the granular layer, numerous GFAP-ir cells with a stellate shaped morphology and short processes that adopt a radial arrangement were observed, these cells resembling us astrocyte-like cells; many of these cells were seen in perivascular location (Fig. 3C). The GFAP expression pattern observed in S34 is similar to that observed in juveniles (Fig. 3D-G).

BLBP and GS expression were detected in the OB in the transition from S31 to S32. At S32 a high BLBP immunoreactivity was observed in the ventricular zone of the olfactory bulb and granular layer, in contrast with the

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

low BLBP expression observed in the glomerular layer (Fig. 4A). Numerous immunoreactive cells with morphology of radial glia are present in the ventricular zone (ependymal cells and/or tanycytes; Horstmann, 1954), and cells with the same morphology are scattered throughout the granular layer (Fig. 4A-A´´). BLBP expression was more restricted in juveniles, where few immunoreactive structures were observed (Figs. 4B-C). No immunoreactive structures were found in the granular layer of juveniles. However, few cells with weak immunoreactivity to BLBP were found in the glomerular layer, where they seem to be arranged surrounding the glomeruli (Figs. 4B', C).

On the other hand, at S32 cell bodies and processes positive for GS were found in the ventricular zone and in the granular and glomerular layer (Fig. 4D-D''). GS immunroeactivity was fainter in the glomerular layer (Fig. 4D') than in the granular layer and ventricular zone, where numerous immunoreactive cells were detected (Fig. 4D''). In juveniles GS immunoreactivity were mostly concentrated in the ventricular zone (ependymal cells or tanycytes; Horstmann, 1954) and in the glomerular layer. At this level numerous cells and processes positive for GS were observed surrounding glomeruli, and even some immunostaining can be appreciated within the glomeruli (Fig. 4F, G). On the contrary, faint immunoreactivity has been detected in the granular layer, where scarce immunoreactive cells were observed (Fig. 4H). In contrast to glomerular cells, these cells exhibited a different morphology.

Double immunofluorescence between GS-GFAP and GS-BLBP.

GS/GFAP double fluorescence revealed a non-overlapping expression pattern of both glial markers. However, some processes positive for both GFAP and GS were observed (Fig. 5A-B). Strikingly, the combination of both markers allowed us to detect some isolated cells with a particular morphology (Figs. 5C-

C’). These cells are rarely found and they possess GS+GFAP- cell bodies with a bulky protrusion from where multiple GS+GFAP+ branches emerge.

On the other hand, GS/BLBP double immunofluorescence revealed a high coexpressing/colocalizing expression pattern both in cells and processes (Fig. 5D). Many double labelled cells can be observed both in the glomerular and granular layer (Figs. 5D-F’’). However, at the level of ON entrance, close to the glomerular layer, some GS+BLBP- and BLBP+GS- cells can be appreciated (Figs. G-G’’’).

Double immunofluorescence for BLBP and PCNA.

In order to determinate if BLBP-ir cells have a proliferative capacity during development we have performed a double BLBP-PCNA immunofluorescence at S32. We have observed that the expression pattern of both markers is highly similar (Figs. 6A, B). Although numerous double immunolabeled cells were detected in the glomerular layer (Figs. 6C-C’’) and granular layer (Figs. 6D-D’’), the higher amount of double-labelled cells was observed in the ON layer (Figs. 6E-E’’).

Discussion

Cartilaginous fishes are good models to study evolution since they occupy a key phylogenetic position as a monophyletic group external to the rest of jawed vertebrates (Coolen et al. 2009; Rodríguez-Moldes et al. 2017). However, despite the phylogenetic distance to mammals, developmental events like migratory streams, morphogenetic processes, and regionalization patterns are strikingly similar (Rodríguez-Moldes et al. 2017). In the present work we have studied the molecular hallmarks of Pax6 expressing cells observed in the ventricular zone of the lateral/ventral pallium of catshark embryos. In addition, we have analysed the expression of several transcription factors implicated in

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

the differentiation of glutamatergic lineage, and the expression pattern of several radial glia markers in the OB of embryos and juveniles.

Pax6 positive cells present in the embryonic telencephalic ventricular zone are proliferating stem cells with molecular hallmarks of radial glia.

Numerous investigations have shown that Pax6 is involved in the development of the olfactory system, regulating neuronal specification, migration and differentiation. (Stoykova and Gruss, 1994; Nomura et al. 2007). Other studies suggest that Pax6 play a similar role in amphibians (Franco et al. 2001; Moreno et al. 2008; Joven et al. 2013). In mammals Pax6 is localized in progenitor radial glial cells during forebrain neurogenesis, including the ventricular zone of the embryonic OB (Götz et al. 1998; Imamura and Greer, 2013). Mouse neural stem cell lines display many hallmarks of radial glia, like bipolar morphology, including BLBP and Pax6 expression (Conti et al. 2005; Pollard et al. 2006).

Previous studies in catshark embryos show that Pax6 is expressed in the developing olfactory epithelium, as in tetrapod vertebrates, and immature neurons positive for Pax6 are present along olfactory axons (Ferreiro-Galve et al. 2012; Quintana-Urzainqui et al. 2012). In addition, after the emergence of the OP (S31) and also previous to the emergence of the OP, numerous Pax6 cells were observed in the ventricular zone of the ventrolateral pallium (present results, Ferreiro-Galve et al. 2012; Quintana-Urzainqui et al. 2012). As the molecular nature of these cells was not addressed and the role of Pax6 in the OB of catshark is not known, we have compared the expression pattern of Pax6 with the expression pattern of stem cell marker (Sox2) and glial markers (GFAP and BLBP) in the embryonic S30 of catshark.

In S30 embryos, numerous Pax6 expressing cells are present in the ventrolateral ventricular zone of the pallium, and differences in the intensity of

USC UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

the Pax6-labelling between positive cells close to the ventricle and cells located far from the ventricle are observed, which is in agreement with previous studies in the catshark (Ferreiro-Galve et al. 2012). *ScSox2* expressing cells are observed at the same location that Pax6 positive cells. Besides, these cells also show immunoreactivity to radial glia markers (GFAP and BLBP) and proliferative activity (PCNA immunoreactivity). Studies with neural stem cell lines indicate that Sox2 is a marker of stem cells and maintain neural stem cells in a proliferative and undifferentiated state. These studies also show that a complete ablation of Sox2 expression produces a loss of proliferative capacity; in addition, low levels of Pax6 are necessary to sustain proliferation and bipolar morphology of neural stem cells, while inactivation of Pax6 reduce the proliferative capacity (Sakurai and Osumi, 2008; Gómez-Lopez et al. 2011).

Our preliminary results show that Pax6 expressing cells located in the ventricular zone of the pallium express *ScSox2* and show proliferative capacity (PCNA) and molecular hallmarks of radial glia (GFAP and BLBP expression) as the mammalian neural stem cells. Further investigations are necessary to elucidate the role of Pax6 in the telencephalic ventricular zone and clarify the nature of the cell progeny originated from the Pax6 positive cells present in the pallial ventricular zone of catshark.

Neurogenic markers related with the differentiation of glutamatergic cell lineage are expressed in the developing OB of catshark.

In the developing neocortex of mammals, the transcription factor Pax6 is expressed sequentially together with others transcription factors (Tbr2 and Tbr1) in progenitor cells and postmitotic neurons of glutamatergic cell lineage (Englund et al. 2005; Hevner et al. 2006). Interestingly, the transcriptional program that operates in the differentiation of glutamatergic phenotype in developing neocortex is also necessary for correct morphogenesis of the OB and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

generation of mitral cells (Bulfone et al. 1998; Imamura and Greer, 2013; Kahoud et al. 2014; Roybon et al. 2015).

In rodents, mitral cells are the first cellular subtype of the OB to be born; these cells are generated around the embryonic day E12 from progenitor cells located in the ventricular zone of the OB (Hinds, 1968). Numerous studies in rodents indicate that Pax6 is required for OB mitral cell specification and differentiation. Progenitor cells of mitral cells express Pax6 (Winppeny et al. 2011; Imamura and Greer, 2013), and in Pax6 mutant mice mitral cells are mislocated (for review see: Nomura et al. 2007). In addition, tracking experiments with BrdU labelling show that mitral cells are generated from Pax6 positive radial glial cells, and postmitotic mitral cell precursors express both Tbr1 and Tbr2. Expression of both transcription factors are essential for the generation of mitral cells and its expression occurs in the same developmental period in which Pax6 expression is down-regulated (Imamura and Greer, 2013). In Tbr2 mutant mice, the amount of mitral cells is reduced and their organization and projections disturbed, which is similar to what happens in Tbr1 mutant mice (Bulfone et al. 1998, 1999; Imamura and Greer, 2013; Kahoud et al. 2014). Moreover, the bHLH transcription factor NeuroD is expressed in the mature glomerular layer, and overexpression of NeuroD leads to the appearance of mature neurons, but knockdown of NeuroD inhibits neuronal differentiation (Boutin et al. 2010). In amphibians, expression of Pax6, Tbr1 and Tbr2 is detected in the developing OB of larvae (Brox et al. 2004; Moreno et al., 2008) and Tbr1 expression is also present in the OB of zebrafish 48 hpf (Mione et al. 2001).

In the present work we have detected *ScTbr2* and *ScNeuroD* expression and Tbr1 immunoreactivity in the primordial OB of S31 of development and, at S32 this expression becomes restricted to territories adjacent to protoglomerular

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

regions. In the catshark, a stream of Pax6 expressing cells can be tracked from the ventricular zone of the pallium to the OP of S31 (present results; Ferreiro-Galve et al. 2012); Curiously, some weak Pax6 positive cells are present in the OP (present results, Ferreiro-Galve et al. 2012). At S32, TH immunoreactive granular and periglomerular cells are also observed (Carrera et al., 2012) and in some cells Pax6 colocalize with TH (Quintana-Urzainqui et al., 2015). The expression pattern of neurogenic markers that we found at S31-32 embryos (present results), seems be adjacent to the Pax6 and TH positive cell populations described previously in the catshark, which suggest us that the territories expressing *ScTbr2*, Tbr1 and *ScNeuroD* may correspond to the beginning of the prospective glomerular layer, where mitral cells are intermingled.

In addition, we have shown that both *ScTbr2* and *ScNeuroD* colocalize with Tbr1, but, as in rodents, it seems that some cells express Tbr1 or Tbr2 only (Imamura and Greer, 2013). In the mammalian developing OB, studies of expression of Tbr2 and Tbr1 show that mitral cells express these factors in an overlapping pattern (Imamura and Greer, 2013), which is in agreement with our results. On the other hand, in mammals NeuroD expression pattern also overlapped Tbr2 expression, but not Tbr1 expression, indicating the existence of subsets of mitral cells (Roybon et al. 2015). In the catshark, the expression pattern of the different neurogenic markers overlapped with that of *ScvGlut1* (present results). In later embryos of rodents, Vglut1 was detected in the mitral cell layer (Ohmomo et al. 2011) and a strong expression of this transporter was also observed in mitral cells of adult reptiles (Sarkar and Atoji, 2018). Some studies in elasmobranchs based on Golgi staining have evidenced the existence of two kind of mitral cells in the OB of the sharks studied (*Sphyrna tiburo, Dasyatis sabina* and *Rhizoprionodon terranovae):* mitral cells with dense and tight arborized dendrites and mitral cells with a loose dendrite arborization (Dryer and Graziadei, 1993). As far as we concern, the existence of more than

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

one type of mitral cells has not been described in S. canicula and inn the present work we were not able to define different subsets of mitral cells with the same markers used in mammals.

Our results together suggest us that some Pax6 positive cells that are present in the pallial ventricular zone invade the OP forming a stream. When these Pax6-expressing cells reach the OP, it seems that they experiment a downregulation of Pax6-expression and begin to upregulate transcription factors related with the differentiation of glutamatergic cells (*ScTbr2, ScNeuroD*, Tbr1); indicating that the developing OB of catshark express the same pallial markers related with the differentiation of glutamatergic cell lineage (see Chapter 1), which suggests us that the transcriptional program that rule glutamatergic neurogenesis in different brain areas has been conserved throughout the vertebrate evolution. Despite these results suggest us that we are tracking mitral cell development, further investigations using BrdU incorporations or cell-tracking experiments are needed to elucidate this matter.

Expression of GFAP, BLBP and GS in later embryos: putative implications of glia in the developing OB

At S32, just at the end of the neurogenic period, numerous GFAP immunoreactive processes were observed in the olfactory nerve, as well as in the region where the olfactory nerve and the OB establish contact, which is in agreement with previous findings in the catshark (Quintana-Urzainqui et al. 2012). Numerous GFAP immunoreactive cells were first observed at S34 in the glomerular layer. Therefore, the appearance of GFAP immunoreactive cells in the OB of catshark occurs at the end of the embryonic period, similar to what was reported in mammals, where first cells to express GFAP were detected at E19 (Bailey et al. 1999).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

At S34 numerous GFAP positive cells with bipolar morphology and highly branched processes were observed forming glomerular shell-like structures, which were also seen in juveniles (present results). GFAP-ir cells with a similar morphology were also described in the OB of mammals (Bailey et al. 1999). In addition, immunoreactivity to GFAP was detected in astrocyte-like cells in the granular layer of the catshark. Although cells with astrocytic morphology have been previously described in the adult telencephalon of *Scyliorhinus canicula* (i.e Kàlmàn and Gould, 2001; Ari and Kàlmàn, 2008a, b), astrocyte-like cells were not observed in other forebrain areas during catshark development (see Chapter 2). In mammals, several morphological categories of astrocytes according their degree of branching and disposal of prolongations have been described in the OB, and they exhibit layer specificity (Bailey and Shipley, 1993; Chiu and Greer, 1996). Whether GFAP-immunoreactive cells found in the OB of the catshark show specificity layer are quite clear but whether may correspond with some of the morphological subtypes described in mammals needs to be explored.

Our results indicate that glomeruli are composed of a shell of glial cells and processes immunoreactive for GFAP and GS. In addition, the neuropil of the glomeruli showed numerous GFAP-ir processes. Curiously, in contrast with mammals, periglomerular cells are scarce in the catshark (Rodríguez-Moldes et al., 1993; Dryer and Graziedi, 1996; Ferrando et al. 2012). Olfactory signals are firstly processed in the olfactory glomeruli, in the catshark, protoglomeruli are recognizable at S31 (Quintana-Urzainqui et al. 2012), which coincides with the expression of GFAP and GS in the glomerular layer (present results). In mammals, glial cells had been related with the formation of glomeruli, and also respond to neuronal activity. In addition, glial processes may act as a barrier to the ingrowth of axons from olfactory epithelium, indicating that glia-neurons interactions play an important role in the organization of the glomeruli (Bailey

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

et al. 1999; Roux et al. 2011). Besides, in mammals during the glomerular formation, mitral cells suffer a refinement that involved changes in the shape and size of their cell body, and also their dendrites undergo morphological modifications (Blanchart et al. 2006). Based on these evidences, further investigations in the catshark are necessary to shed light about the function of the periglomerular glial cells during development of the OB.

On the other hand, BLBP/GS immunohistochemistry was observed in cells and processes from S32 to juveniles. At S32 both glial markers colocalized in the same cells, mainly located in the granular layer. However, in juveniles the number of BLBP positive cells decreased drastically and only a few positive cells were observed in the glomerular layer. In contrast, numerous GS-ir processes were located in the glomerular layer of juveniles. In glutamatergic synapses most of the glutamate released to the synaptic cleft is uptaken by astrocytes, where by the action of GS is transformed into glutamine, and the activity of this enzyme increase postnatally (Battú et al. 2005). The expression pattern of GS observed in the catshark is in agreement with previous results in rodents (Okere and Haba, 2000) and is correlated with sites of glutamatergic synapses.

At S32 most of the BLBP positive cells present in the OB showed proliferative activity (PCNA-ir). It is striking the high amount of proliferative cells expressing BLBP observed in the olfactory nerve layer (present results). In mammals, olfactory axons from olfactory sensory neurons are directed toward the glomerular layer through the olfactory nerve. These axons are accompanied in their migration by a population of migratory cells, that collectively are named migratory mass (MM), which is formed by several cell populations including neurons, and a class of peripheral glial cell restricted to the olfactory system, the olfactory ensheathing cells (OECs) (Valverde et al. 1992; Miller et al. 2010). In

mammals, these cells derived from olfactory placode (Chuah and Au, 1991; Miller et al. 2010). However, it was recently shown that OECs derived from neural crest (Barraud et al. 2018). Curiously, numerous investigations have shown that BLBP is a marker of rodent OECs (Murdoch and Roskams, 2007; Blanchart et al. 2011; Barraud et al. 2018). In a previous study in the catshark (Quintana-Urzainqui et al. 2012) it was shown that like in mammals (Astic et al. 1998), amphibians (Huang et al. 2005; Lazzari et al. 2016) and teleost fishes (Lazzari et al. 2013, 2014), OECs express GFAP. All these findings together support the idea previously suggested by Quintana-Urzainqui et al. (2012) that the OECs are already present in the most ancient radiation of jawed vertebrates. In mammals, cells of the MM show proliferative activity and give rise to different cell populations (neurons, glia and OECs), and also OECs progenitors cells seems to enter the central nervous system (Blanchart et al. 2011; Geller et al. 2017). Additional studies with different markers of OECs will help us to elucidate the molecular identity of the OECs in the catshark, and other experimental approaches would be required to determine the progeny of the proliferative BLBP positive cells detected in the OB of the catshark

Conclusions

The OB of the catshark has shown to be an intriguing model to study neurogenesis. We found that proliferative-Pax6 positive cells with stem cell and radial glial properties are present before the emergence of the OB in the pallial ventricular zone. Glutamatergic cells of the OB, which are mainly represented by mitral cells, express a battery of transcription factors in the same way as in mammals, which seem to indicate that the transcription program of glutamatergic cells specification/differentiation have been conserved throughout the scale of vertebrates. Further investigations are necessary to elucidate whether some Pax6-expressing progenitor cells present in the pallium

might be the source of mitral cells that would colonize the future OB. We have also found different subsets of glial cells, some of them with astrocytic-like shape and with a proliferative potential, which seems to be organized in a layer-dependent way. Besides, we have observed numerous proliferating cells that express OECs markers such as BLBP in the olfactory nerve layer.

The conserved transcription program of glutamatergic cells, as well as the large differences in the glial architecture not only with mammals, but also with other brain areas of the same specie makes the olfactory system of the catshark a good system to study the putative implications of peripheral and central glial cells in the development of the central nervous system in basal vertebrates. This study paves the way for further investigations about the development of the olfactory system in the catshark from an evo-devo perspective.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

References

Ari C, Kálmán M (2008a). Glial architecture of the ghost shark (Callorhinchus milii, Holocephali, Chondrichthyes) as revealed by different immunohistochemical markers. *J Exp Zool B Mol Dev Evol* 310:504-19.

Ari C, Kálmán M (2008b) Evolutionary changes of astroglia in Elasmobranchii comparing to amniotes: a study based on three immunohistochemical markers (GFAP, S-100, and glutamine synthetase). *Brain Behav Evol* 71:305-24.

Astic L, Pellier-Monnin V, Godinot F (1998). Spatio-temporal patterns of ensheathing cell differentiation in the rat olfactory system during development. *Neuroscience* 84:295-307

Bailey MS, Puche AC, Shipley MT (1999). Development of the OB: evidence for glia-neuron interactions in glomerular formation. *J Comp Neurol* 415:423-48.

Bailey MS, Shipley MT (1993). Astrocyte subtypes in the rat OB: morphological heterogeneity and differential laminar distribution. *J Comp Neurol* 328:501-26.

Ballard WW, Mellinger J and Lechenault (1993). A series of normal stages for development of *Scyliorhinus canicula*, the lesser spoted dogfish (Chondrichthyes: Scyliorhinidae). *J exp zoo* 267:318-336.

Blanchart A, De Carlos JA, López-Mascaraque L (2006). Time frame of mitral cell development in the mice OB. *J Comp Neurol* 496:529-43.

Blanchart A, Martín-López E, De Carlos JA, López-Mascaraque L (2011). Peripheral contributions to OB cell populations (migrations towards the OB). *Glia* 59:278-92.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Barraud P, St John JA, Stolt CC, Wegner M, Baker CV (2018). Olfactory ensheathing glia are required for embryonic olfactory axon targeting and the migration of gonadotropin-releasing hormone neurons. *Biol Open* 2:750-9.

Battú CE, Godinho GF, Thomazi AP, de Almeida LM, Gonçalves CA, Kommers T, Wofchuk ST (2005). Ontogenetic changes in glial fibrillary acid protein phosphorylation, glutamate uptake and glutamine synthetase activity in olfactory bulb of rats. *Neurochem Res* 30:1101-8

Boutin C, Hardt O, de Chevigny A, Coré N, Goebbels S, Seidenfaden R, Bosio A, Cremer H (2010). NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. *Proc Natl Acad Sci U S A* 107:1201-6

Brox A, Puelles L, Ferreiro B, Medina L (2004). Expression of the genes Emx1, Tbr1, and Eomes (Tbr2) in the telencephalon of Xenopus laevis confirms the existence of a ventral pallial division in all tetrapods. *J Comp Neuro*l 474:562-77.

Brill MS, Ninkovic J, Winpenny E, Hodge RD, Ozen I, Yang R, Lepier A, Gascón S, Erdelyi F, Szabo G, Parras C, Guillemot F, Frotscher M, Berninger B, Hevner RF, Raineteau O, Götz M. (2009). Adult generation of glutamatergic OB interneurons. *Nat Neurosci* 12:1524-33

Bulfone A, Smiga SM, Shimamura K, Peterson A, Puelles L, Rubenstein JL. (1995). T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron* 15:63-78

Bulfone A, Martinez S, Marigo V, Campanella M, Basile A, Quaderi N, Gattuso C, Rubenstein JL, Ballabio A. (1999). Expression pattern of the Tbr2

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

(Eomesodermin) gene during mouse and chick brain development. *Mech Dev* 84:133-8

Bulfone A, Wang F, Hevner R, Anderson S, Cutforth T, Chen S, Meneses J, Pedersen R, Axel R, Rubenstein JL (1998). An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. *Neuron* 21:1273-82.

Chuah MI, Au C (1991). Olfactory Schwann cells are derived from precursor cells in the olfactory epithelium. *J Neurosci Res* 29:172-80

Chiu K, Greer CA (1996). Immunocytochemical analyses of astrocyte development in the OB. *Brain Res Dev Brain Res* 95:28-37.

Coolen M, Sauka-Spengler T, Nicolle D, Le-Mentec C, Lallemand Y, Da Silva C, Plouhinec JL, Robert B, Wincker P, Shi DL, Mazan S (2009). Evolution of axis specification mechanisms in jawed vertebrates: insights from a chondrichthyan. *PLoS One* 2:e374

Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3:e283.

Curto GG, Nieto-Estévez V, Hurtado-Chong A, Valero J, Gómez C, Alonso JR, Weruaga E, Vicario-Abejón C (2014). Pax6 is essential for the maintenance and multi-lineage differentiation of neural stem cells, and for neuronal incorporation into the adult OB. *Stem Cells Dev* 23:2813-30.

Dellovade TL, Pfaff DW, Schwanzel-Fukuda M (1998). OB development is altered in small-eye (Sey) mice. *J Comp Neurol* 402:402-18.

Díaz-Guerra E, Pignatelli J, Nieto-Estévez V, Vicario-Abejón C (2013). Transcriptional regulation of OB neurogenesis. *Anat Record* 296:1364-82.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Dryer L, Graziadei PPC (1993). A pilot study on morphological compartmentalization and heterogeneity in the elasmobranch OB. *Anat Embryol* 188:41-51.

Dryer L, Graziadei PPC (1996). Synaptology of the olfactory bulb of an elasmobranch fish, *Sphyrna tiburo*. *Anat. Embryol* 193, 101–114.

Englund C, Flink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T and Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci* 25:247-51

Ferrando S, Bottaro M, Pedemonte F, De Lorenzo S, Gallus L, Tagliafierro G (2007) Appearance of crypt neurons in the olfactory epithelium of the skate Raja clavata during development. *Anat Rec* 290:1268–1272

Ferrando S, Gallus L, Gambardella C, Amaroli A, Cutolo A, Masini MA, Vallarino M, Vacchi M (2012). Neuronal nitric oxide synthase (nNOS) immunoreactivity in the olfactory system of a cartilaginous fish. *J Chem Neuroanat* 43:133-40.

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012). Dynamic expression of Pax6 in the shark olfactory system: evidence for the presence of Pax6 cells along the olfactory nerve pathway. *J Exp Zool B Mol Dev Evol* 318:79-90.

Fishelson , Baranes A (1997). Ontogenesis and cytomorphology of the nasal olfactory organs in the Oman shark, Iago omanensis (Triakidae), in the Gulf of Aqaba, Red Sea. *Anat Rec* 249:409–421

Franco MD, Pape MP, Swiergiel JJ, Burd GD (2001). Differential and overlapping expression patterns of X-dll3 and Pax-6 genes suggest distinct roles

in olfactory system development of the African clawed frog *Xenopus laevis*. *J Exp Biol* 204(Pt 12):2049-61

Geller S, Lomet D, Caraty A, Tillet Y, Duittoz A, Vaudin P (2017).Rostro-caudal maturation of glial cells in the accessory olfactory system during development: involvement in outgrowth of GnRH neurites. *Eur J Neurosci* 46:2596-2607.

Gong Q, Shipley MT (1995). Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce the formation of the OB. *Neuron* 14:91-101.

Gómez-López S, Wiskow O, Favaro R, Nicolis SK, Price DJ, Pollard SM, Smith A (2011). Sox2 and Pax6 maintain the proliferative and developmental potential of gliogenic neural stem cells in vitro. *Glia* 59:1588-99.

Götz M, Stoykova A, Gruss P (1998). Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* 21:1031-44

Hevner RF, Hodge RD, Daza RA and Englund C (2006). Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci. Res* 55:223-33

Hinds JW (1968). Autoradiographic study of histogenesis in the mouse OB. I. Time of origin of neurons and neuroglia. *J Comp Neurol* 134:287-304

Huang Q, Zhao S, Gaudin A, Quennedey B, Gascuel J (2005). Glial fibrillary acidic protein and vimentin expression in the frog olfactory system during metamorphosis. *Neuroreport* 16:1439-42

Huilgol D, Tole S (2016). Cell migration in the developing rodent olfactory system. *Cell Mol Life Sci* 73:2467-90

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Imamura F, and Greer CA (2013). Pax6 regulates Tbr1 and Tbr2 expressions in OB mitral cells. *Trends Neurosci* 377-84.

Imamura F, Ayoub AE, Rakic P, Greer CA (2011). Timing of neurogenesis is a determinant of olfactory circuitry. *Nat Neurosci* 14:331-7.

Joven A, Morona R, González A, Moreno N (2013). Spatiotemporal patterns of Pax3, Pax6, and Pax7 expression in the developing brain of a urodele amphibian, *Pleurodeles waltl. J Comp Neurol* 521:3913-53.

Kahoud RJ, Elsen GE, Hevner RF, Hodge RD (2014). Conditional ablation of Tbr2 results in abnormal development of the OBs and subventricular zone-rostral migratory stream. *Dev Dyn* 243:440-50.

Kálmán M, Gould RM (2001). GFAP-immunopositive structures in spiny dogfish, Squalus acanthias, and little skate, Raia erinacea, brains: differences have evolutionary implications. *Anat Embryol (Berl)* 204:59-80

Kohwi M, Osumi N, Rubenstein JL, Alvarez-Buylla A (2005). Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the OB. *J Neurosci* 25:6997-7003

Lazzari M, Bettini S, Franceschini V (2016).Immunocytochemical characterisation of ensheathing glia in the olfactory and vomeronasal systems of *Ambystoma mexicanum* (Caudata: Ambystomatidae). *Brain Struct Funct* 221:955-67.

Lazzari M, Bettini S, Franceschini V (2014).Immunocytochemical characterisation of olfactory ensheathing cells of zebrafish. *J Anat* 224:192-206

Lazzari M, Bettini S, Franceschini V (2013).Immunocytochemical characterization of olfactory ensheathing cells in fish. *Brain Struct Funct* 218:539-49.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Lim DA, Alvarez-Buylla A (2016). The Adult Ventricular-Subventricular Zone (V-SVZ) and OB (OB) Neurogenesis. *Cold Spring Harb Perspect Biol* 8:a018820

Méndez-Gómez HR, Vergaño-Vera E, Abad JL, Bulfone A, Moratalla R, de Pablo F, Vicario-Abejón C (2011). The T-box brain 1 (Tbr1) transcription factor inhibits astrocyte formation in the OB and regulates neural stem cell fate. *Mol Cell Neurosci* 46:108-21.

Miller AM, Treloar HB, Greer CA. (2010). Composition of the migratory mass during development of the olfactory nerve. *J Comp Neurol* 518:4825-41

Mione M, Shanmugalingam S, Kimelman D, Griffin K (2001). Overlapping expression of zebrafish T-brain-1 and eomesodermin during forebrain development. *Mech Dev* 100:93-7.

Mizuguchi R, Naritsuka H, Mori K, Mao CA, Klein WH, Yoshihara Y (2012).Tbr2 deficiency in mitral and tufted cells disrupts excitatory-inhibitory balance of neural circuitry in the mouse OB. *J Neurosci* 32:8831-44

Moreno N, Morona R, López JM, Dominguez L, Muñoz M, González A (2008). Anuran olfactory bulb organization: embryology, neurochemistry and hodology. *Brain Res Bull* 75:241-5.

Mueller T, Wullimann MF, Guo S (2008). Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa), and GAD67 gene expression. *J Comp Neurol* 507:1245-57.

Mueller T, Wulliman MF (2016). Atlas of early zebrafish brain development. Elsevier ISBN: 978012417269.

Murdoch B, Roskams AJ (2007). Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. *J Mol Histol* 38:581-99.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Nomura T, Osumi N (2004). Misrouting of mitral cell progenitors in the Pax6/small eye rat telencephalon. *Development* 131:787-96

Nomura T, Haba H, Osumi N (2007). Role of a transcription factor Pax6 in the developing vertebrate olfactory system. *Dev Growth Differ* 49:683-90

Ohmomo H, Ehara A, Yoshida S, Shutoh F, Ueda S, Hisano S (2011). Temporally distinct expression of vesicular glutamate transporters 1 and 2 during embryonic development of the rat olfactory system. *Neurosci Res* 70:376-82.

Olude MA, Ogunbunmi TK, Olopade JO, Ihunwo AO. (2014). The OB structure of African giant rat (Cricetomys gambianus, Waterhouse 1840) I: cytoarchitecture. *Anat Sci Int* 89:224-31.

Olude MA, Mustapha OA, Aderounmu OA, Olopade JO, Ihunwo AO (2015). Astrocyte morphology, heterogeneity, and density in the developing African giant rat (*Cricetomys gambianus*). *Front Neuroanat* 9:67.

Okere CO, Kaba H (2000). Region-specific localization of glutamine synthetase immunoreactivity in the mouse OB: implications for neuron-glia interaction in bulbar synaptic plasticity. *Brain Res* 857:308-12

Osorio J, Mueller T, Retaux S, Vernier P, Wullimann MF (2010). Phylotypic expression of the bHLH genes Neurogenin2, Neurod, and Mash1 in the mouse embryonic forebrain. *J Comp Neurol* 518:851-71

Pollard SM, Conti L, Sun Y, Goffredo D, Smith A (2006). Adherent neural stem (NS) cells from fetal and adult forebrain. *Cereb Cortex* 16 Suppl 1:i112-20.

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E (2012). Developmental, tract-tracing and immunohistochemical study of the peripheral

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

olfactory system in a basal vertebrate: insights on Pax6 neurons migrating along the olfactory nerve. *Brain Struct Funct* 219:85-104.

Quintana-Urzainki I, Rodríguez-Moldes I, Mazan S and Candal E (2015). Tangential migratory pathways of subpallial origin in the embryonic telencephalon of sharks: evolutionary implications. *Brain Struct. Funct* DOI 10.1007/s00429-014-0834-5.

Rodríguez-Moldes I, Manso MJ, Becerra M, Molist P, Anadón R (1993). Distribution of substance P-like ir in the brain of the elasmobranch *Scyliorhinus canicula. J Comp Neurol* 335: 228–244.

Rodríguez-Moldes I, Santos-Durán GN, Pose-Méndez S, Quintana-Urzainqui I, Candal E (2017). The Brains of Cartilaginous Fishes. In: Kaas J (ed.), Evolution of Nervous Systems 2e. vol. 1, pp. 77–97. Oxford: Elsevier. ISBN: 9780128040423.

Roybon L, Mastracci TL, Li J, Stott SR, Leiter AB, Sussel L, Brundin P, Li JY (2015). The Origin, Development and Molecular Diversity of Rodent OB Glutamatergic Neurons Distinguished by Expression of Transcription Factor NeuroD1. *PLoS One* DOI: 10.1371/journal.pone.0128035.

Roux L, Benchenane K, Rothstein JD, Bonvento G, Giaume C (2011). Plasticity of astroglial networks in olfactory glomeruli. *Proc Natl Acad Sci U S A* 108:18442-6.

Sakurai K, Osumi N (2008). The neurogenesis-controlling factor, Pax6, inhibits proliferation and promotes maturation in murine astrocytes. *J Neurosci* 28:4604-12.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Sarkar S, Atoji Y (2018). Distribution of vesicular glutamate transporters in the brain of the turtle (Pseudemys scripta elegans). *J Comp Neurol* 526:1690-1702.

Sánchez-Farías N, Candal E (2015). Doublecortin is widely expressed in the developing and adult retina of sharks. *Exp Eye Res* 134:90-100.

Spassky N, Heydon K, Mangatal A, Jankovski A, Olivier C, Queraud-Lesaux F, Goujet-Zalc C, Thomas JL, Zalc B (2001). Sonic hedgehog-dependent emergence of oligodendrocytes in the telencephalon: evidence for a source of oligodendrocytes in the OB that is independent of PDGFRalpha signaling. *Development* 128:4993-5004.

Stoykova A, Gruss P (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J Neurosci* 14(3 Pt 2):1395-412

Valverde F, Santacana M, Heredia M (1992). Formation of an olfactory glomerulus: morphological aspects of development and organization. *Neuroscience* 49:255-75

Wasowicz M, Ward R, Repérant J (1999). An investigation of astroglial morphology in torpedo and scyliorhinus. *J Neurocytol* 28:639-53

Winpenny E, Lebel-Potter M, Fernandez ME, Brill MS, Götz M, Guillemot F, Raineteau O (2011). Sequential generation of OB glutamatergic neurons by Neurog2-expressing precursor cells. *Neural Dev* 6:12

Yopak KE, Lisney TJ, Collin SP (2015). Not all sharks are "swimming noses": variation in OB size in cartilaginous fishes. *Brain Struct Funct.* 220:1127-43.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 1. Primary and secondary antibodies used.

Table1. Primary and secondary antibodies used.

Primary Antibody	Source	Working dilution	Secondary Antibody	Source	Working dilution
Pax6	Policlonal rabbit anti-Pax6 Covance (Cat nº PRB-278P)	1:300	**Goat anti-rabbit HRP coupled**	Dako, Glostrup, Denmark	1:200
PCNA	Monoclonal mouse anti-PCNA Sigma Cat. Nº. P8825	1:500	**Goat anti-mouse HRP coupled**	Dako, Glostrup, Denmark	1:200
GFAP	Polyclonal rabbit anti-GFAP Dako Cat. Nº. Z033429	1:500	**488-cojugated donkey anti-mouse**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
GS	Monoclonal mouse anti-GS Millipore Cat. Nº. MAB302	1:500	**546-conjugated donkey anti-rabbit**	Alexa Fluor Molecular Probes, Eugene, OR	1:200
BLBP	Polyclonal rabbit anti-BLBP Millipore Cat. Nº. ABN14	1:300			
Tbr1	Policlonal rabbit anti-Tbr1 Chemicon (Cat. nº AB9616) Millipore (Cat. nº AB10554)	1:200			

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Figure 1. Transverse sections showing the expression pattern of progenitor markers in the ventricular zone of the telencephalic walls of S30 of development. **(A)** Schema showing the main divisions of the developing brain of a S30 of development. The square represents the portion of the pallium studied at this stage of development. **(B)** Photomicrograph showing the expression pattern of Pax6 in the lateral portions of the pallium. Note two different intensities of immunolabeling. **(C)** Photomicrograph showing the expression pattern of *ScSox2*. **(D-D'')** Double immunofluorescence BLBP/PCNA in the lateral portions of the pallium showing double labelled cells (stars). **(E-E'')** Double immunofluorescence GFAP/PCNA in the lateral portions of the pallium showing double labelled cells (stars). **(F)** Panoramic of the telencephalon showing the expression pattern of Pax6 in a S31 of development. **(F')** Detail of the expression pattern of Pax6 showing a stream of Pax6 positive cells running from the ventricular zone to the OP. Note that Pax6 positive cells in the OP show low levels of Pax6. Scale bars: 200 µm (F-F') 25µm (B-E''). Abbreviations: DP, dorsal pallium; Hab, habenula; OE, olfactory epithelium; ON, olfactory nerve; OP, olfactory primordium; PT, pretectum; SP, subpallium; VLP, ventrolateral pallium.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

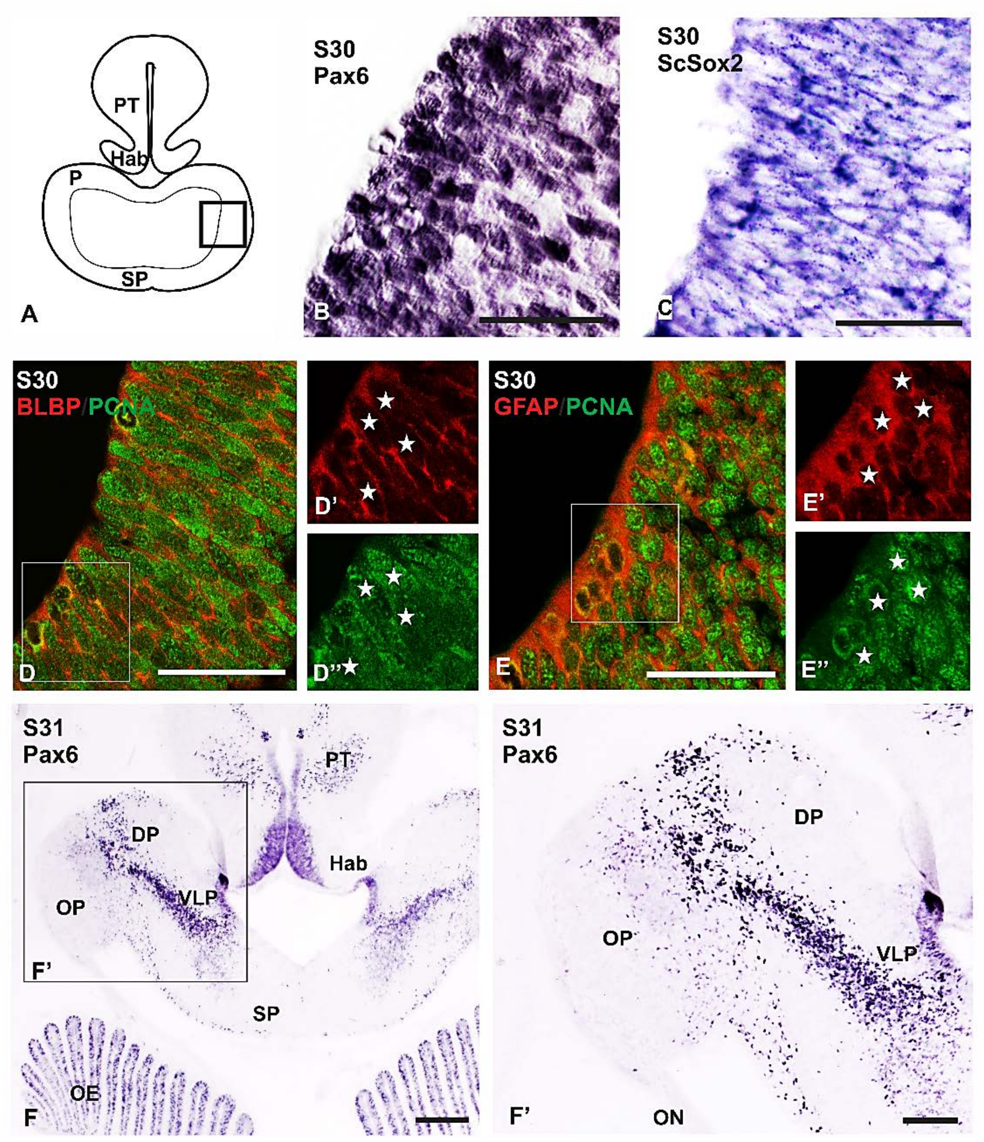


FIGURE 1

Figure 2. Transverse sections showing the expression pattern of the neurogenic markers *ScTbr2, ScNeuroD*, Tbr1 and *ScvGlut1* in the OP of S31 and S32 of development. In S31, *ScTbr2* (**A**) and *ScNeuroD* (**B**) are expressed as a specific cell band close to the future glomerular layer, meanwhile Tbr1 is expressed in all regions of the OP (**C**). Dotted lines shows the limit of the OP. In S32 embryos *ScTbr2* (**D**), *ScNeuroD* (**E**) and Tbr1 (**F**) are expressed in a similar pattern. Note that Tbr1 seems to label a wider territory compared with *ScTbr2* and *ScNeuroD*. Dotted lines shows the limit of the OB. Asterisks show protoglomerulli. **(G-G'')** Double ISH-IHC between *ScTbr2*-Tbr1 **(G)** in the lateral **(G')** and ventral **(G'')** portions of the distal OB, showing double-labelled cells. Note that in the lateral portion Tbr1 is more abundant **(G')**, in contrast to the ventral portion, where *ScTbr2* is more abundant **(G'')**. **(H-H'')** Double ISH-IHC between *ScNeuroD*-Tbr1 showing an almost total colocalization of both neurogenic markers. **(I)** Photomicrograph showing the expression pattern of *ScvGlu*t, coincident with the expression of *ScTbr2, ScNeuroD* and Tbr1. Abbreviations: GL, granular layer; OP, olfactory primordium; pGlom, protoglomeruli; VZ, ventricular zone. Scale bars: 100 µm (A, B, C, D, E, F, G, H, I), 50 µm (G', G'', H').

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

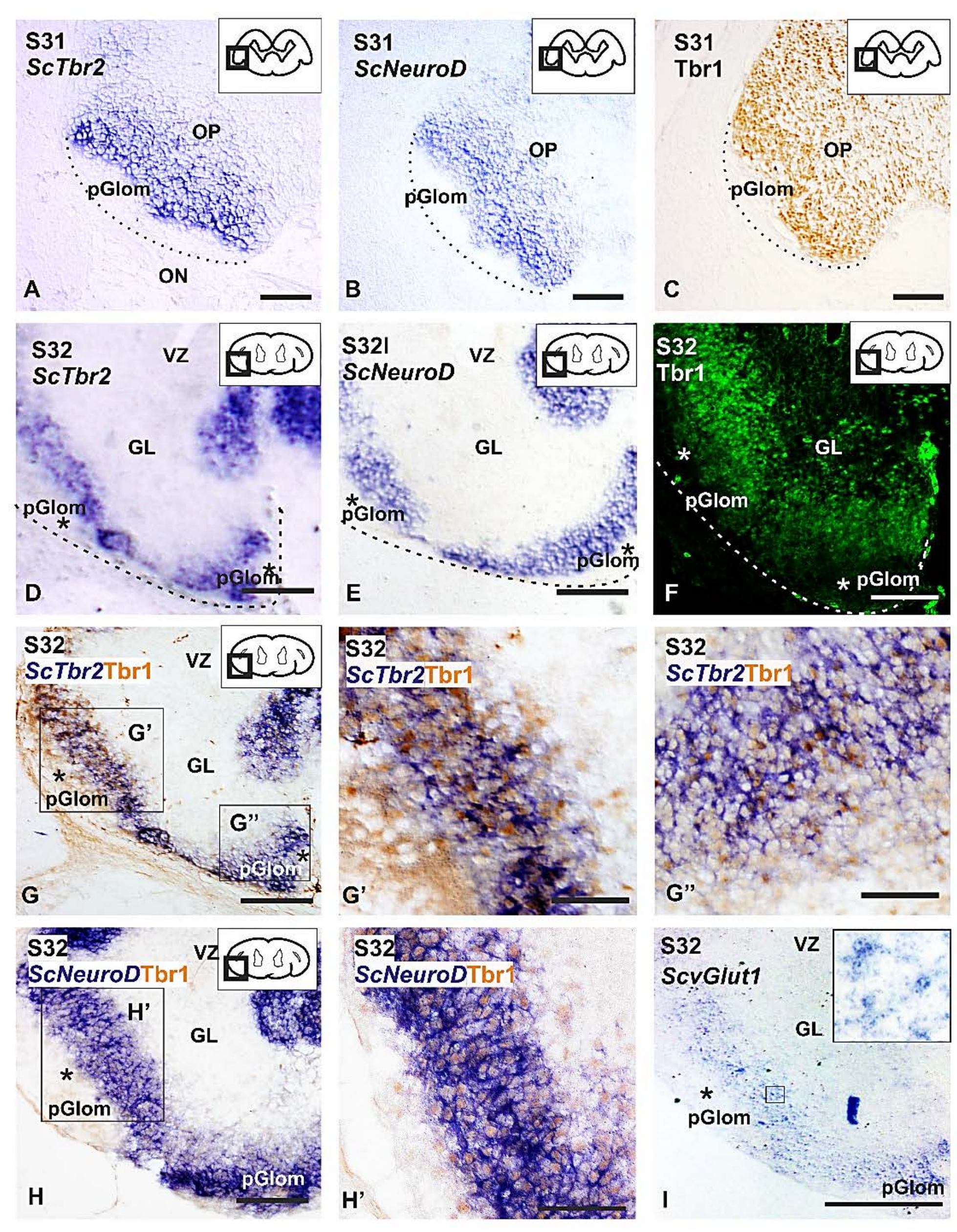
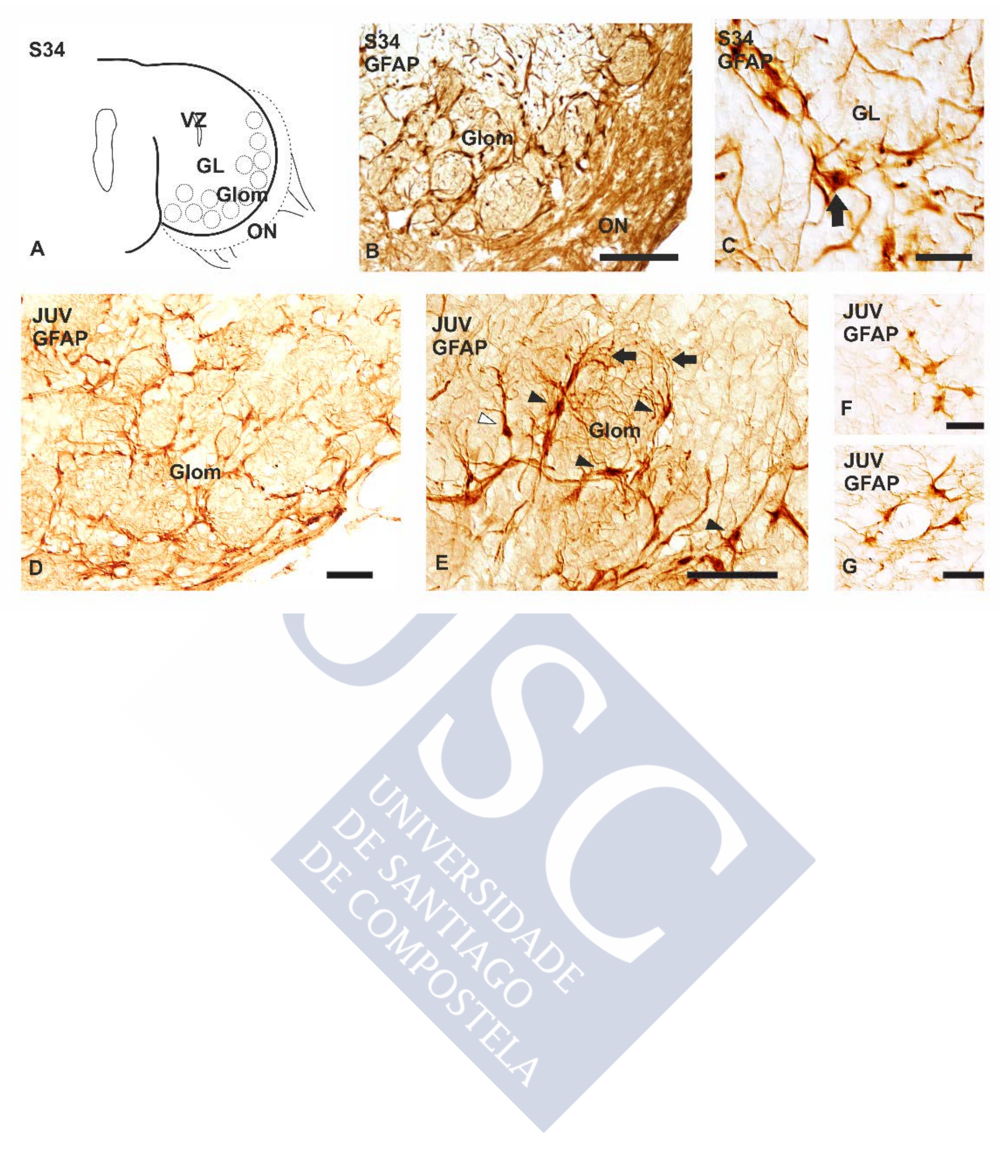


FIGURE 2

Figure 3. Transverse sections of the OB in S34 of development and posthatching/juveniles showing the expression of GFAP. (A) Schema of the OB of a S34 of development. **(B)** Panoramic of the glomerular layer showing GFAP positive cells surrounding glomeruli. Note also positivity for GFAP in the ON. In this stage of development small and stellate-like cells can also be visualized in the granular layer **(arrow in C)** and also perivascular immunoreactivity**.** In juveniles, GFAP positive cells can be observed in the glomerular layer surrounding glomeruli forming a glial shell-like structure **(D, E)** and numerous positive cells were found in the granular layer **(F, G).** Positive cells in the glomerular layer have high ramified branches that invade the neuropil of glomeruli **(arrows in E).** Note that these cells exhibit fusiform **(black arrowhead)** or tufted **(empty arrowhead)** morphology. In the granular layer positive cells have astrocytic-like shape and were usually seen surrounding blood vessels **(F, G).** Abbreviations: GL, granular layer; Glom, glomerular layer; ON, olfactory nerve; VZ, ventricular zone. Scale bars: 500 µm (D), 100 µm (B), 50 µm (B, E), 25 µm (C), 10 µm (F, G).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA



UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

FIGURE 3

Figure 4. Transverse sections of the OB from S32 of development to posthatching/juveniles showing the expression of BLBP and GS. (A-A'') Photomicrographs at different magnifications showing the expression pattern of BLBP in S32 embryos. Note the high amount of positive cells in the granular layer (**arrows in A'**) compared to the glomerular layer (**arrows in A''**). In juveniles the expression of BLBP is considerably lower and restricted to a few cells in the glomerular layer **(arrows in B', C). (D-D'')** Photomicrographs at different magnifications showing the expression pattern of GS at S32. Scattered positive cells for GS has been found in the glomerular (**arrows in D'**) and in the granular layer (**arrows in D''**) Note the similitudes with BLBP expression pattern. In juveniles, GS expression has been abundantly found in the glomerular layer as a shell of cells and processes that surrounds glomeruli **(F and arrows in G)** and as small highly branched cells widely distributed in the granular layer **(arrows in H).** Abbreviations: GL, granular layer; Glom, glomerular layer; OV, olfactory ventricle. Scale bars: 200 µm (B) 100 µm (A, D, F) 50 µm (A', A'', B', C, D', D'', G, H).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

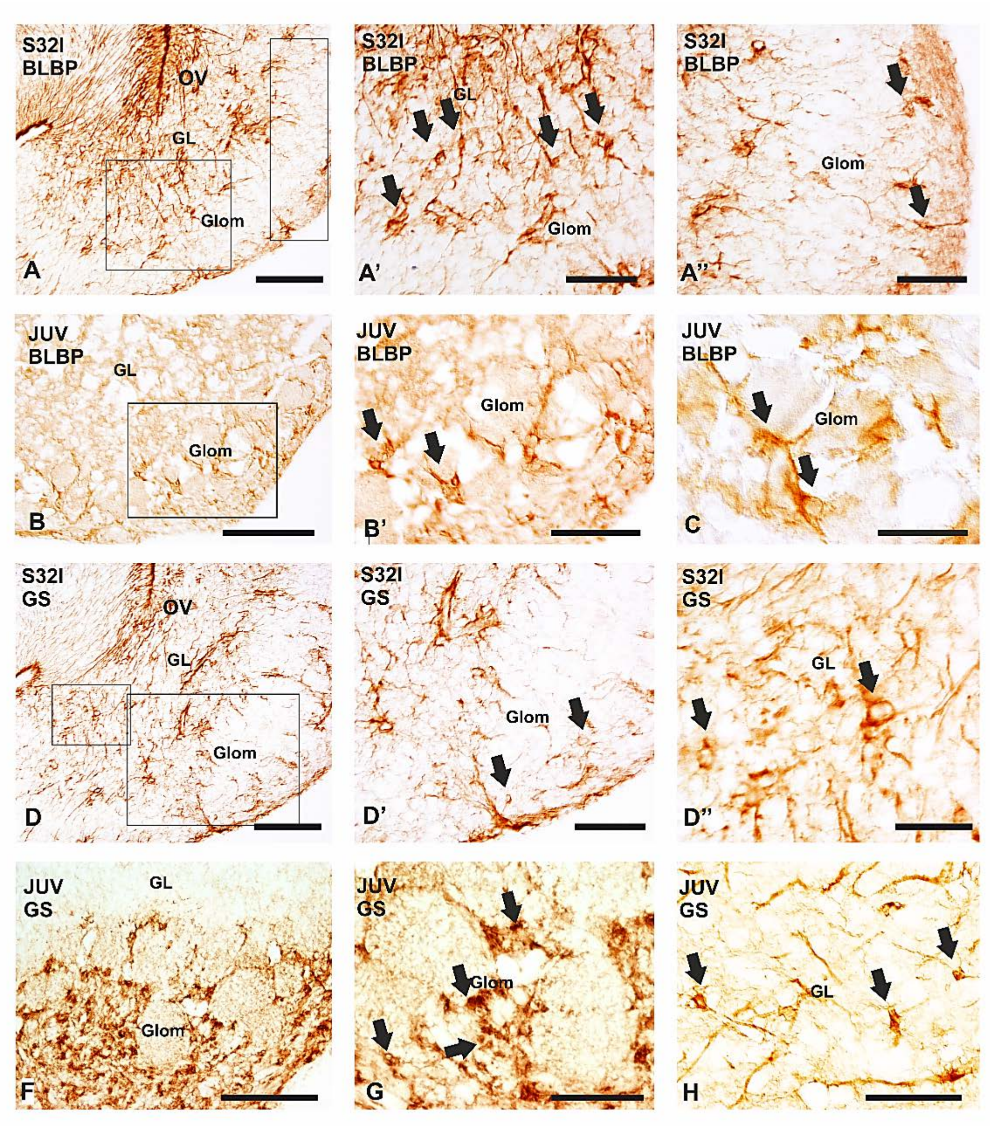


FIGURE 4

Figure 5. Double fluorescence between GS-GFAP (A-C'') and GS-BLBP (D-G'') showing double-labelled cell in the OB of a S32 of development. (A-B) Panoramic views of the OB showing double fluorescence GS/GFAP in the glomerular layer **(A)** and in the granular layer **(B).** Note that colocalization of both factors is restricted to some isolated processes. **(C-C'')** Detail of a cell that show a special morphology positive for GFAP and GS**. (D-G'')** Photomicrographs at different magnifications of the OB showing double fluorescence GS/BLBP. Note that most part of glial cells in the granular layer are double-labelled **(D-F''),** in contrast to the few cells in the glomerular layer that only express one of glial marker **(D, G-G'').** Abbreviations: GL, granular layer; Glom, glomerular layer. Scale bars: 200 µm (D), 100 µm (B), 50 µm (A), 10 µm (C-C'', E-G'').

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

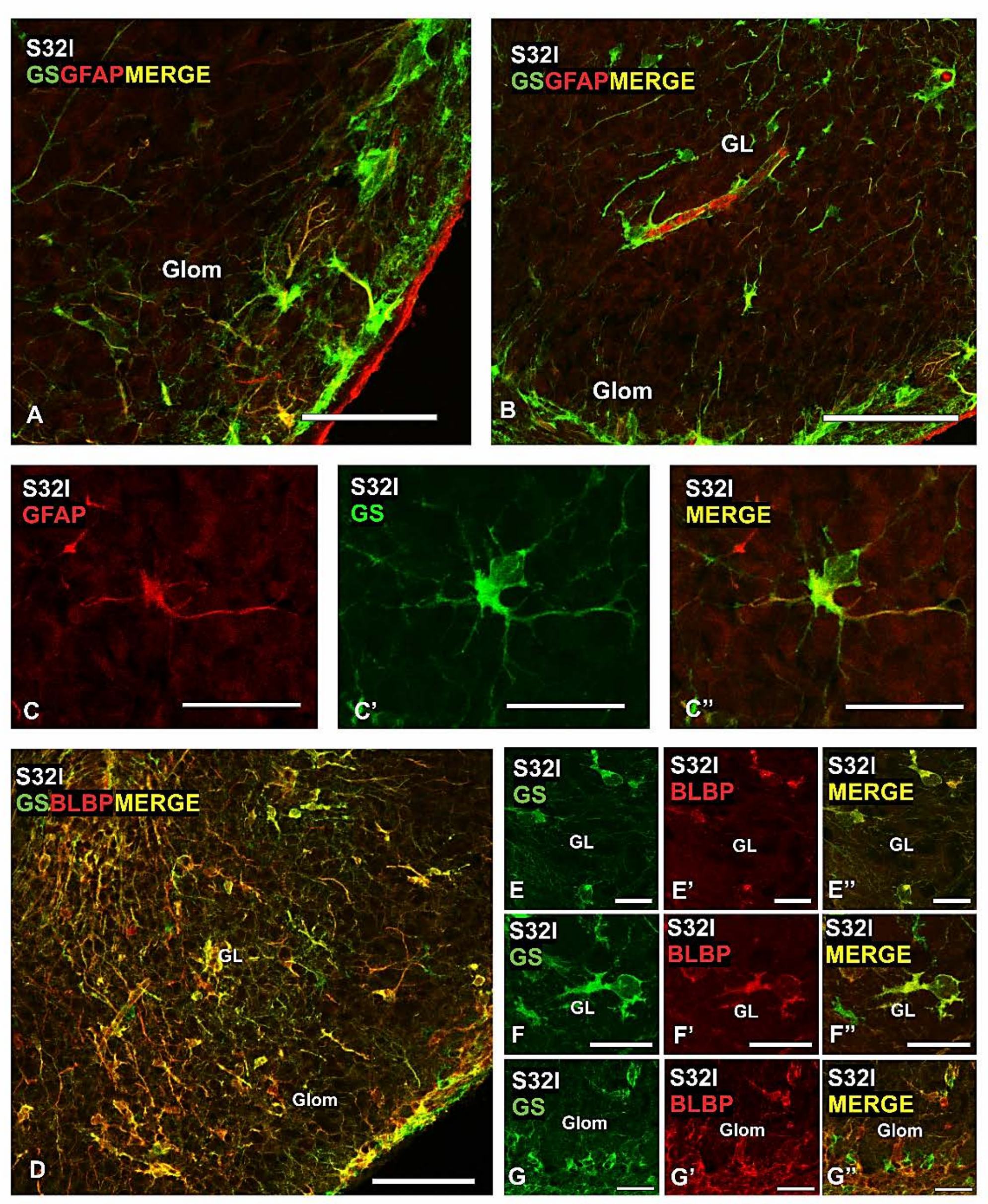


FIGURE 5

Figure 6. Double fluorescence between BLBP and PCNA showing double-labelled cell in the OB of a S32 of development. (A-B) Panoramic views at different levels of the OB showing coexpression between BLBP and PCNA. **(C-E'')** Details at higher magnification of glomerular (**C-C''),** granular **(D-D''),** and olfactory nerve layer **(E-E'')** showing double-labelled cells. Note that the vast majority of cells are double-labelled and that they are specially concentrated in the olfactory nerve layer. Abbreviations: GL, granular layer; Glom, glomerular layer; ONL, olfactory nerve layer. Scale bars: 200 µm (A, B), 25 µm (C-E'').

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

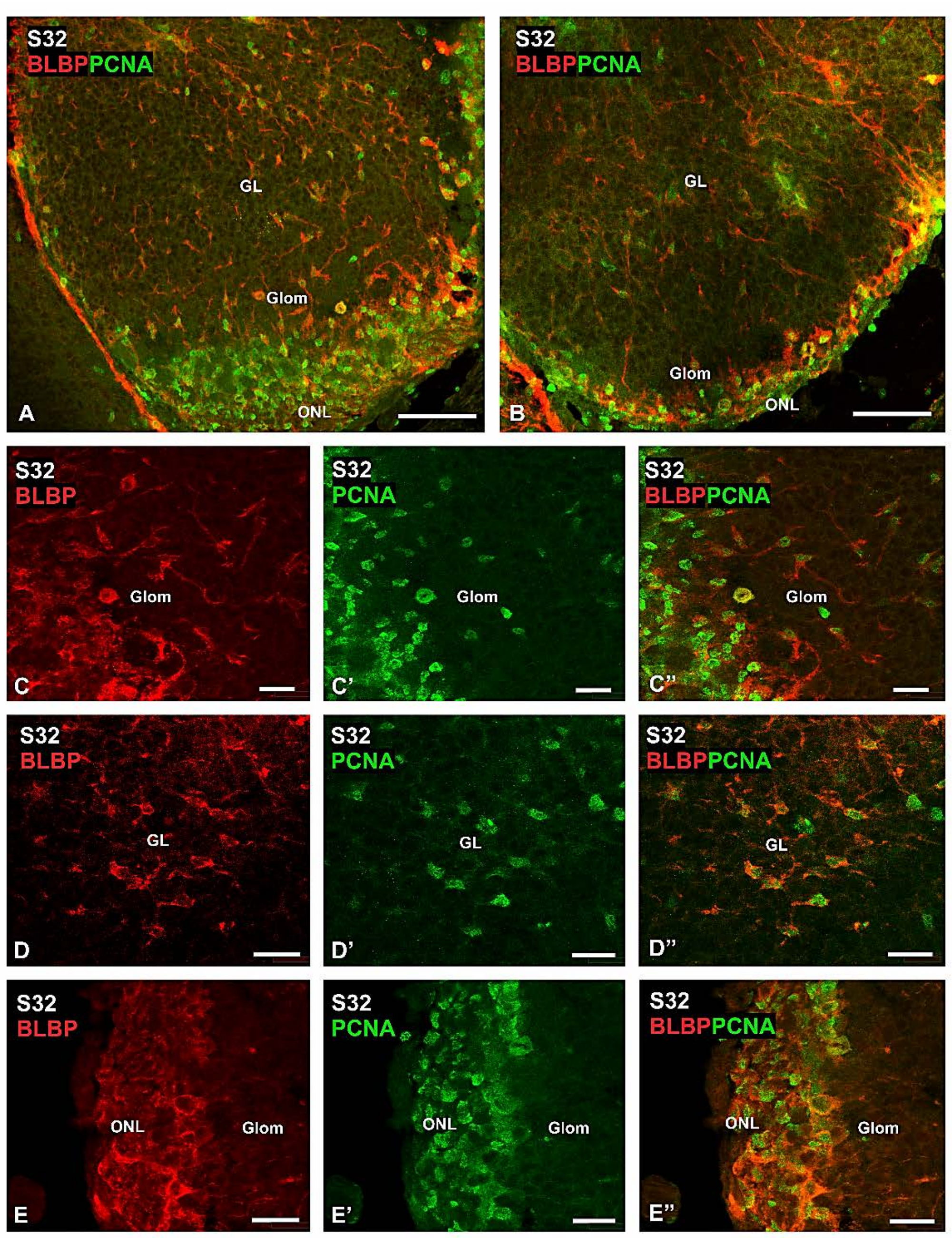


FIGURE 6

GENERAL DISUSSION

GENERAL DISCUSSION

The telencephalon of vertebrates has been the focus of extensive studies because its high cytoarchitectonic variability across the scale of vertebrates and also because of its capacity for adult neurogenesis even in mammals (reviewed by Medina and Abellán, 2009; Ming and Song, 2011; Grandel and Brand 2013, Bond et al. 2015; Lim and Alvarez-Buylla, 2016). Comparisons with other vertebrate groups revealed two significant facts. First, the number of neurogenic niches increases as we descend in the evolutive tree (reviewed by Ganz and Brand, 2016). Second, high numbers of progenitor radial cells persist in the zebrafish (a teleost usually used as a representative of all other fishes; reviewed by Than-Trong and Bally-Cuif, 2015). The high neurogenic capacity of fishes and the amount of persistent progenitor radial glial cells in adult zebrafish individuals made this species a perfect candidate to study neurogenesis in an evo-devo context (reviewed by Than-Trong and Bally-Cuif, 2015; and Ganz and Brand, 2016). However, studies in elasmobranch fishes, which possesses a high ratio of telencephalon/brain size and show a protracted period of embryonic development, evidenced some conserved processes regarding neuronal migratory pathways in the developing telencephalon that had been largely overlooked in zebrafish (Quintana-Urzainqui et al. 2015).

The scarcity of developmental information about chondrichthyans may explain in part why they were not traditionally contemplated in comparative analyses of the nervous system. The use of the zebrafish as a model organism of fish has yielded an incredibly large amount of data that have helped to identify conserved developmental processes and some derived traits that might be indicative of evolutionary trends. These animal model has mainly chosen for their technical advantages, such as easy maintenance or short generation time, rather than their ability to represent phylogenetic diversity. Bearing in mind that

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

fishes form the largest vertebrate group, that there exist great differences between cartilaginous and bony fishes, and that bony fishes, but not cartilaginous fishes, went through an extra duplication of their genomes, the widespread use of a single species as the canonical model to represent all fishes, is constraining the possibility of gaining knowledge about the diversity of fish brains. Thus, to deepen our understanding of vertebrate brain evolution and fish diversity in the context of neuroscience, studies in cartilaginous fishes are crucial (Rodríguez-Moldes et al. 2017).

Up to date, some studies have been performed to shed light into the development of telencephalon in *S. canicula* by using immunohistochemistry and *in situ* hybridization against neurochemical and transcription factors (Carrera et al. 2008; Ferreiro-Galve, 2010; Quintana-Urzainqui et al. 2012; Quintana-Urzainqui et al. 2015; Rodríguez-Moldes et al. 2017). These studies were focused on early regionalization of the telencephalon and migratory pathways. Present work corresponds to the first monographic study focused on neurogenesis of the telencephalon from different perspectives.

Previous studies form our group have identified that GABAergic cells are born in the subpallial ventricular zone of sharks and migrate towards the pallium during the neurogenic period as it happens in mammals and other species of vertebrates (Carrera et al. 2008; Quintana-Urzainqui et al. 2015). However, studies focused on the neurogenic process in the pallium itself have not been addressed until now (Chapter 1). We found that apical progenitors in the ventricular zone exhibit the classical morphology of radial glial cells and express typical molecular markers of progenitor cells as S*cSox2*, GFAP and Pax6. In addition, we have explored the existence of a subventricular zone (SVZ) during the development of the telencephalon, based on the definition of this structure stablished in the Boulder Committee Report of 1970 (Angevine et

al. 1970) and updated by Martínez-Cerdeño et al. 2016, which include: timing (must be a transitory structure), position (subventricular), expression of Tbr2 and presence of active mitotic cells that, at difference of apical progenitors, does not divide in the ventricular zone. Studies performed in other species suggest that the pallial SVZ appears in evolution in the transition from reptiles to mammals (Martínez-Cerdeño et al. 2006). However, recent studies pointed the possibility that these kind of subventricular intermediate progenitor cells (IPCs), might have appeared early in evolution (amphibians: Moreno and Gonzalez, 2017). Here we show that sharks possess traits of a primitive SVZ. The existence of SVZ-like in sharks might be related to the large size of the telencephalon. In addition, we have found that the main transcription factors (*ScTbr2-ScNeuroD*-Tbr1) that rule glutamatergic neurogenesis in the pallium of mammals are already present in sharks. These transcription factors are expressed sequentially during the process of maturation of pallial new-born cells in the cortex of mammals (reviewed by Hevner et al. 2006), a fact that has been related to layer-related fate specification (Hevner et al. 2006). However, in the pallium of catsharks, these transcription factors show an overlapping rather than a sequential pattern of expression, which can be related with the absence of layering in the pallium of this species.

We have then investigated the glial cytoarchitecture and how it changes during development and postnatally. Does exist a relationship between the radial glia and proliferation when the pallium is already mature? Developmental studies about radial glial cells are barely inexistence in fishes, which have been mainly carried out in teleost fishes. For that, we have analyzed the expression pattern of well-known radial glial markers such as GFAP, BLBP and GS in the telencephalon of *S. canicula* form early (S22) to post-hatching individuals (Chapter 2). We have found that the expression of these three markers began at different times of development; GFAP immunoreactivity begin in the early

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

period of development (before S26), while BLBP expression starts in the intermediate period (around S28), coinciding with the beginning of the neurogenic period. In contrast, GS immunoreactivity has been appreciated in the telencephalon in the transition from S31 to S32, coinciding with late development and the maturity period. During embryonic development of catshark, the GFAP, BLBP and GS labelled cells with radial glial morphology. The expression pattern of these markers is persistent in juveniles (present results) and, at least, GFAP and GS expression label ependymoglia (or tanycytes, Horstmann, 1954) in posthatching fish and adults of this specie (Wasovicz et al. 1999; Ari and Kàlmàn, 2008). Additionally, both during late development and in juveniles, GS positive cells have been found far away from the ventricular zone in cells with a non-radial morphology. These cells possess and eccentric nuclei, relatively large size and particular branching, which resembles morphologically to oligodendrocytes. Curiously, they are located in portions of the telencephalon where myelinated tracts are found (Smeets et al, 1983). This fact let us suspect that they could belong to the oligodendrocyte lineage. However, further studies with markers of oligodendrocytes are needed to solve this matter.

We have also investigated if the same populations of radial glial cells express GFAP, BLBP and GS in late stages of development. We found that, in the pallium, different subsets of radial glial cells can be found based on the combinatory expression of these markers. However, the vast majority express the three of them. In addition, we have investigated whether this populations of ventricular radial glia retain a proliferative capacity in late embryos by performing double-immunofluorescence against PCNA. We found that all subdivisions of the pallium exhibit proliferating cells and that most of them possess a radial glial nature. Relatively fewer numbers of proliferating cells are found in the subpallium.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Once we knew that radial glial cells maintain their proliferative capacity during the whole development of the telencephalon, we aimed to identify main cell types in neurogenic niches in the postnatal telencephalon. In mammals three main cell types are found in the telencephalic niches: radial glial cells (adult neural stem cells; B cells), IPCs (C cells) and neuroblasts (A cells; first described by Doetsch et al. 1997). These cells can be recognized by the differential expression of radial glial, progenitor and neuronal lineage markers in the telencephalon of different species of vertebrates such as birds (reviewed by Barnea and Pravasudov, 2011), reptiles (reviewed by González-Granero, 2011), amphibians (Simons et al. 2008; Kirkham et al. 2014) and teleost fishes (reviewed by März et al. 2010). In order to perform a detailed analysis of the neurogenic potential and the main cell types in the postnatal telencephalon of catshark (juveniles and adults) we have analyzed the proliferation pattern by using PCNA immunohistochemistry. Besides, we have used other markers in combination with PCNA to distinguish different cell types in proliferative areas of the juvenile/adult brain, like Sox2 (stem cells); GFAP, BLBP and GS (radial glia); and neuronal fate markers such as Dlx2 (GABAergic lineage), Tbr2 (Glutamatergic lineage) and DCX (neuronal lineage; Chapter 3). We found that the whole telencephalic ventricle exhibits proliferating cells, specially the presumptive ventral pallial subdivision where the ventricular zone is wider than the others. Also, we were able to identify different cell types including radial glial progenitor cells (B cells, positive for glial markers/PCNA), IPCs (C cells, positive for Dlx2/PCNA) and migratory neuroblasts (A cells, DCX/PCNA), but further studies are needed in order to deepen the knowledge about different types of cells mediating adult neurogenesis in the telencephalon of sharks. In addition, as studies in lampreys did not show PCNA populations in adult individuals (Villar-Cheda et al. 2006), we can point to an evolutionary origin of adult neurogenesis in the transition from agnathans to gnathostomes.

Most part of the studies in the telencephalon are focused on the telencephalic hemispheres. However, the OB also exhibits glutamatergic projecting neurons (mitral cells) that follow a similar neurogenic program to that of the cortex (Imamura and Greer, 2013). Besides, the OB astroglial system exhibits diverse amounts of cells with different morphology showing layer specificity (Bailey and Shipley, 1993; Gong and Shipley, 1995; Bailey et al. 1999). In line with previous chapters, we decide to study the development of the OB by investigating the expression pattern of glutamatergic neurogenic factors (Pax6, *ScTbr2, ScNeuroD* and Tbr1) and the distribution pattern of radial/astroglial markers (BLBP, GFAP, GS) (Chapter 4). We found that radial glial cells positive for BLBP, GFAP, Pax6 and the stem cell marker *ScSox2* can be found previously to the emergence of the OB (at S30). Besides, at the time where the primordium of the OB appears in S31, a stream of Pax6 positive cells were observed between the ventricular zone and the OP. At the same developmental period, transcription factors involved in mitral cell development (*ScTbr2*, *ScNeuroD,* Tbr1) were first expressed in the OP. These factors are still expressed at S32 in a territory that may correspond to the prospective glomerular layer (where mitral cells are intermingled). These transcription factors have an overlapping expression pattern, as in the pallium of *S. canicula* (Chapter 1), and the olfactory bulbs in mammals (Imamura and Greer, 2013). However, two subpopulations of mitral cells can be found in mammals based on these markers: Tbr2+NeuroD-Tbr1+ and Tbr2+NeuroD+Tbr1-. Despite in some species of elasmobranch fishes two subpopulations of mitral cell have been found (Dyer and Graziadei, 1993), we were not able to identify these subpopulations by using these molecular markers. On the other hand, from S32 onwards, glial cells with radial glial nature or astroglial features can be observed in the OB of catshark. The three glial markers studied began to be expressed at the same developmental period, in contrast to what it happens in the telencephalon (Chapter 2), pointing

to some differences between the glial system of the telencephalic hemispheres and the OB. However, different cells with astrocytic morphologies, which were not present in the telencephalon of this species (see Chapter 2), can be appreciated especially in the OB of posthatching individuals. The process of maturation and gliogenesis of these cells seems to occur both during embryonic development and postnatally.

The OB of fishes shows endogenous proliferation (Zupanc and Horschke, 1995; Wullimann and Knipp, 2000; Mueller and Wullimann, 2003; Grandel et al, 2006). Curiously, a considerable number of BLBP positive cells (adventricular cells) have been found in S32 embryos of catshark. BLBP expression has been related with neurogenic/gliogenic potential (Pinto and Götz, 2007). For that, we decided to investigate if they retain a proliferative potential by using double-immunofluorescence against PCNA. We found that most part of PCNA positive cells are also positive for BLBP, evidencing a neurogenic potential in astrocyte-like cells in sharks. Curiously, a considerable number of double-labelled cells can be appreciated in the ON entrance, suggesting a placodal origin. Glial cells BLBP positive with a particular phenotype are generated in the olfactory placode and migrate along the ON during development in mammals (Miller et al. 2010). These cells are called olfactory ensheathing cells and have regenerative properties. Whether these cells invade the OB of sharks in late stages of development or whether they have the ability to generate different neural or glial phenotypes deserves further investigation.

Final considerations

The discovery of a rudimentary SVZ, the high proliferative capacity of radial glia and astroglial cells in the telencephalon and OB, the adult neurogenic potential and other particularities of this specie clearly highlight the importance of studying basal vertebrates as sharks to understand the evolution of vertebrates. Besides, all of this characteristic made the catshark an excellent model to study neurogenic processes both during development and postnatally. In addition, sharks seem to possess a particular glial system in the OB that could be used to explore other glial cell functions and their implications during telencephalic development.

However, some other questions remain unresolved; do developing IPCs share more molecular markers with mammals, or they express only the basic ones? Are adventricular GS-BLBP positive cells truly oligodendrocytes? What are the final destinations of adult newborn cells in the telencephalon? What are the origin and role of proliferating glial cells in the OB? We hope that this study paves the way for further investigations on the field of neurogenesis and help to shed light into the evolution of the telencephalon of vertebrates.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

References

Angevine JB, Bodian D, Coulombre AJ, Edds MV, Hamburger V, Jacobson M, Lyser KM, Prestige MC, Sidman RL, Varon S, Weiss PA (1970). Embryonic vertebrate central nervous system: revised terminology. *Anatomical Record* 166:257–261.

Ari C, Kálmán M (2008). Evolutionary changes of astroglia in Elasmobranchii comparing to amniotes: a study based on three immunohistochemical markers (GFAP, S-100, and glutamine synthetase). *Brain Behav Evol* 71:305-24.

Bailey MS, Shipley MT (1993). Astrocyte subtypes in the rat olfactory bulb: morphological heterogeneity and differential laminar distribution. *J Comp Neurol* 328:501-26.

Barnea A, Pravosudov V (2011) Birds as a model to study adult neurogenesis: bridging evolutionary, comparative and neuroethological approaches. *Eur J Neurosci* 34:884-907.

Bond AM, Ming GL, Song H (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell* 17:385-95.

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R, Rodríguez-Moldes I (2008). Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. *Brain Res Bull* 75(2-4):405-9.

Doetsch F, García-Verdugo JM, Alvarez-Buylla A (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 17:5046-61.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Dryer L, Graziadei PPC (1993). A pilot study on morphological compartmentalization and heterogeneity in the elasmobranch olfactory bulb. *Anat Embryol* 188:41-51.

Ferreiro-Galve S (2010). Brain and retina regionalization in sharks: study based on the spatiotemporal expression pattern of Pax6 and other neurochemical markers. PhD.

Ganz J, Brand M (2016). Adult Neurogenesis in Fish. *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a019018.

Grandel H, Kasli J, Ganz J, Wenzel I, Brand M (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: Origin, proliferation dynamics, migration and cell fate. *Dev Biol* 295:263-277.

Grandel H, Brand M (2013). Comparative aspects of adult neural stem cell activity in vertebrates. *Dev Genes Evol* 223:131-47.

González-Granero S, Lezameta M, García-Verdugo JM (2011). Adult neurogenesis in reptiles. In: Seki T, Sawamoto K, Parent JM, Alvarez-Buylla A (eds) Neurogenesis in the adult brain, vol. I. Springer, pp- 169-189.

Hevner RF, Hodge RD, Daza RA, Englund C (2006). Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci Res* 55:223-33.

Horstmann E (1954). Die Faserglia des Selachiergehirns. *Z Zellforsch Mikrosk Anat* 39:588-617.

Imamura F and Greer CA (2013). Pax6 regulates Tbr1 and Tbr2 expressions in olfactory bulb mitral cells. *Trends Neurosci* 37(2):77-84.

Kirkham M, Hameed LS, Berg DA, Wang H, Simon A (2014). Progenitor cell dynamics in the Newt Telencephalon during homeostasis and neuronal regeneration. *Stem Cell Reports* 2:507-19.

Kriegstein A, Noctor S, Martínez-Cerdeño V (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat Rev Neurosci* 7:883-90

Lim DA, Alvarez-Buylla A (2016). The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a018820.

Martínez-Cerdeño V, Noctor SC and Kriegstein AR (2006). The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb Cortex* 16 Suppl 1:152-61

Martínez-Cerdeño V, Cunningham CL, Camacho J, Keiter JA, Ariza J, Lovern M and Noctor SC (2016). Evolutionary origin of Tbr2-expressing precursor cells and the subventricular zone in the developing cortex. *J Comp Neurol.* doi: 10.1002/cne.23879.

März M, Chapouton P, Diotel N, Vaillant C, Hesl B, Takamiya M, Lam CS, Kah O, Bally-Cuif L, Strähle U (2010). Heterogeneity in progenitor cell subtypes in the ventricular zone of the zebrafish adult telencephalon. *Glia* 58:870-88.

Medina L, Abellán A (2009). Development and evolution of the pallium. *Semin. Cell Dev Biol* 20:698-711.

Miller AM, Treloar HB, Greer CA. (2010). Composition of the migratory mass during development of the olfactory nerve. *J Comp Neurol* 518:4825-41

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Ming GL, Song H (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70:687-702.

Moreno N, González A (2017). Pattern of Neurogenesis and Identification of Neuronal Progenitor Subtypes during Pallial Development in *Xenopus laevis Front Neuroanat* 11:24.

Mueller T, Wullimann MF (2003). Anatomy of neurogenesis in the early zebrafish brain. *Dev Brain Res* 140:137-155.

Pinto L, Götz M (2007). Radial glial cell heterogeneity--the source of diverse progeny in the CNS. *Prog Neurobiol* 83:2-23.

Quintana-Urzainqui I, Sueiro C, Carrera I, Ferreiro-Galve S, Santos-Durán G, Pose-Méndez S, Mazan S, Candal E, Rodríguez-Moldes I (2012). Contributions of developmental studies in the dogfish Scyliorhinus canicula to the brain anatomy of elasmobranchs: insights on the basal ganglia. *Brain Behav Evol* 80:127-41

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E (2014a). Developmental, tract-tracing and immunohistochemical study of the peripheral olfactory system in a basal vertebrate: insights on Pax6 neurons migrating along the olfactory nerve. *Brain Struct Funct* 219:85-104.

Quintana-Urzainqui I, Anadón R, Candal E, Rodríguez-Moldes I (2014b). Development of the terminal nerve system in the shark *Scyliorhinus canicula*. *Brain Behav Evol* 84:277-87

Quintana-Urzainqui I, Rodríguez-Moldes I, Mazan S, Candal E (2015). Tangential migratory pathways of subpallial origin in the embryonic telencephalon of sharks: evolutionary implications. *Brain Struct Funct* 220:2905-26.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Rodríguez-Moldes I, Santos-Durán GN, Pose-Méndez S, Quintana-Urzainqui I, Candal E (2017). The Brains of Cartilaginous Fishes. In: Kaas J (ed.), Evolution of Nervous Systems 2e. vol. 1, pp. 77–97. Oxford: Elsevier. ISBN: 9780128040423.

Simmons AM, Horowitz SS, Brown RA (2008). Cell proliferation in the forebrain and midbrain of the adult bullfrog, *Rana catesbeiana. Brain Behav Evol* 71:41-53.

Smeets WJAJ, Nieuwenhyus R, Roberts BL (1983). The central nervous system of cartilaginous fishes. Ed. Springer-Verlag.

Than-Trong E, Bally-Cuif L (2015). Radial glia and neural progenitors in the adult zebrafish central nervous system. *Glia* 63:1406-28.

Villar-Cheda B, Pérez-Costas E, Meléndez-Ferro M, Abalo XM, Rodríguez-Muñoz R, Anadón R, Rodicio MC (2006). Cell proliferation in the forebrain and midbrain of the sea lamprey. *J Comp Neurol* 494:986-1006.

Wasowicz M, Ward R, Repérant J (1999). An investigation of astroglial morphology in torpedo and scyliorhinus. *J Neurocytol* 28:639-53.

Wullimann MF, Knipp S (2000). Proliferation pattern changes in the zebrafish brain from embryonic thorugh early postembryonic stages. *Anat Embryol* 202:385-400.

Zupanc GK, Horschke I (1995). Proliferation zones in the brain of adult gymnotifrom fish: a quantitative mapping study. *J Comp Neurol* 353:213-33.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CONCLUSIONS

GENERAL CONCLUSIONS

1. The expression pattern of progenitor and glial markers at the beginning of the neurogenic period revealed that apical progenitors and radial migration are present in sharks and therefore, their presence must be highly conserved throughout evolution.

2. We have identified a transitory subventricular band of *ScTbr2*-expressing cells during embryonic development, some of which also expressed mitotic markers, indicating that the existence of basal progenitors should be considered an ancestral condition rather than a novelty of mammals or amniotes.

3. The main transcriptional program specifying the glutamatergic lineage in the pallium (Pax6, Tbr2, NeuroD, Tbr1) was already present in cartilaginous fishes during embryonic development. Contrary to that reported in mammals, we do not observe a segregation of these transcription factors in different cell populations, indicating that this non-zonal distribution pattern might be the ancestral condition and may be linked to the lack of layering in the telencephalon of anamniotes.

4. Radial glial markers such as GFAP, BLBP and GS are sequentially expressed in radial glial cells mainly located in the telencephalic ventricular zone of early, intermediate and late embryos, respectively, and its expression becomes persistent in the postnatal telencephalon.

5. During late embryonic development, BLBP and GS, are also expressed in adventricular cells with morphological features of oligodendrocytes.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

6. In late embryos, heterogeneous subpopulations of radial glial cells are identified in the ventricular surface of the telencephalic hemispheres based on the expression pattern of GFAP, BLBP and GS, and the vast majority of these cells show proliferative capacity.

7. Proliferation markers are expressed in the ventricular zone of the telencephalic hemispheres in juvenile and adult catsharks, being relatively more abundant in the ventral pallium. The absence of proliferation in adult lampreys suggests an evolutionary origin of adult neurogenesis in the transition from agnathans to gnathostomes.

8. We have characterized different types of proliferating cells in the telencephalic ventricular zone of juveniles, which were interpreted as stem radial glial progenitors, intermediate progenitor cells and migratory neuroblasts, underlying the conserved nature of different progenitor types in the adult brain of gnathostome vertebrates.

9. Pax6 expressing cells present in the pallial ventricular zone exhibited molecular hallmarks of radial glial cells and proliferative activity before the emergence of the OB, which suggest they might be the source of mitral cells during development of this structure.

10. The expression pattern of *ScvGlut1* in the developing OB overlaps with the expression of a battery of transcription factors *(ScTbr2, ScNeuroD*, Tbr1) involved in the specification/differentiation of glutamatergic cells in the mammalian pallium, which suggest that this transcriptional program has been conserved throughout the scale of vertebrates.

11. Radial glial markers such as GFAP, BLBP and GS begins to be expressed simultaneously in the developing OB in the transition from S31 to S32 and their expression persist in juveniles.

12. Numerous cells coexpress BLBP and GS in all regions of the OB in late embryos, which is in contrast with that observed in posthatching specimens where BLBP expression decreases and GS expression increases drastically in the glomerular layer.

13. Numerous GFAP expressing cells with bipolar and astrocyte-like morphology are observed in different regions of the OB of juveniles, which point to a higher degree of morphological heterogeneity of glial cell populations in the OB compared with the telencephalic hemispheres.

14. In late embryos, most of the BLBP positive cells present in the granular, glomerular and olfactory nerve layers of the OB show proliferative activity, suggesting that these cells could be the source of neural cells populations of the OB.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Resumen en castellano

RESUMEN EN CASTELLANO

El telencéfalo es la parte del cerebro responsable de procesos cognitivos superiores, así como del procesamiento y la asociación de información sensorial y motora. Uno de los procesos más importantes en el que intervienen es la memoria y el aprendizaje, aunque también es esencial para aspectos relacionados con la supervivencia, como el sentido del olfato. Es conocida como una de las estructuras cerebrales más plásticas y también como la estructura que cambia más morfológicamente a nivel evolutivo. Dada la diversidad de morfologías y subdivisiones que presenta, muchos estudios se han centrado en la comprensión de los mecanismos morfogenéticos básicos (regionalización temprana y patrones específicos de neurogénesis, diferenciación y migración celular) que subyacen a esta variación. Los primeros estudios genéticos entre especies han demostrado que el telencéfalo de vertebrados se divide en dos regiones: palio (dorsal) y subpalio (ventral), separados por el límite palio-subpalio. Además, los estudios desde peces a mamíferos han confirmado que el palio se subdivide en 4 subdivisiones: palio dorsal, palio medial, palio ventral y palio lateral, cada uno de ellos con sus respectivos derivados, como por ejemplo la corteza cerebral o el hipocampo en mamíferos. El conocimiento de cómo surgen estas estructuras como resultado de variaciones en la velocidad y duración del proceso de neurogénesis, en la especificación/diferenciación de diferentes tipos neuronales o en la dirección de la migración celular, es esencial para comprender la gran variabilidad en la citoarquitectura del telencéfalo.

Curiosamente, el telencéfalo (incluidos los bulbos olfatorios) de mamíferos es una de las pocas estructuras que presenta neurogénesis activa en etapas adultas. El número de estudios que se han centrado en el análisis de este proceso es extenso en mamíferos, pero los mecanismos que lo subyacen en otros grupos de

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

vertebrados todavía están pobremente caracterizados. Hasta la fecha, varios grupos se han estudiado fuera del orden de los mamíferos, como aves, reptiles, anfibios y peces teleósteos, pero el organismo más estudiado es el pez cebra, que, contrariamente a los mamíferos, posee un gran número de nichos neurogénicos y un gran número de células gliales progenitoras en el adulto. Sin embargo, la falta de estudios en más vertebrados basales como tiburones (peces cartilaginosos) excluye cualquier conclusión sobre los componentes obligatorios (conservados) versus específicos (divergentes) de los nichos germinales y la heterogeneidad en las poblaciones de células progenitoras, tanto durante la embriogénesis como en el adulto. Los peces cartilaginosos o condríctios, representan una antigua radiación de vertebrados considerados como el grupo hermano de gnatóstomos con esqueleto óseo que dio lugar a los vertebrados terrestres. La posición filogenética de este grupo de vertebrados basales como un grupo externo a todos los demás vertebrados con mandíbulas vivientes (gnatóstomos) hace que los condríctios sean esenciales para evaluar la organización ancestral del cerebro de los vertebrados con mandíbula. El modelo animal principal que representa los peces cartilaginosos es la pintarroja o *Scyliorhinus canicula*.

El objetivo del presente trabajo es estudiar los mecanismos morfogenéticos básicos (patrones específicos de neurogénesis y gliogénesis que dependen de diferentes tipos de células progenitoras) que conforman el telencéfalo de la pintarroja. Con este objetivo, analizamos el patrón de expresión de varios marcadores de diferentes tipos de células progenitoras y células neuronales y gliales en el telencéfalo en desarrollo y adulto mediante el uso de técnicas de inmunohistoquímica o inmunofluorescencia e hibridación *in situ*. Nuestros resultados sugieren que los cambios en los mecanismos morfogenéticos básicos a lo largo de la evolución pueden contribuir a las variaciones interespecíficas en la morfología, el tamaño y la función del telencéfalo.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CAPÍTULO 1

La parte dorsal del telencéfalo en desarrollo es una de las áreas del cerebro que ha sufrido los cambios más drásticos durante la evolución de vertebrados. Durante el desarrollo de mamíferos, el palio dorsal generará localmente neuronas piramidales que construirán el sistema principal de conexiones punto a punto de la corteza. Estas células se especifican y luego se diferencian mediante la expresión de una secuencia bien conocida de factores de transcripción (Pax6, Tbr2, NeuroD y Tbr1). Se cree que el aumento evolutivo en la complejidad del palio dorsal de los mamíferos se logró en parte por la aparición de un nuevo nicho neurogénico en la zona subventricular embrionaria (SVZ). Aquí, un nuevo tipo de progenitores amplificadores (progenitores basales) derivados de células gliales radiales (progenitores apicales) y que expresan Tbr2, experimentan una segunda ronda de divisiones, que se cree que ha contribuido a la expansión del neocortex, al aumento de la complejidad e incluso ha sido relacionado con la girificación. En consecuencia, la existencia de una SVZ palial se ha considerado clásicamente exclusiva de los mamíferos. Sin embargo, la falta de estudios en vertebrados basales imposibilita cualquier conclusión clara sobre el origen evolutivo de la SVZ y los mecanismos neurogénicos que regulan el desarrollo palial.

En este capítulo exploramos la neurogénesis palial en un vertebrado basal, el tiburón *Scyliorhinus canicula*, a través del estudio de los patrones de expresión de varios marcadores neurogénicos, que incluye marcadores de glia radial, marcadores proliferativos y marcadores de linaje glutamatérgico entre otros. Encontramos que los progenitores apicales y la migración radial están presentes en los tiburones y, por lo tanto, su presencia debe estar altamente conservada a lo largo de la evolución. Sorprendentemente, detectamos una banda

subventricular de células que expresan *ScTbr2*. Algunas de estas células expresan marcadores mitóticos y experimentaron segundas rondas de proliferación (PH3 y BrdU), lo que indica que la existencia de progenitores basales debería considerarse una condición ancestral en lugar de una novedad de mamíferos o amniotas. Sin embargo, la cantidad de células mitóticas encontradas en esta posición subventricular parece ser mucho menor que en los mamíferos. Parece plausible que lo que estamos observando en el cerebro de *S. canicula* sean los primeros pasos hacia la formación de progenitores intermedios organizados en una zona definida en el palio de los vertebrados. En base a esto, hemos sugerido que, como los peces cartilaginosos son los primeros vertebrados en desarrollar pallium evaginado con ventrículos grandes y claros, es concebible que la expansión del palio en los tiburones con respecto a las lampreas se deba a la aparición de un nuevo tipo de progenitor amplificador. Finalmente, observamos que el programa transcripcional para la especificación de células glutamatérgicas (Pax6, Tbr2, NeuroD, Tbr1) también está presente en los tiburones. Sin embargo, la segregación de estos marcadores en diferentes tipos de células aún no está clara. En este contexto, hemos discutido si este hecho puede estar relacionado con la falta de estratificación en anamniotas.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CAPÍTULO 2

Las células gliales radiales (RGCs, de sus siglas en inglés) son la primera población celular de naturaleza glial que aparece durante la ontogenia cerebral. Inicialmente se pensó que tenían un papel estructural durante el desarrollo al contribuir a construir andamios para la migración neuronal. Hoy en día, se sabe que estas células realizan numerosas funciones en el cerebro de los vertebrados, incluido un papel esencial en los procesos neurogénicos, tanto durante el desarrollo como en etapas adultas, como células primarias progenitoras (células

tronco). La capacidad proliferativa de estas células, tanto en el desarrollo como en la edad adulta, ha sido tema de interés durante las últimas décadas. En contraste con los mamíferos donde las RGCs están restringidas a áreas ventriculares específicas en el cerebro adulto, las RGCs son el elemento glial predominante en los peces adultos. Estas células se han caracterizado en el cerebro en desarrollo de peces teleósteos. Sin embargo, los estudios de desarrollo sobre las RGCs en peces cartilaginosos son escasos. Por lo tanto, en el presente trabajo hemos estudiado en detalle los patrones de expresión de los marcadores de RGCs, incluida la proteína ácida fibrilar glial (GFAP), la proteína cerebral de unión a lípidos (BLBP) y la glutamina sintasa (GS) en los hemisferios telencefálicos de la pintarroja (*Scyliorhinus canicula*) desde los embriones tempranos hasta estadios postnatales.

GFAP, BLBP y GS se detectan por primera vez, respectivamente, en embriones tempranos, intermedios y tardíos. Los embriones tempranos (hasta S26) pertenecen a un período de desarrollo en el que se logra progresivamente la morfología elemental básica y se produce la expansión del neuroepitelio. Por otro lado, los embriones intermedios (de S27 a S31) pertenecen a un período de desarrollo donde se está produciendo neurogénesis activa; y los embriones tardíos (S32 en adelante) pertenecen a una etapa de madurez. Se desconoce si la expresión GFAP, BLBP y GS está vinculada a diferentes períodos de desarrollo o su función en esos períodos, pero es factible pensar que existe una relación directa entre ambos hechos. La expresión de estos marcadores gliales se observó en células con morfología de glía radial tapizando los ventrículos telencefálicos, así como en sus procesos radiales y pies terminales en la superficie pial, y su expresión continúa en células ependimarias y/o tanicitos en juveniles tempranos. El patrón de inmunorreactividad, similar para GFAP, BLBP y GS en la zona ventricular en etapas tardías de desarrollo, nos llevó a explorar la posibilidad de que los tres marcadores gliales estuvieran marcando las mismas

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

poblaciones gliales. La inmunofluorescencia doble y el recuento celular han demostrado que la gran mayoría (entre 80 y 85%) de las células expresan los tres marcadores, dejando una pequeña población de células que expresan un solo un marcador, evidenciando un cierto grado de heterogeneidad en la zona ventricular del palio. Además, la mayoría de las RGCs en etapas posteriores de desarrollo han demostrado la coexpresión del marcador de proliferación PCNA. Curiosamente, se han observado diferencias en el potencial proliferativo entre la parte dorsal y ventral, siendo mayor en el palio dorsal.

También se observaron algunas células inmunorreactivas a BLBP y GS situadas lejos de la zona ventricular y que se asemejan morfológicamente a los oligodendrocitos. Las fibras en el telencéfalo de la pintarroja son en su mayoría amielínicas, excepto en los haces compactos que atraviesan el palio medial y el palio dorsal centromedial. Curiosamente, también hemos observado células similares positivas para GS en el romboencefalo de esta especie asociada con tractos mielinizados de gran diámetro, como el fascículo longitudinal medial. La presencia deun tipo de célula GS positivo que se parece morfológicamente a oligodendrocitos en regiones que contienen fibras mielinizadas, sugiere la posibilidad de que sean realmente oligondedrocitos.

La alta capacidad proliferativa de los peces cartilaginosos y la presencia persistente de RGCs después del nacimiento merece más investigaciones para determinar si las RGCs progenitoras similares a las encontradas en los embriones también están presentes en el cerebro adulto (véase el Capítulo 3).

CAPÍTULO 3

La neurogénesis es un proceso de múltiples pasos por el cual las células progenitoras se convierten en neuronas diferenciadas bajo el control de factores

intrínsecos y extrínsecos. El estudio de la neurogénesis adulta ha captado enormemente la atención en los últimos años con el objetivo de comprender los mecanismos de plasticidad implicados en procesos de formación de memoria y toma de decisiones, adaptación a nuevos entornos o con el objetivo de desarrollar nuevos tratamientos basados en células para enfermedades neurodegenerativas. Los sitios activos de neurogénesis adulta existen desde peces a mamíferos, aunque, en el cerebro de mamíferos adultos, el número y la extensión de las áreas neurogénicas se reduce considerablemente en comparación con vertebrados no mamíferos. En el cerebro de los mamíferos, la neurogénesis adulta tiene lugar principalmente en dos regiones del telencéfalo: la zona subgranular (SGZ) del giro dentado del hipocampo y la zona subventricular (SVZ) de las paredes laterales de los ventrículos telencefálicos. En el nicho telencefálico adulto de los mamíferos, las neuronas recién nacidas se generan a partir de células madre neurales adultas, también llamadas, en general, progenitores adultos. Hoy en día, se acepta que las células progenitoras adultas en mamíferos se pueden subdividir en progenitores radiales y no radiales. Los progenitores radiales son células que tienen la capacidad de autorrenovación, muestran un mantenimiento prolongado del estado inmaduro y pueden generar diferentes tipos de neuronas. Los progenitores radiales a veces se dividen y generan progenitores no radiales. Estos progenitores expresan GFAP, BLBP y Sox2, entre otros. Por otro lado, los progenitores no radiales son células progenitoras intermedias. Las células progenitoras intermedias están dividiendo activamente, carecen de prolongaciones radiales y expresan marcadores de proliferación y linaje neuronal que dependen de su futuro fenotipo (los progenitores GABAérgicos expresan el marcador *Dlx2* y los progenitores glutamatérgicos expresan el factor de transcripción *Tbr2*). Los progenitores intermedios sufren mitosis generando más progenitores

intermedios o dos neuroblastos migratorios. Estos neuroblastos salen del nicho neurogénico y migran a sus destinos finales en el cerebro.

La neurogénesis adulta también se ha estudiado en el telencéfalo de otras especies de vertebrados no mamíferos como aves, reptiles, anfibios y peces teleósteos. Se han encontrado altas similitudes entre los mamíferos y estos grupos con respecto a los principales tipos de células en los nichos neurogénicos y los marcadores moleculares que expresan. Sin embargo, la organización de diferentes tipos de células dentro del nicho es diferente entre los vertebrados. Los estudios comparativos han evidenciado que la capacidad neurogénica se vuelve más restringida a las partes anteriores del cerebro durante el curso de la evolución. Además, los peces son el grupo de vertebrados con mayor potencial neurogénico. Sin embargo, la mayoría de los estudios en peces se han realizado en teleósteos modernos como el pez cebra, y casi ninguno se realizó en peces cartilaginosos. El uso del pez cartilaginoso *Scyliorhinus canicula* como modelo amplía el marco comparativo a una especie que muestra una actividad altamente neurogénica y una glía radial persistente en el telencéfalo adulto.

En este trabajo, estudiamos el patrón de proliferación en el telencéfalo juvenil y adulto de *S. canicula* utilizando anticuerpos contra el marcador de proliferación PCNA. Hemos caracterizado las zonas inmunorreactivas de PCNA mediante el uso de marcadores de células madre (*ScSox2*), marcadores gliales (GFAP, BLBP y GS), marcadores de células progenitoras intermedias (*ScDlx2 y ScTbr2*) y marcadores para neuroblastos migratorios (DCX).

Este trabajo representa el primer estudio en peces cartilaginosos que ha investigado la neurogénesis adulta mediante la caracterización del nicho neurogénico telencefálico. En base al patrón de expresión de estos marcadores, demostramos la existencia de diferentes subtipos celulares dentro de las zonas inmunorreactivas de PCNA, incluidas células madre no gliales, progenitores

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

gliales, células similares a progenitores intermedios y neuroblastos migratorios, que se distribuyeron ampliamente en la zona ventricular del palio, lo que sugiere que los principales tipos de células que constituyen el nicho neurogénico en los mamíferos ya están presentes en peces cartilaginosos. Hemos demostrado que el ventrículo telencefálico de la pintarroja exhibe altos niveles de proliferación, como en los peces teleósteos, pero a diferencia de las lampreas, lo que indica un origen evolutivo de la neurogénesis adulta en la transición de agnatos a gnatostomos. También hemos comparado estos resultados con los realizados en otros grupos de vertebrados y, a pesar de los diferentes enfoques experimentales, los principales tipos celulares encontrados en los nichos neurogénicos en la pintarroja son los mismos que los encontrados en esos grupos. Sin embargo, no pudimos encontrar progenitores intermedios del linaje glutamatérgico basado en la expresión de *ScTbr2*. No encontramos expresión de *ScTbr2* ni en la zona ventricular ni en las porciones subventriculares de los ventrículos telencefálicos, sino más bien en la zona intermedia, siendo especialmente abundante en el palio ventral. De hecho, la expresión de Tbr2 se ha utilizado previamente para identificar el subdominio palial ventral en anfibios. La ausencia de expresión de *ScTbr2* en la zona ventricular o en las porciones subventriculares sugiere que los progenitores intermedios (que se encuentran en/cerca del ventrículo) no expresarían *ScTbr2* y que la expresión de este factor de transcripción está regulada en los neuroblastos migratorios, lo que evidencia la necesidad de marcadores específicos para progenitores intermedios glutamatérgicos. Se necesitan más estudios que utilicen seguimiento del linaje celular para aclarar este punto.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CAPÍTULO 4

En mamíferos, el desarrollo del bulbo olfatorio (OB) está relacionado con la expresión de factores de transcripción implicados en la especificación/diferenciación de células glutamatérgicas. Además, las interacciones neurona-glía son necesarias para el correcto desarrollo del OB. Durante la morfogénesis del OB de ratón, las neuronas de proyección (glutamatérgicas, células mitrales) son las primeras en nacer y lo hacen a partir de células progenitoras paliales Pax6 positivas. El programa de transcripción involucrado en la especificación y diferenciación de células mitrales del OB es bien conocido en mamíferos, y curiosamente es el mismo que opera en la especificación/diferenciación de neuronas glutamatérgicas paliales. En el Capítulo 1, mostramos que los marcadores glutamatérgicos paliativos están presentes en el pallium en desarrollo de la pintarroja. Sin embargo, falta información sobre los mecanismos moleculares que operan en el desarrollo de la OB en la pintarroja. En los mamíferos, la expresión de estos factores de transcripción ocurre antes de la formación/maduración de los glomérulos en los roedores y también antes de que se logre la organización madura del OB. Las interacciones glía-neurona desempeñan un papel importante en diversos aspectos del desarrollo del OB, incluida la formación de los glomérulos. Sin embargo, como sucede con los marcadores neurogénicos, la información sobre el desarrollo de las células gliales en el OB de los tiburones es casi inexistente.

Con el propósito de arrojar luz sobre el desarrollo del sistema olfativo central en un contexto evo-devo, los objetivos del presente trabajo son: (1) caracterizar las células inmunorreactivas Pax6 presentes en la zona ventricular de los

embriones de palio dorsal/lateral de la pintarroja; para lograr este objetivo, hemos combinado marcadores para Pax6, células madre (Sox2), células gliales radiales (GFAP, BLBP) y células proliferantes (PCNA); (2) estudiar el patrón de expresión de factores de transcripción relacionados con la diferenciación de células glutamatérgicas (tales como Tbr2, NeuroD y Tbr1) en embriones tardíos de pintarroja usando inmunocitoquímica y técnicas de hibridación *in situ*; y (3) estudiar el patrón de expresión de varios marcadores de glía radial durante el desarrollo embrionario y en individuos postnatales usando anticuerpos contra GFAP, BLBP y GS. También hemos evaluado la capacidad proliferativa de las células gliales radiales mediante el uso de anticuerpos contra PCNA. Hemos encontrado que, previamente a la aparición del primordio olfatorio (OP) en la pintarroja, la zona ventricular que daría lugar al OB presentaba células que expresaban Pax6 y mostraban características moleculares de la glía radial. Más adelante en el desarrollo, cuando aparece el OP, se observó una corriente de células Pax6 positivas entre la zona ventricular y el OP. En el mismo período de desarrollo, los factores de transcripción implicados en el desarrollo de las células mitrales (*ScTbr2*, *ScNeuroD*, Tbr1) se expresaron por primera vez en el OP. Más adelante en el desarrollo, estos factores de transcripción se expresan en una banda celular próxima a la región protoglomerular, que coincide con el patrón de expresión de *ScVglut1*, un marcador de células mitrales. Además, usando anticuerpos contra marcadores de glía radial (GFAP, BLBP y GS), observamos tipos de células gliales morfológicamente diferentes en diferentes dominios del OB en desarrollo; algunos de ellos mostraron morfología de la glía radial, pero también se observaron células similares a astrocitos. Curiosamente, la capa glomerular es la región del OB donde se observaron numerosos cuerpos celulares y procesos de naturaleza glial formando un armazón glial glomerular, sugiriendo un papel para estas células en la formación de los glomérulos. Durante el desarrollo, también se detectaron numerosas células proliferantes de

BLBP en el OB, pero fueron especialmente abundantes en la capa del nervio olfativo. Estas células pueden corresponder a las células de la glía olfatoria envolvente, lo que sugiere que las células gliales con orígenes diferentes residen en el OB en desarrollo de la pintarroja.