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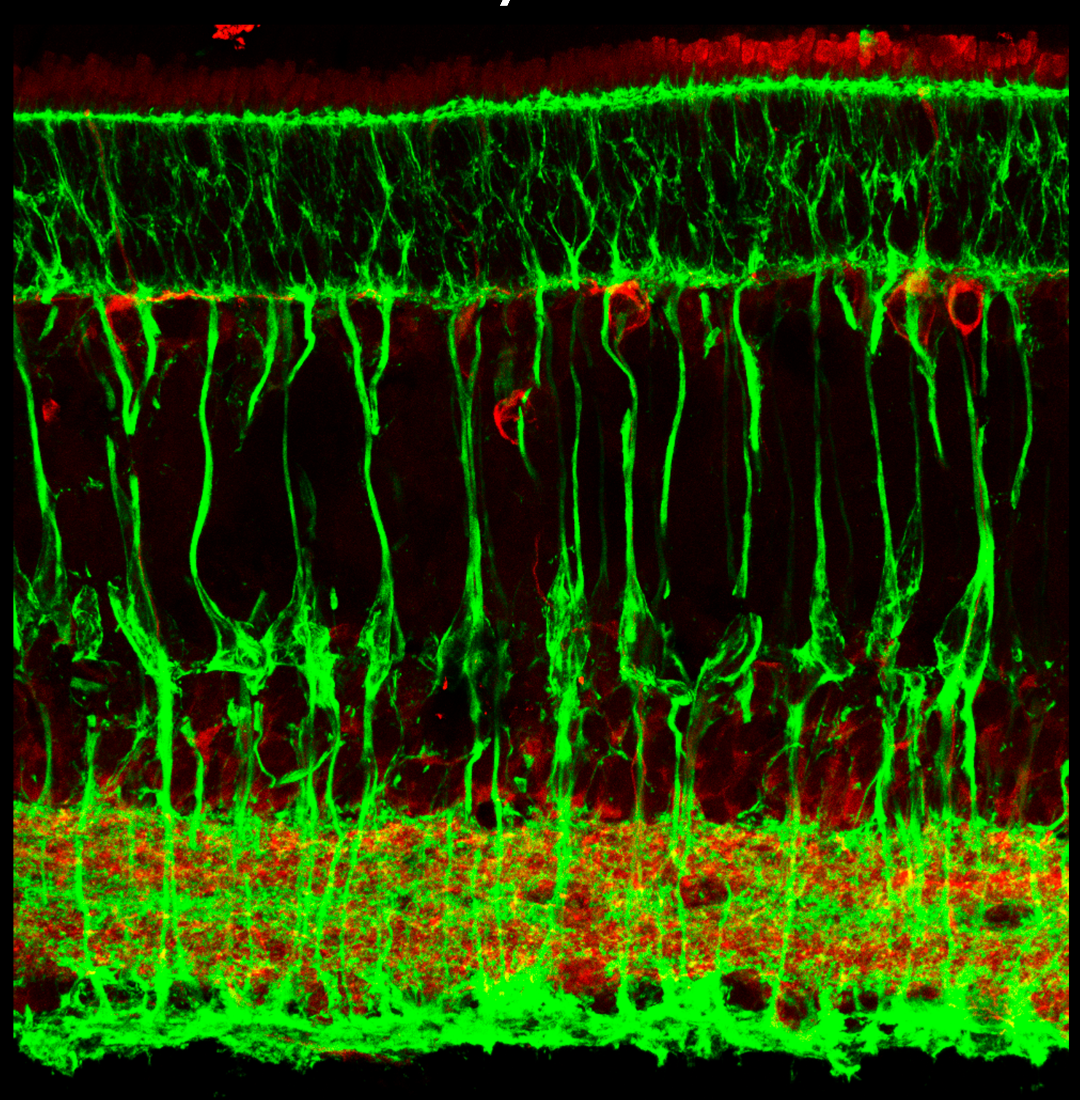
The retina of sharks as a model for studying radial glia development and cell positioning during the neurogenesis of the central nervous system



Doctoral Thesis

NURIA SÁNCHEZ FARÍAS

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The retina of sharks as a model for studying radial glia development and cell positioning during the neurogenesis of the central nervous system

MEMORIA

Que para optar al Grado de Doctor presenta

Nuria Sánchez Farías

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Autorización de la directora de la tesis doctoral

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Autoriza la presentación de la tesis indicada, considerando que reúne los requisitos exigidos en el artículo 34 de Regulación de los Estudios de Doctorado, y que como Directora de la misma no incurren las causas de abstención establecidas en la ley 30/1992.

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Y para que así conste expedimos el presente certificado en Santiago de Compostela, a 3 de diciembre de 2015.

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It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change.

Ch, Darwin

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CONTENTS

General introduction 1

1. Unsolved questions in neurogenesis 3
    1.1. Neurogenesis and neuronal progenitors in the developing mammalian brain 3
    1.2. What about the mammalian adult brain? 6
    1.3. Then, is CNS regeneration possible? What we learned from anamniotes 9

2. The retina: a single place to tackle long-lasting *constitutive* neurogenesis and *regenerative* neurogenesis 12
    2.1. Some general aspects of the anatomy of the eye and development of the retina 12
    2.2. Cell positioning during neurogenesis 15
    2.3. Adult constitutive neurogenesis 18
    2.4. Regenerative neurogenesis 21

3. An evolutionary approach for understanding neurogenesis 25

4. The retina of *S. canicula* 30
    4.1. What is known so far about the histogenesis of the retina in *S. canicula* 30
    4.2. The retina of *S. canicula* as a model 36

References 37
Abbreviations 50

Rationale and aims 53

Abbreviations 59

Material and methods 61

1. Animals 63

2. Staging embryos 64

3. Tissue processing 64

4. Immunohistochemistry 66

5. Antibodies used: controls and specifity 70

6. Image acquisition 74

References 75
Abbreviations 78

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Chapter 1: Spatiotemporal organization of doublecortin immunoreactivity in the retina of *Scyliorhinus canicula* **79**

1. Introduction 81

2. Material and methods 85

3. Results 86
  3.1. Distribution of DCX in the developing retina of *S. canicula* 87
  3.2. DCX immunoreactivity in the mature retina 88

4. Discussion 90
  4.1. Neuroepithelial, non-layered retina 90
  4.2. Progressive layering of the retina. Synaptogenesis 92
  4.3. Mature organization 93
  4.4. DCX in the CMZ 94

5. Conclusions 94

References 96
Abbreviations 104
Tables and figures 105

Chapter 2: Doublecortin and neurogenesis in the developing and mature retina of sharks: an immunohistochemical comparative study with proliferation and differentiation markers **115**

1. Introduction 117

2. Material and methods 120

3. Results 121
  3.1. *First developmental period* (stages 26 to 29). Neuroepithelial non-layered retina 121
  3.2. *Second developmental period*. Progressive layering of the retina 123
  3.3. *Third developmental period*. Layering extended central to peripheral retina 124
  3.4. Retinal pigment epithelium 125

4. Discussion 126
  4.1. DCX was first detected in the endfeet of proliferating cells of the neuroblastic retina 126
  4.2. DCX immunoreactivity during cell cycle withdrawal 127
  4.3. DCX immunoreactivity in mature cells 129
  4.4. DCX in the RPE 132

5. Conclusions 133

References 134
Abbreviations 140

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Tables and figures 141

Chapter 3: Identification of radial glial progenitors in the retina of *Scyliorhinus canicula* **157**

1. Introduction 159

2. Material and methods 163

3. Results 163
3.1. Stages 26-29 163
3.2. Stages 30-32 165
3.3. Stages 33-34 167
3.4. Juveniles and adults 169

4. Discussion 170
4.1. GFAP as a marker for astrocytes 171
4.2. GFAP in neuroepithelial (pre-neurogenic) retina 173
4.3. GFAP immunoreactivity in early RGCs 174
4.4. GFAP during the layering process. Transition from RG to Müller glia 175
4.5. GFAP in the CMZ and CE 177
4.6. GFAP in Müller cells increases with age 179
4.7. GFAP in other structures related with the eye 180

5. Conclusions 180

References 182
Abbreviations 192
Tables and figures 193

General discussion **207**

References 217
Abbreviations 224

Resumen **225**

Conclusions **241**

Appendix: Retinal regeneration in sharks **249**

1. Retinal regeneration and diseases 251

2. Potential sources of neurogenesis in the adult retina of different vertebrates 254
2.1. Regeneration from the CMZ 255
2.2. Regeneration from the CE 256
2.3. Regeneration from the RPE 257

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.4. Regeneration from the MG 258

3. Fishes vs. Mammals 261
3.1. *Scyliorhinus canicula* as a possible model for studying retinal regeneration 262

4. Accessing the retina through the pupil in *S. canicula* 263
4.1. Pupil dilatation assay 264
4.2. Preliminary data and discussion 266

5. An experimental approach 268
5.1. Experimental light treatment design 269
5.2. Preliminary data and discussion 271

6. Training stay in the MOKALAB: Laboratory of the Professor Mike O. Karl. Center for Regenerative Therapies TU Dresden (CRTD) 276
6.1. Lab Project: Identify chemical strategies to stimulate MG neurogenic competence and prevent reactive gliosis in the mammalian retina 276
6.2. Brief summary of the rotation 277
Aim 1: Find out which mitogens differentially stimulates MG cell proliferation and de-differentiation in mouse retina *ex-vivo* 278
Aim 2: Stimulate the generation of MG-derived progeny 279

References 281
Abbreviations 293

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. Unsolved questions in neurogenesis

1.1. Neurogenesis and neural progenitors in the developing mammalian brain

Neurogenesis is defined as the process by which primary progenitor cells give rise to new neurons and glia. Neural stem cells (NSCs) are the most primitive cells in the central nervous system (CNS), defined by their ability for self-renewal (long-term proliferation in an undifferentiated stage) and by their potential for differentiation to form specialized cells (De Filippis and Binda, 2012; Götz et al., 2015).

During development, NSCs gradually change in potential under the control of intrinsic and extrinsic factors and generate different types of neurons and glial cells at different times. In the mammalian brain, NSCs give rise to other NSCs and/or to transient-amplifying cells termed

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progenitors, which show decreased potential to proliferate and increased fate restriction to generate specific types of neurons and glial cells (De Filippis and Binda, 2012; **see also Fig. 1**). NSCs are characterized by the expression of markers such as Sox2, glial fibrillary acidic protein (GFAP) and Nestin, among others, but so far no specific combination of markers has been identified to definitely distinguish a NSC from a neural progenitor cell (PC; De Filippis and Binda, 2012). Two types of PCs have been described in the developing CNS of mammals. The first PCs are neuroepithelial cells (NECs), that have only limited self-renewal ability *in vivo* (though they possess limitless self-renewal potential *in vitro*) and divide symmetrically to generate more NECs (Götz et al., 2015). As the epithelium thickens, NECs suffer a transition to radial glial cells (RGCs), the second type of PCs, which divide asymmetrically a limited number of times to generate neurons and glial cells, including oligodendrocytes and astrocytes (reviewed in Götz et al., 2015; Kriegstein and Álvarez-Buylla, 2009; Paridaen and Huttner, 2014).

Figure 1. Schematic overview of neurogenesis in the vertebrate CNS. This scheme summarizes the main events in neurogenesis and gliogenesis in the cerebral cortex of the mammalian brain, but it is applicable to other CNS regions. NSCs are the primary progenitor cells at different developmental stages because they can initiate different cell lineages. Neurons and glia ultimately derive from a pseudostratified epithelium formed by NECs. At first, NECs divide to generate more NECs. Some of them elongate and change their identity into radial glial cells (RGCs in the Figure). RGCs can generate neurons or intermediate progenitor cells (IPCs), which can give rise to neurons or glial cells. RGCs show apical-basal polarity. The apical domain of RGCs (down) is in contact with the ventricle while the basal domain (up) is in contact with the basal lamina and blood vessels. Taken from Paridaen and Huttner (2014).

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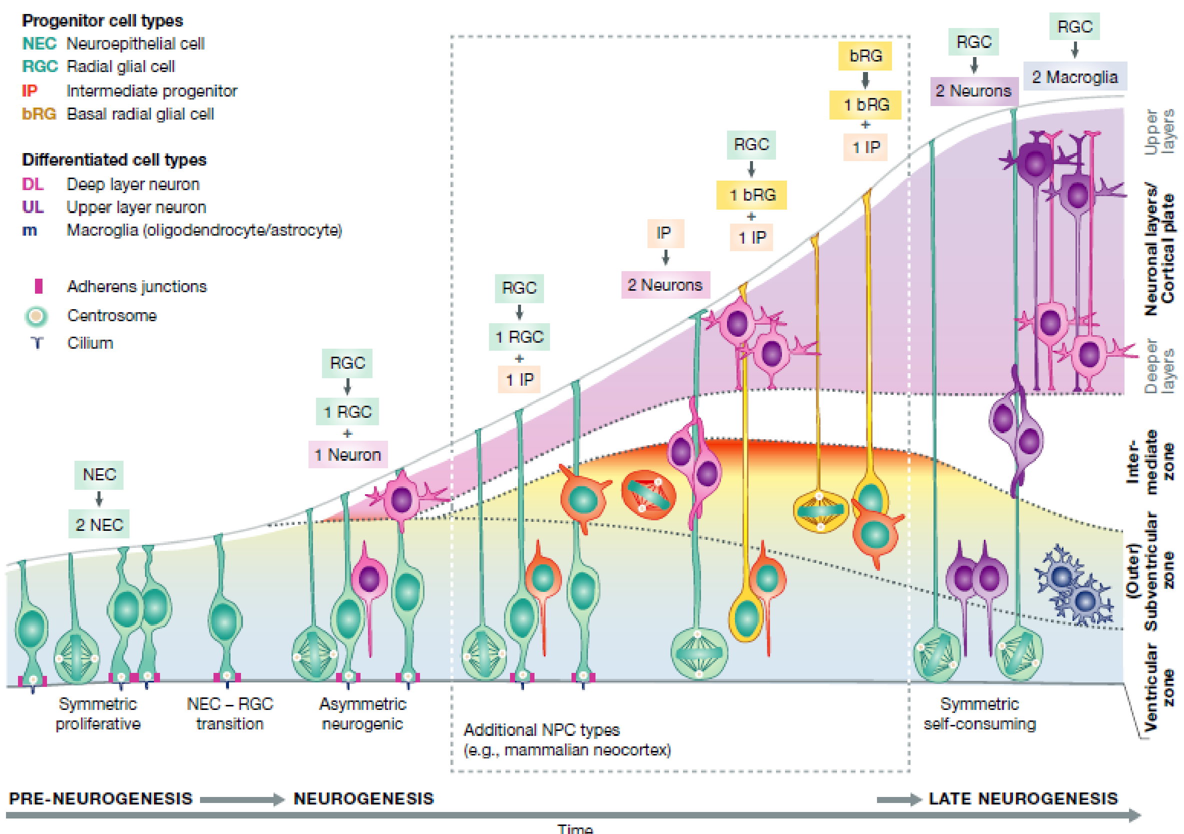

Figure 1.

RGCs are characterized by their radial morphology spanning from the basal to the apical surface of the neural tube, their apico-basal polarity and the expression of glial cell markers usually found in astroglial cells (nicely reviewed in Than-Trong and Bally-Cuif, 2015; see also Alvarez-Buylla and Lim, 2004; Denham et al., 2005; Doetsch, 2003; Ever and Gaiano, 2005; Galli et al., 2003; Ming and Song, 2005). However, any of these traits alone define a RGC, since radial morphology and polarity also characterize NECs and many RGC markers are shared with NECs, astrocytes and/or ependymal cells (Than-Trong and Bally-Cuif, 2015).

In mammals, RGCs are lost in most brain regions at the end of neurogenesis (**Fig. 1**), either by symmetric self-consuming neurogenic divisions or by producing or becoming glial cells, such as astrocytes or ependymal cells (Götz et al., 2015; Kriegstein and Alvarez-Buylla, 2009; Paridaen and Huttner, 2014).

1.2. What about the mammalian adult brain?

Despite this neurogenic decline, a subset of PCs are found in restricted regions of the brain (*germinal niches*) that maintain the potential to generate neurons in the adulthood (**Fig. 2**): the subventricular zone (SVZ) of the lateral ventricles (Doetsch, 2003; Doetsch et al., 1999; Lois and Alvarez-Buylla, 1993; Ming and Song, 2011; Morrens et al., 2012), the subgranular zone of the dentate gyrus of the hippocampus (Cameron and McKay, 2001; Christie and Cameron, 2006; Ehninger and Kempermann, 2008; Eriksson et al., 1998; von Bohlen Und Halbach, 2007), the olfactory bulb (Lepousez et al., 2013), the subcallosal zone (Seri et al., 2006) and the postnatal cerebellum (Lee et al., 2005).

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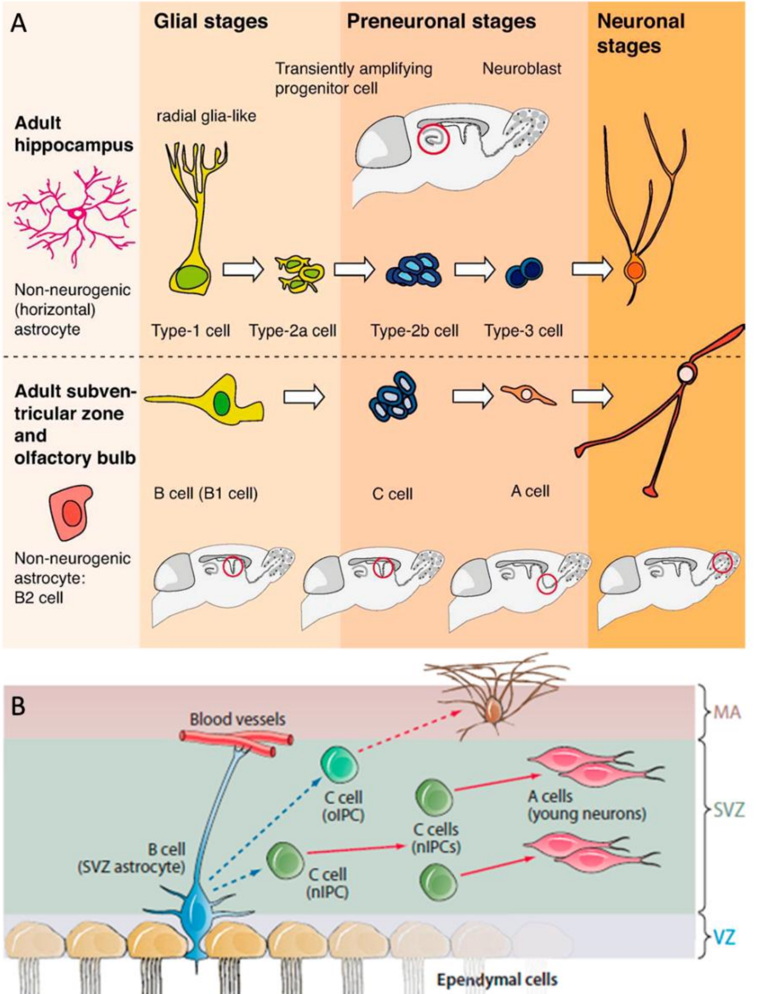

Figure 2. Germinal niches in the adult mammalian CNS. Adult NSCs persist in two main regions in the postnatal brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the hippocampus. These adult NSCs can produce not only glial cells but also neurons. Type-1 cells represent the NSCs in the subgranular zone of the hippocampus. Type-1 cells generate cells with high proliferative capacity (Type-2a cells) that generates Type-2b cells/neuronal IPCs. These Type-2b cells give rise to immature neuroblasts (Type-3 cells) (**see Fig. 2A**). NSCs of the lateral ventricles correspond to B cells/SVZ astrocytes which produce C cells/neuronal IPCs. C cells generate immature neuroblasts (A cells) which migrate through the rostral migratory stream to the olfactory bulb. B cells also produce oligodendrocytes (o) through oIPCs (**see Fig. 2A, B**). Note that both, Type-1 cells and B1 cells display a characteristic radial morphology, showing an epithelial apico-basal organization. Modified from Morrens et al. (2012) and Kriegstein and Alvarez-Buylla (2009); respectively. MA, mantle; VZ, ventrivular zone.

Postnatal progenitors, usually referred as adult NSCs, can produce glial cells and also neurons (Alvarez-Buylla and Lim, 2004; Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2005). These adult NSCs are known as B1 cells in the SVZ or as Type-1 cells in the subgranular zone of the hippocampus (**see Fig. 2**), and tanycytes bordering the hypothalamic ventricle (for a review see Than-Trong and Bally-cuif, 2015). These adult NSCs displays features and markers of both RGCs and mature astrocytes, which prompted the hypothesis that postnatal NSCs 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 RGCs in the embryo, namely pre-B1 cells, which set aside from those generating neurons (**Fig. 3A**). These cells remain quiescent until they become reactivated at different ages in the postnatal brain to produce distinct cohorts of neurons. Interestingly, postnatal NSCs inherit the positional information of their embryonic progenitors, which determines the types of neurons and glia they can produce (Kriegstein and Alvarez-Buylla, 2009).

Quiescent neurogenesis can be activated following injury in other non-neurogenic regions such as the cerebral cortex (Gould et al., 1999; Magavi et al., 2000; Taverna et al., 2014), the spinal cord (Matsumura et al., 2010; Sabelström et al., 2013), and the eye (Ahmad et al., 2004; Wohl et al., 2012). While the adult CNS of mammals is able to functionally integrate transplanted neurons (for a revision see Becker and Becker, 2015), it appears that progenitors fail to generate appropriate cells for endogenous repair. Indeed, endogenous progenitors in the mammalian adult CNS do not replace lost neurons in neurodegenerative diseases and, after injury, they generate a glial scar that contributes to inhibitory environments for axon regrowth instead of replacing lost neurons. Therefore, RGCs are mostly transient in mammals (they largely differentiate at postnatal stages) and postnatal NSCs appear incapable

of endogenous reparation, which is behind the low regenerative capacity of the mammalian brain.

1.3. Then, is CNS regeneration possible? What we learned from anamniotes

PCs with radial morphology, apico-basal polarity and the expression of glial markers similar to that found in the radial glia (RG) of mammals, are broadly found in the developing CNS of anamniote vertebrates (amphibians and fishes). Contrasting with the situation in mammals, RG are widely maintained in the adult CNS of anamniote vertebrates, particularly in fishes and salamanders (Becker and Becker, 2015; Than-Thong and Bally-Cuif, 2015), which additionally are able to CNS regeneration. Recent studies have focused on how these vertebrates activate progenitors, regenerate particular cell types, and functionally integrate them into the mature CNS. Some developmental pathways are recapitulated during regeneration, whereas others are distinctive of the regeneration process (Becker and Becker, 2015).

By far, the greatest number of proliferation zones has been reported to date in teleost fishes (Sîrbulescu and Zupanc, 2011; Zupanc and Sîrbulescu, 2011), which possess neurogenic compartments in nearly all brain regions and also preserve their capacity for continual neurogenesis throughout adulthood (Grandel et al., 2006). The most common explanation for this is that teleosts have indeterminate growth, and therefore the majority of brain and sensory structures continue to enlarge by the addition of newborn neurons during senescence. However, in most instances adult neurogenesis is a selective process limited to a subset of neurons in some of the neurogenic niches, which argue against neurogenesis being an additive process for the single purpose of growth (Lindsey et al., 2014; Than-Trong and Bally-Cuif, 2015).

Among teleosts, the zebrafish, a fast-developing fish species, has become a popular model to approach the understanding of postnatal neurogenesis and neuronal repair. In zebrafish, the expression of RGC markers is initiated very early in development, making more difficult than in mammals to identify the transition from NECs to RGCs. Other RGC markers switch on as development progresses, and get a mature state similar to that described for RGCs in the developing CNS of mammals. However, contrary to that found in mammals, both NECs (which was unexpected) and RGCs are found in the adult brain (nicely reviewed in Than-Trong and Bally-Cuif, 2015; **see Fig. 3B**). In recent years it has been found that cells with NEC features are retained in some areas of the adult zebrafish CNS and can act as adult neural progenitors in constitutive and/or regenerative neurogenesis. The cellular hierarchies involving NECs during adult neurogenesis, i.e, if they are at the origin of adult RG progenitors, remains unsolved (Than-Trong and Bally-Cuif, 2015). On their part, RGCs in the adult brain of zebrafish continue to be morphologically similar to RGCs in the developing mammalian brain and fulfill similar roles as primary progenitors in the generation of neurons and glia, and also as guide for the migration of young neurons along their radial processes. Moreover, these RGCs were also identified as prominent progenitors under regenerative conditions.

Figure 3. Comparison between different types of progenitors found during embryonic and adult neurogenesis in the SVZ of the lateral ventricles in mammals **(A)** and the retina of zebrafish **(B).** Some markers frequently used to identify these cell types are indicated. Markers that are no longer expressed are in grey. In the SVZ of mammals **(A)** pre-B1 cells in the embryo set aside a subpopulation of RGCs and remain quiescent until they become reactivated in the postnatal brain to produce distinct cohorts of neurons and glial cells. In zebrafish **(B)**, both NECs and RGCs can be identified during retinogenesis. Cells with radial morphology persist in the peripheral retina of the adult to give rise to different types of neurons and a type of RGC termed Müller cell. In the central retina, Müller glial cells divide infrequently, with an asymmetric self-renewing division, to give rise to proliferating progenitors that generate rod photoreceptors. After retinal damage, Müller glia can divide to give rise to progenitors of different types of neurons (not shown). Data after: Lenkowski and Raymond, 2014; Than-Trong and Bally-Cuif, 2015.

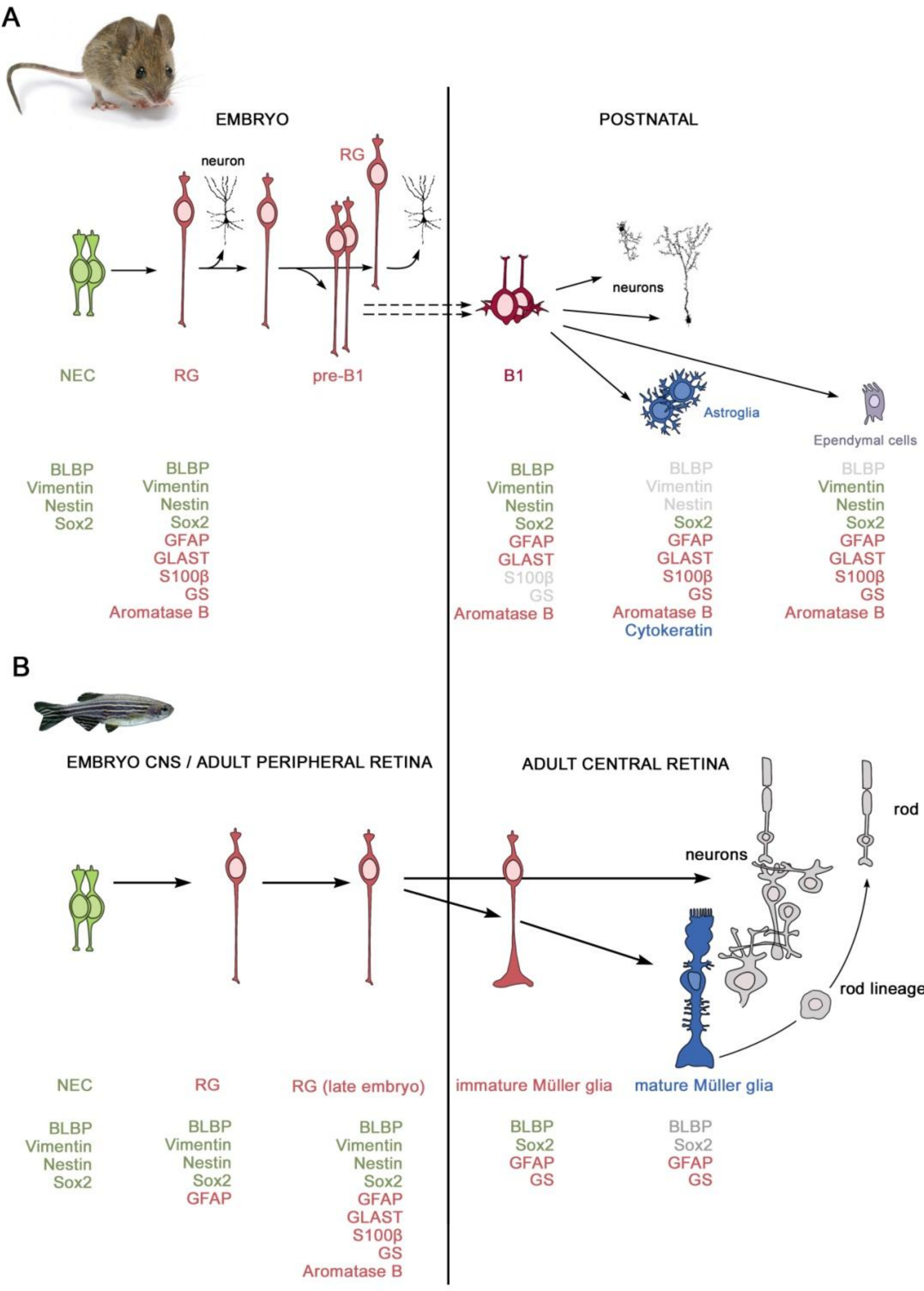

Figure 3.

2. The retina: a single place to tackle long-lasting *constitutive* neurogenesis and *regenerative* neurogenesis

Among all neurogenic systems in the CNS of zebrafish, one of the best studied to date is the retina because of the presence of high rates of long-lasting adult neurogenesis and because of the occurrence of reparation processes involving RGCs (Bernardos et al., 2007; Goldman, 2014; Lenkowski and Raymond, 2014; Otteson and Hitchcock, 2003).

2.1. Some general aspects of the anatomy of the eye and development of the retina

The first events of the eye development take place within the anterior neural plate, which originates from the dorsal ectoderm. During neurulation, induction signals come from distinct domains and act on the neural plate to form the neural tube. Proliferation and differentiation of epithelial cells located at the rostral end of the neural tube lead to the formation of three vesicles: forebrain, midbrain and hindbrain (Gilbert, 2013). During neurulation, optic pits become defined in the lateral wall of the forebrain and each one forms an optic vesicle, connected to the forebrain by the optic stalk (**Fig. 4A**). Later, each optic vesicle invaginates to form a double-layered optic cup (see for example Lamb et al., 2007; Saha et al., 1992; Wilson and Houart, 2004). These layers will form the retina, most of the ciliary body (except the muscles that control the shape and position of the lens), and a part of the iris. The remaining part of the ciliary body and the iris will develop from the mesenchyme, derived principally from neural crest cells. The lenses, in turn, originate from the surface ectoderm (**Fig. 4B**; see for example Adler and Canto-Soler, 2007; Sivak and Sivak, 2000).

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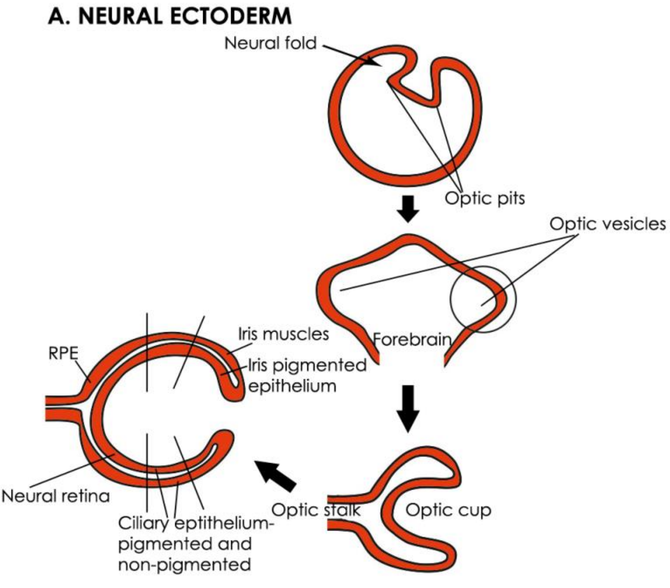

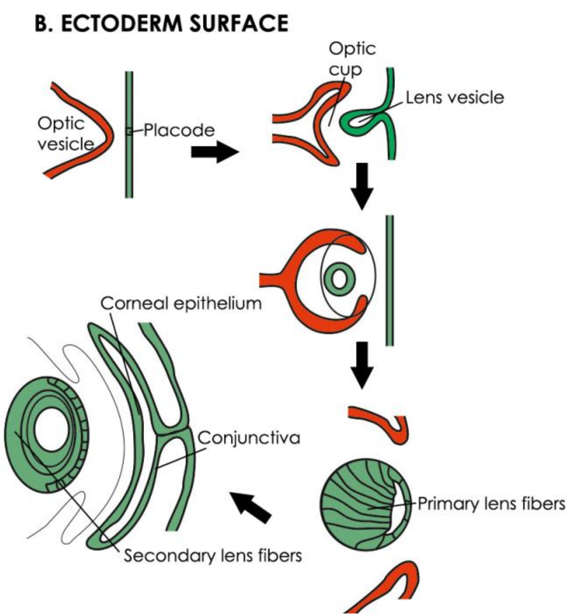

Figure 4. Schematic drawings of the sequence of the eye development. The eye is an extension of the CNS. (**A**) The neural ectoderm is the most significant player in the formation of a functional eye from an undifferentiated neuroepithelium. The sequence represents the development of the optic cup and the structures derived from it. (**B**) This sequence shows the formation of the lens vesicle, the epithelium of the cornea and the conjunctiva, all derived from the ectoderm surface. Adapted from Sivak and Sivak (2000).

Cells of the inner layer of the optic cup proliferate and develop into the neural retina. Epithelial cells present in the outer layer of the optic cup synthesize melanin and constitute the retinal pigment epithelium (RPE) which plays indispensable roles for the normal function and survival of the neural retina (Chiba, 2014; Strauss, 2005).

Cell specification of embryonic cells that will produce the retina begins during early embryo development. In the formation of the neural retina several genes and interactions between the eye field and the mesoderm located bellow, give rise to the retinal stem cells (RSCs). External as well as internal cues that initiate neurogenesis have been deeply reviewed in Schmidt et al. (2013). Initially, the neural retina is composed of undifferentiated multipotent progenitors from which all retinal cell types derive (Bassett and Wallace, 2012; Turner et al., 1990). *Retinal progenitor cells* are multipotent and retain the ability to produce different cell types until the last cell division. During this process, cells gradually exit the cell cycle and differentiate. Six types of retinal neurons and one specialized type of RGC, the Müller glia (MG), arise from a pool of multipotent retinal progenitor cells in a particular order that is relatively conserved across vertebrates, though some differences exist in the order wherein these neurons are generated (Bassett and Wallace, 2012; Jadhav et al., 2009; Kitambi and Malicki, 2008; Livesey and Cepko, 2001; Peterson et al., 2001; Young, 1985). In most vertebrate species, retinal cells close to the vitreal (basal) region differentiate first, whereas cells close to the scleral (apical, ventricular) region differentiate last. In addition, retinogenesis proceeds in a central-peripheral gradient. Retinal lamination occurs when neuronal precursors exit the cell cycle and become positioned within appropriate layers. Then, they differentiate, acquire the morphology and specific molecular markers characteristic of the mature cell (Ferreiro-Galve et al., 2010b; Harahush et al., 2009; Malicki, 2004; Marquardt and Gruss, 2002; Olson et al., 1999; Rapaport et al., 2004) and establish synapses within plexiform layers. The inner plexiform layer (IPL)

separates the ganglion cell layer (GCL; which contains ganglion cells) from the inner nuclear layer (INL, which contains amacrine, bipolar and horizontal cells). The outer plexiform layer (OPL) separates the INL from the outer nuclear layer (ONL, which contains the nuclei of photoreceptor cells). The nuclei of Müller glial cells (MGCs) are located in the INL while their processes span the retina from the apical to the basal surface. Axons from cells located in the GCL will form the optic nerve (ON), which connects the eye to the brain (**see below in Box 3**).

2.2. Cell positioning during neurogenesis

Neurogenesis not only relates to proliferation, but also to differentiation, migration, targeting and synaptic integration processes that lead to the production of functional mature cells. Defects in the migration of neurons to their final positions produce several developmental malformations (reviewed in Ohshima, 2015; Ross and Walsh, 2001). Several studies have demonstrated that neuronal migration is a critical process in neural development of different regions of the CNS, including the retina (Baye and Link, 2008; Hatten, 1999). It has been reported that postmitotic neuronal precursors receive signals to facilitate their migration to acquire their right final positions (Malicki, 2004).

Multiple modes of migration generate the definitive laminar structure of the neural retina. Interkinetic nuclear migration (IKNM) and nuclear translocation are two mechanisms of neuronal migration during retinogenesis, which are closely related with cell cycle phases (Baye and Link, 2007, 2008; Ohnuma and Harris, 2003). In the neuroepithelial retina, the nuclei of PCs move up and down along the apical-basal axis coordinated with the cell cycle. This process, known as IKNM (**Fig. 5**), is a common characteristic of developing vertebrate neuroepithelia and was first described in 1935, based on histological observations of Sauer in the neural tube (Sauer, 1935). During this process, NECs elongated in shape

span the entire thickness of the neuroepithelial retina from the vitreal (basal) to the ventricular (apical) surface, with nuclei located at different levels depending on the phase of the cell cycle (**Fig. 5A**). Nuclei in M phase are close to the apical surface, near the RPE, whereas nuclei in S phase are located in basal positions. The different positions of the nuclei of PCs give the pseudostratified appearance typical of the neuroepithelia of vertebrates (Baye and Link, 2007, 2008; Del Bene, 2011; Del Bene et al., 2008; Sauer, 1935, 1936; Sauer and Walker, 1959; Spear and Erickson, 2012a, 2012b). IKNM was also described for RG (**Fig. 5B**). IKNM in RGCs does not extend into the portion of the cell that traverses the neuronal layers, but is confined to the portion of the cell residing in the ventricular zone (VZ) and, when present, the SVZ (reviewed in Taverna and Huttner, 2010). Despite recent studies aimed to understand the essence of IKNM, the developmental function and molecular mechanisms underlying this process remains controversial. It is well known that microtubules (MTs) and microtubule associated proteins (MAPs) are involved in basal-to-apical nuclei movement and positioning (**Fig. 5C**). In MT-based IKNM, the nuclei of neural progenitors move, as a cargo, along MT tracks mediated by minus-end-directed motor MAPs (Baye and Link, 2008). The motor MAP dynein interacts with several proteins, including dynactin and Lis1 among others, to direct the movement of the nucleus toward the minus end of the MTs (reviewed in Norden et al., 2009). In addition to the dynein system, other classes of motor proteins, notably actomyosin, are also involved in IKNM (**Fig. 5D**; for a review see Taverna and Huttner, 2010). Apical-to-basal IKNM has received less attention, but it appears to depend on either plus end-directed motor MAPs of the kinesin type (**Fig. 5C**) or directional myosin-II-dependent constriction (**Fig. 5D**).

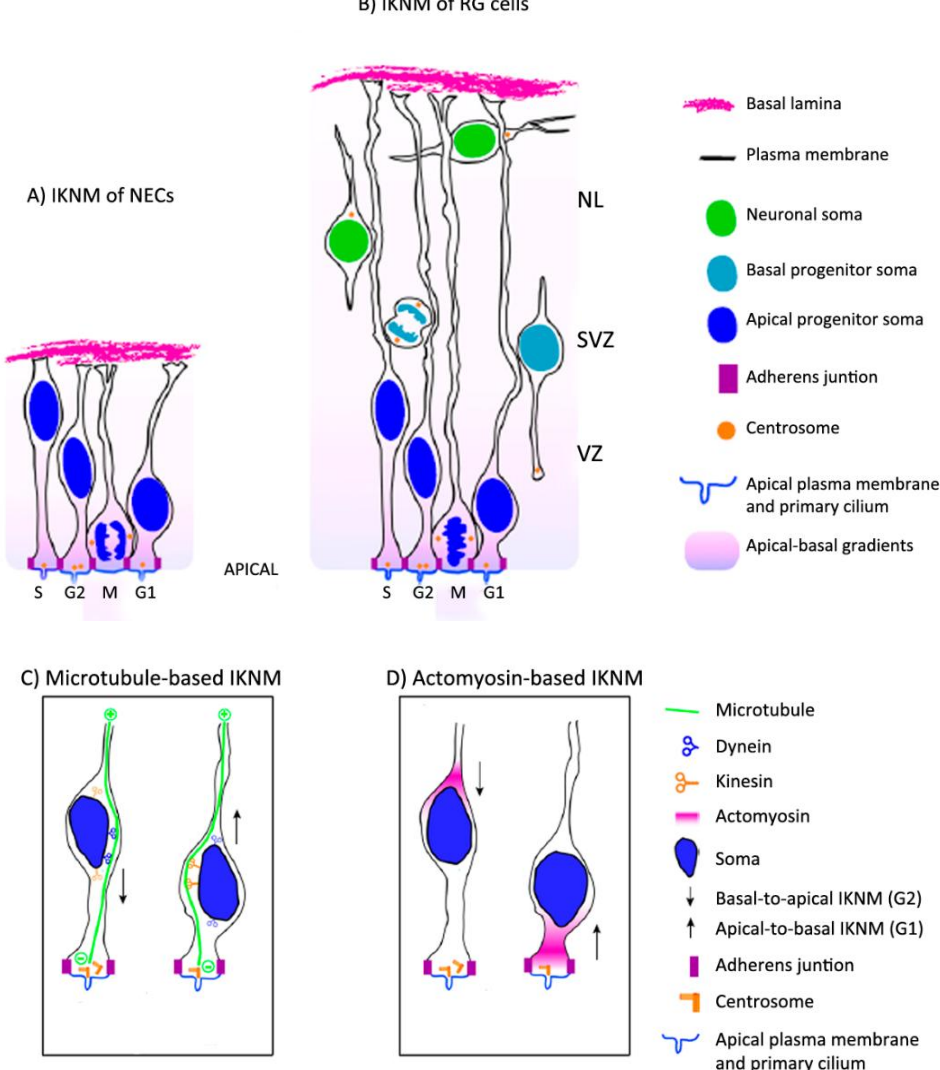

Figure 5. IKNM processes of apical neural progenitors. Modified from Taverna and Huttner (2010).

Interestingly, recent studies have reported the importance of IKNM in the determination of cell fate of neuron precursors (Del Bene et al., 2008; Latasa et al., 2009; Murciano et al., 2002). Baye and Link have proposed a correlation between neurogenic cells divisions and the dynamic of IKNMs within the retinal neuroepithelium (Baye and Link, 2007). Several retinal cells use this type of cell migration to acquire their

appropriate laminar position (Baye and Link, 2007, 2008; Braisted et al., 1994; Del Bene et al., 2008; Lenkowski and Raymond, 2014; Norden et al., 2009; Spear and Erickson, 2012a, 2012b).

Apart from IKNM, later, following cell cycle exit, some neuron precursors migrate by means of nuclear translocation, in which both apical and basal cytoplasmatic processes are maintained and begin to retract when the soma gets to the appropriate laminar position (Baye and Link, 2008; Ghashghaei et al., 2007; Nadarajah et al., 2001). Nuclear translocation has been reported in precursors of retinal ganglion cells (GCs) and bipolar cells (BCs) (reviewed in Baye and Link, 2008; Morgan et al., 2006; Snow and Robson, 1994). As in the IKNM, several kinases such as Ckd5, which binds to the microtubule-binding protein Doublecortin, have been shown to regulate nuclear translocation (Tanaka et al., 2004). After migration, cells will differentiate and assembly in a laminar network that comprises a determined number of cell types.

2.3. Adult constitutive neurogenesis

Constitutive neurogenesis in the adult retina of zebrafish originates from two different supplies, the ciliary marginal zone (CMZ) and the MG (Raymond et al., 2006).

The CMZ has been defined as a circumference of PCs at the edge of the retina located between the neural retina and the ciliary epithelium (CE), which persist until the adulthood to generate all retinal cell subtypes, including the MG (Raymond et al., 2006). The CMZ has been also morphologically defined by the lack of layering that characterizes the central (differentiated, mature) retina. Interestingly, the CMZ is a reflection of the embryonic retinal development. The spatio-temporal pattern of expression of various stem cell and progenitor markers within the CMZ of zebrafish (Raymond et al., 2006) revealed that the CMZ is arranged in

concentric rings of gradually increased commitment, i.e., NSCs are located in the most peripheral zone, nearby the CE, while progressively more fate-restricted progenitors are successively located toward the central (differentiated) retina, a fact that had been previously found in the larval retina of *Xenopus* (Perron et al., 1998). Thereupon, the CMZ in zebrafish can be divided into three regions which reflect the temporal sequence of retinogenesis: the CMZ-peripheral close to the CE, wherein multipotent progenitors (NSCs) are located; the CMZ-middle, containing proliferating progenitors; and the CMZ-central, where postmitotic but undifferentiated cells are found (Amato et al., 2004; Raymond et al., 2006; Wheman et al., 2005).

The fact that this region recapitulates the events which took place during retinogenesis makes the CMZ an ideal system to follow neurogenic processes even in the adult. This spatially ordered arrangement of NSCs, NECs and increasingly committed cells could help to follow the progeny of individual PCs from the embryo into postnatal life, which has been technically challenging in the brain of mammals due to the long migration that separates the final progeny from PCs (e.g. interneurons of the olfactory bulb from PCs within the walls of the lateral ventricles).

However, whether the CMZ in the adult retina completely fulfill the definition of a NSC niche is today a matter of debate. Many questions are still unsolved regarding this point. The first is to clarify what cells within the CMZ actually conform NSC definition (self-renewal and multipotency at the single cell level) to support continuous neurogenesis. Indeed, a shocking trait of the NECs in the CMZ is its high proliferating rate, a feature that does not match with the long-lasting maintenance of stem cells. Various hypotheses have been proposed on this regard, including the existence of a small number of quiescent cells within the pool, of quiescence phases within each individual cell in the pool, or of a continuous renewal of the pool from a different quiescent source (see Than-Trong and Bally-Cuif, 2015). Identifying relevant markers of quiescent

NSCs *versus* rapidly-dividing progenitors will provide insight on the cellular hierarchies of the germinal niche. This leads to a second important question, that is whether NSCs are multipotent at the single cell level or rather at the population level, as has been recently defined for adult neural stem cells (aNSCs) in mammals (Götz et al., 2015). Interestingly, CMZ cells in the adult retina express BLBP, a RGC/astrocyte marker found in mammals in NECs and RGCs during development and in B1 progenitors during adulthood (Giachino et al., 2014).

On its hand, MGCs, which are morphologically similar to RGCs in the developing brain of mammals, represent the main glial population of the mature vertebrate retina. They are radial shaped, with their cell bodies situated in the INL and their processes spanning the retina from the apical (close to the RPE) to the basal surface. Müller cells express classical RG markers such as GFAP and glutamine synthetase (GS). Due to the continuous growth of the retina from the peripheral CMZ, young MG (which additionally expresses the brain lipid binding protein (BLBP) is located close to the CMZ while more mature MG (BLBP-negative) are progressively located in the central retina. MG participating in constitutive neurogenesis are largely quiescent but can be activated by various signaling molecules and environmental stimuli. During constitutive neurogenesis MG proliferate at low frequency to generate a small population of amplifying rod precursors that migrate into the ONL along non-proliferating MG, and therefore they are the primary source of new rods in the central retina (Bernardos et al., 2007). The fact that MGCs become rapidly quiescent after they are generated from the CMZ and that, after constitutive activation they give rise only to photoreceptors, has led to the hypothesis that the chief contribution of the CMZ cells to generate all types of adult-born neurons, does not imply an obligatory transition through the RG state.

2.4. Regenerative neurogenesis

Nowadays, loss of retinal neurons and associated degeneration diseases are a global health problem and the main reason of vision loss in the aging human. About 45 million people worldwide are blind, and it is estimated that over 76 million will suffer vision loss or blindness by 2020 (www.vision2020.org), most of them due to age-related macular degeneration (Schmeer et al., 2012). Unfortunately, mammals do not possess self-endogenous regeneration (see above), and therefore, retinal GCs and photoreceptors cannot be restored after they are lost in glaucoma or in age-related macular degeneration, respectively.

Retinal regeneration mechanisms have been extensively investigated in the last fifteen years (Baker and Brown, 2009; Centanin and Wittbrodt, 2014; Enzmann et al., 2009; Karl and Reh, 2010; Lin et al., 2014; Ramsden et al., 2013; Reh and Fischer, 2001; Reh and Levine, 1998; Tibbetts et al., 2012; Tucker et al., 2014). The retina of mammals possesses a low regenerative potential related with the restriction of some cell-signaling pathways (see above). Indeed, several groups have reported that regeneration mechanisms can be stimulated in the mammalian retina using growth factors, transcription factors or amino acids, though the proliferative response of the MG to injury is very limited (Karl and Reh, 2010). In this context, an important strategy would be identifying the limitations of the regeneration processes in mammals using other animal models (**Fig. 6**).

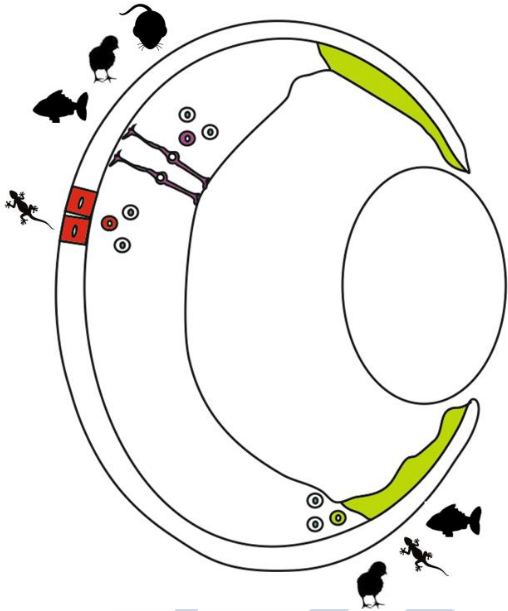

Figure 6. Schematic drawing to show the main cell sources of regeneration in postnatal vertebrate retinas. The retina can regenerate from the RPE, the MG and/or from the CMZ (an analogous region of the SVZ in the adult brain; see above). PCs (in green) present in the CMZ of amphibians and fishes give rise to most of the mature retina. However in birds, PCs of the CMZ give rise to only a small part of the retina. In amphibians, cells of the RPE (in red) can dedifferentiate and regenerate new neurons upon damage. In fishes, and to a lesser extent in birds and rodents, MG (in purple) dedifferentiate and regenerate new neurons upon retinal damage. Adapted from Karl and Reh (2010).

The chick retina is capable of limited constitutive neurogenesis through the addition of new neurons at the peripheral edge of the retina. The addition potential of this region in chick is somewhat restricted as it can generate some cell types (BCs and amacrine cells), but not others (GCs or photoreceptors), and it is not induced following retinal injury. The non-pigmented epithelium of the ciliary body in chick contains quiescent stem cells, and MG can also generate new neurons after retinal damage (**see more details in Table 1 in the Appendix of this thesis;** Fischer, 2005; Fischer and Bongini, 2010; Fischer and Reh, 2000, 2003; Tood et al., 2015). In amphibians, after retinal damage, cells of the RPE lose their

pigmentation, dedifferentiate and generate new neurons through trans-differentiation. While this capacity of the RPE for trans-differentiation has not been described in fishes, they can replace lost neurons following damage by dedifferentiation of MG to a PC (Amato et al., 2004; Hitchcock and Raymond, 2004; Moshiri et al., 2004; Otteson and Hitchcock, 2003; Raymond et al., 2006; Thummel et al., 2008; Yoshii et al., 2007).

Indeed, in teleost fishes (**Fig. 7**), most pathways involved in RG (Müller glia) activation have been detected in regenerative contexts (for a deep review in the field see Than-Trong and Bally-Cuif, 2015).

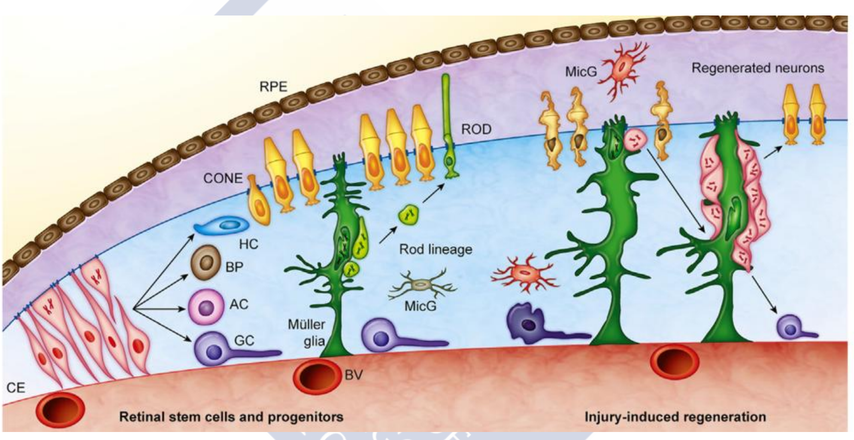

Figure 7. Development of new neurons from the two niches in the intact and regenerating adult retina of teleost fishes. The CMZ, next to CE, represents the first neurogenic niche (in red). Retinal PCs (asterisks) of the CMZ can generate all retinal cell types except rod photoreceptor cells. The second neurogenic niche cells in uninjured retinas is constituted by the MG (in green) in the central retina, which are somatic retinal stem cells supporting rod lineage that give rise to rod photoreceptors. Moreover, upon damage, the MG can dedifferentiate producing PCs. This neurogenic niche, induced by retinal lesions, gives rise to all retinal cell types that have been affected. Taken from Lenkowski and Raymond (2014). ACs, amacrine cells; BCs, bipolar cells; BV, blood vessels; CE, ciliary epithelium; GCs, ganglion cells; HCs, horizontal cells; MicG, microglia; RPE, retinal pigment epithelium.

Activation of these pathways in regenerative contexts includes some features of reactive gliosis, such as transient up-regulation of BLBP and GFAP together with stimulation of MG nuclei re-entering in the cell cycle. Moreover, activation in this context is also sufficient to induce nuclei migration from the INL to the ONL, which is an essential condition for retinal regeneration (for a review see Raymond et al., 2006). Later on, damaged-activated MGCs enter dedifferentiation and/or reprogramming processes (including GFAP down-regulation) that allow them to serve as PCs that, at the population level, are able to generate different retinal neurons. Contrary to that described in the mammalian retina, the result of this damage-induced activation is not the production of new MGCs or the generation of a glial scar, but rather the establishment of a germinal niche with the morphological and molecular characteristics of the neurogenic CMZ (Lenkowski and Raymond, 2014; Raymond et al., 2006). In fact, self-renewing and multipotent cell divisions were observed in clonal analyses of the MG under regenerative conditions (Nagashima et al., 2013). However, differences also exists which leads to the statement that regenerative neurogenesis is inherently different from constitutive neurogenesis in the anamniote CNS (Becker and Becker, 2015).

The knowledge of the mechanisms underlying the neurogenesis in the retina, especially in retinas with endogenous regeneration capacity, is essential to understand the potential and limitations of retinal regeneration in mammals, including humans. These mechanisms not only include proliferation and differentiation processes, but also migration and cell positioning.

3. An evolutionary approach for understanding neurogenesis

While studying these aspects and comparing them with the situation in mammals, one main goal is to be able to differentiate the mandatory (conserved) versus specific (diverged) components of the adult germinal niches, the heterogeneity of the NSC state and progenitor commitment and the regulation of the neurogenic process at the population scale. This comparison will be crucial to design strategies to stimulate adult neurogenesis in mammals. Importantly, although there may be a few elusive characteristics common to all NSCs in mammals and zebrafish, important differences exist across species and in the order and place where neurons are produced, where they migrate and where they are ultimately incorporated.

Recent findings have evidenced that the continuation of neurogenesis during adulthood is highly region specific and species specific (Grandel and Brand, 2013). Thus, key questions in the field are related to the regulation of region-specific continuation of neurogenesis and the developmental mechanisms that determine whether cells continue to generate neurons throughout the organism's lifetime or instead stop at some point.

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). A survey of the literature on this topic (see Lindsey and Tropepe, 2006; Schmidt et al., 2013) highlights the fact that most of the investigations examining the presence of adult neurogenesis have taken place in tetrapod vertebrates (especially in mammals) and teleost fishes. Strikingly, other vertebrate

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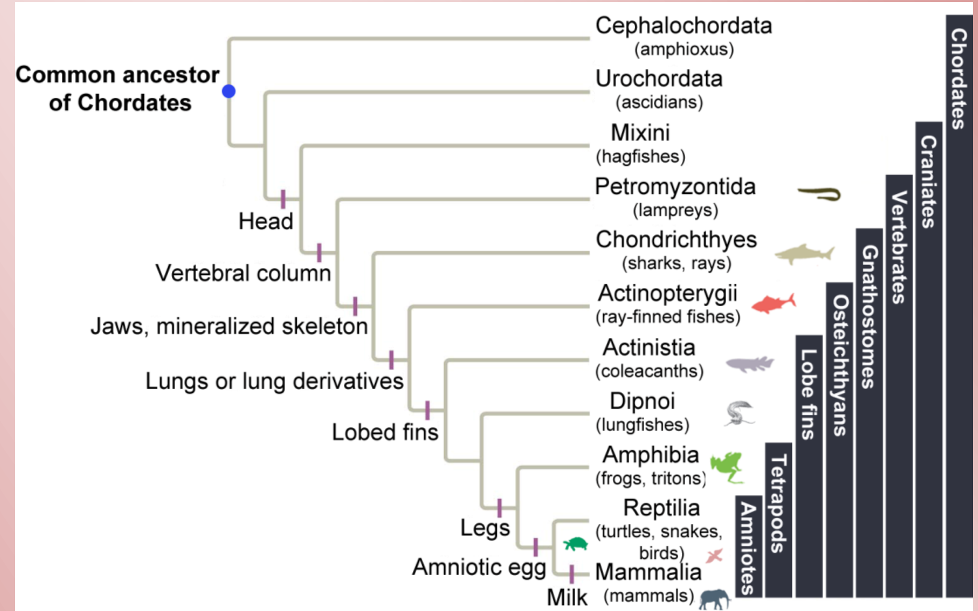
classes, such as cartilaginous fishes, in which adult neurogenesis has been found (Leonard et al. 1978) are not typically used for this kind of analyses. Cartilaginous fishes (rays and sharks) represent an ancient radiation of vertebrates (**BOX 1**) and are currently considered the sister group of bony fishes (jawed vertebrates with bony skeleton), which demonstrate that adult neurogenesis is a phylogenetically ancient trait of gnathostome vertebrates that was present before the split of cartilaginous fishes from the radiation that give rise to bony fishes and tetrapods, including mammals (Grandel and Brand, 2013).

This key position makes cartilaginous fishes an essential comparative reference that may provide insight into the origin of the major characteristics of jawed vertebrates, which will allow us to distinguish between ancestral and derived characteristics in comparative analyses. Among elasmobranch fishes, the lesser spotted dogfish, *Scyliorhinus canicula* (*S. canicula*), appears to offer the most potential as a model species for study (**BOX 2**), since its phylogenetic position among elasmobranch fishes confirms it as an informative representative of the clade (Coolen et al., 2009). Therefore, 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. Taking this approach will yield insight into questions such as why phylogenetically basal vertebrates display more neurogenic compartments than more recently diverged vertebrates (i.e. mammals), or what changes in the neurogenic process have occurred that could be at the basis of this decrease in neurogenesis throughout evolution.

BOX 1. Chondrichthyes are at the base of the gnathostome lineage

Cartilaginous fishes (class Chondrichthyes) occupy a key phylogenetic position at the base of the vertebrate evolutionary tree, representing the earliest stages of the evolution on jawed vertebrates (Gnathostomata).



Vertebrate phylogeny. Chondrichthyes are at the base of the gnathostome lineage.

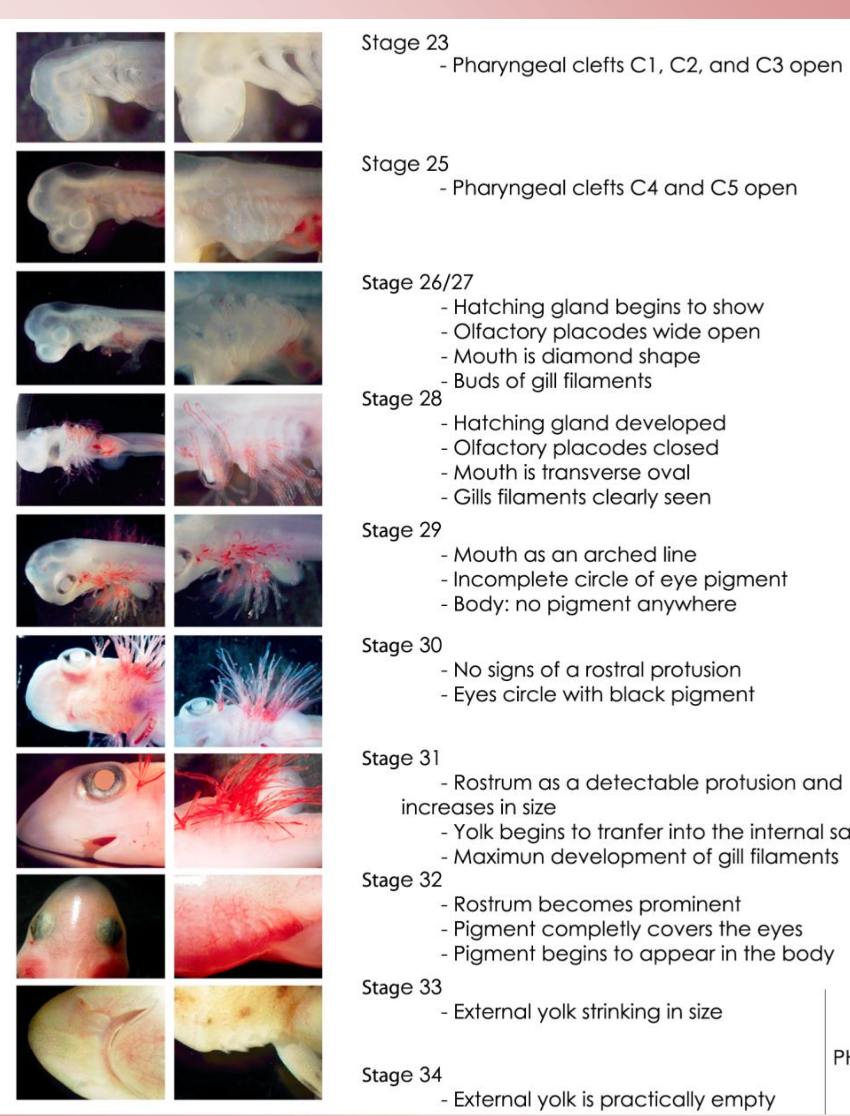
Chondrichthyes are the sister group of bony fishes (class Osteichthyes). Cartilaginous fishes have existed for over 400 million years. They are divided into two major radiations that diverged over 350 million years ago into two subclasses: Elasmobranchii (including sharks, skates, and rays), and Holocephali (elephant sharks and chimaeras) (Collin, 2012; Compagno, 1977, 1990; Delarbre et al., 1998; Kikugawa et al., 2004; Lisney et al., 2012; Northcutt, 1977; Renz et al., 2013; Rodríguez-Moldes, 2011; Smeets, 1998; Yopak, 2012). There are about 1100 species of living elasmobranchs widely distributed in diverse habitats, from some freshwater rivers to the ocean depths (Camhi et al., 1998; Hart et al., 2006). Skates and rays are characterized by their flat bodies and are grouped in the superorder Batoidea, while most of sharks show streamlines bodies and they are divided into two groups: squalomorphs and galeomorphs. Sharks with flattened bodies are included in the squatinomorphs group (reviewed in Smeets, 1998). For about 400 species of cartilaginous fishes are sharks (Camhi et al., 1998).

BOX 2. *Scyliorhinus canicula*, a model species for elasmobranch fishes

S. canicula (Linnaeus, 1758), also known as catshark or lesser spotted dogfish, is a relative small shark of about 60 cm which belongs to the Family *Scyliorhinidae*. It is very common on the Northeast Atlantic coast, from the Norway and British Islands to the south of Senegal, including the Mediterranean Sea with some variations between subspecies. This species is usually found on sandy, coralline, algal, gravel or muddy bottoms, mainly between 10-100 m depth and up to 400 m depth in the Mediterranean. Its basic feeding consists on a variety of invertebrates as mollusks, crustaceans, small cephalopods, and small bony fishes FishBase (Compagno, 1984; Rodríguez-Cabello et al., 2004; Springer, 1979; see also www.fishbase.org).

S. canicula is an oviparous species. Its eggs are laid inside a protective case which are deposited in pairs (one per oviduct) throughout the year, and anchored on to the macroalgae or other fixed surfaces with the tendrils that have in both ends. In captivity, the embryo hatch from the egg after 5-11 months, depending on the water temperature (Compagno, 1984; Ellis and Shackley, 1997). The different embryonic developmental stages before hatching have been characterized by Ballard et al. 1993. They described 34 stages considering distinctive features in the embryo such as the number of pharyngeal sets, presence of gill filaments, mouth shape, eye and body pigment, total length, or the size of the yolk sac (Ballard et al., 1993). Newly hatched sharks are about 8-10 cm in length (Serena, 2005). From that moment, they are considered as "juveniles", although they may require a year or more to become sexually mature.

Because of its several advantages, in recent years *S. canicula* has been extensively used in evolutionary developmental studies (reviewed in Coolen et al., 2009). These advantages include the easy maintaining of the eggs and post-hatching specimens under laboratory conditions, and the readily staging of the embryos through the translucent capsule, which allows the selection of the required developmental stages. Moreover, the big size of the embryos and the extended embryonic period (5-11 months depending on the water temperature), compared with other fish model species, permits monitoring different developmental processes, evidencing details that can be neglected in other species with faster development.



External features at different developmental stages in *S. canicula*. External featrures as described in Ballard et al., 1993. Microphotographs taken from Carrera, I. (2008).

4. The retina of *S. canicula*

4.1. What is known so far about the histogenesis of the retina in *S. canicula*

It has been traditionally assumed that sharks have poorly eyesight in comparison with olfaction, which seems to be the most important of their senses. However, recent studies have demonstrated the relative importance of vision and olfaction, which depend on the different environmental conditions, habitats, and life styles of each species. Indeed, sharks have well-developed eyes positioned on both sides of the head adapted for a wide range of light levels (**Fig. 8**), and a large area in the brain to process visual information.

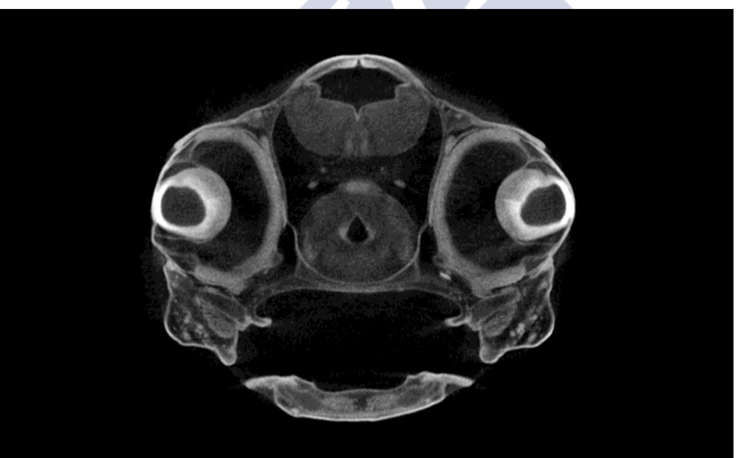


Figure 8. Transversal tomographic section of a stage 32 embryo of *S. canicula* imaged with micro-CT. The eyes are placed on both sides of the head. The embryo was fixed in 4% paraformaldehide, preserved in methanol and rinsed in 1% phosphotungstic acid in methanol. The image was taken with a Bruker Skyscan 1172 microtomograph at the University of Santiago de Compostela Marine Biology Station of "A Graña"; courtesy of Santiago Pereira and María Candás.

In terrestrial vertebrates, the cornea is the primary refractive element of the eye, but its refractive ability is extremely reduced in aquatic vertebrates because of the refractive index of the aqueous humor (**Fig. 9**). Light enters the eye through a gap in the iris, the pupil, which is mobile in most elasmobranchs in contrast to the fixed pupils of teleosts. Sharks usually have slit-shaped pupils that are horizontal in the case of *S. canicula*. In this species, the pupil has the ability to become completely closed under very bright conditions (**see more details in the Appendix of this thesis**). It has been reported that the pupillary response of *S. canicula* to intense white light was much more sensitive than in other species typical from more bright environments. Sharks possess lenses variable in shape. In the aquatic eye, lenses are the main refractive elements.

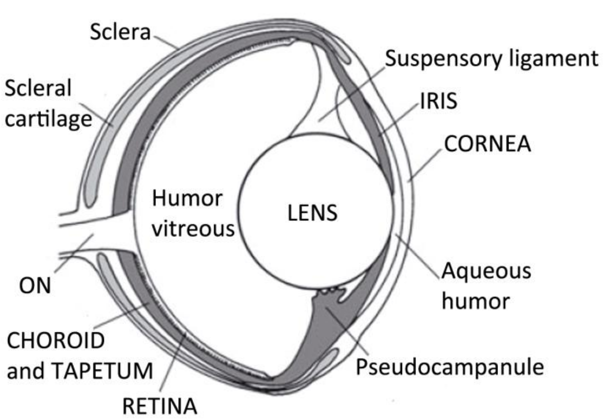


Figure 9. Schematic drawing of the eye structure of elasmobranchs. Modified from Lisney et al. (2012). ON, optic nerve.

S. canicula possesses a non-occlusive blue-green *tapetum*. The *tapetum* is a specialized structure typical of animals that live in scotopic conditions, which lies in the choroid behind the retina to increase the visual sensitivity by reflecting the light back toward the photoreceptors. Its structure is formed by reflective material (crystalline guanine)

accumulated in cells of the RPE (**see Box 1 in the Appendix** and **Fig. 10;** reviewed in Bozzano et al., 2001; Hart et al., 2006; Lisney et al., 2012; Litherland et al., 2009; Nicol, 1961; Raymond et al., 2006; Sivak and Sivak, 1999).

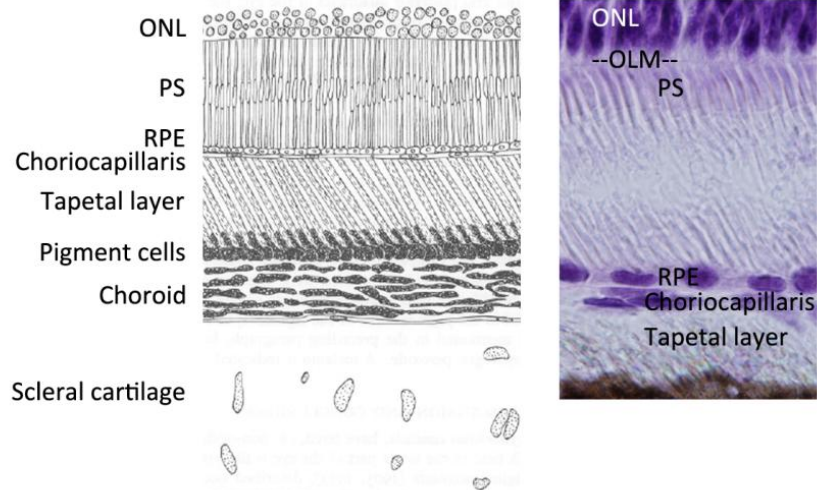


Figure 10. The *tapetum* and the RPE in *S. canicula*. The RPE contains non-pigmented cells that lie next to the photoreceptor segments (PS). Cells with processes (migratory cells) are observed close to the choroid. OLM, outer limiting membrane; ONL, outer nuclear layer; PS, photoreceptor segments; RPE, retinal pigment epithelium. Image on the left modified from Nicol (1961).

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

In *S. canicula*, optic vesicles appear for the first time during stage 17. At stage 20, the lens placodes pressure into the optic vesicles. Later, the optic vesicles will give rise to the optic cup. By stage 24, the lenses begin to separate from the surface ectoderm and press the eyeballs. At stage 29, the eyeballs show an incomplete circle of pigment, even if there is not present in any other region of the body. This circle of black pigment will be completed during stage 30. At stage 32, the pigment covers entirely the eyeballs and begins to appear in the skin (Ballard et al., 1993; O'Neill et al., 2007). When the embryo emerges from the egg, the neural retina is fully differentiated and synaptic connections are established (Bejarano-Escobar et al., 2012; Ferreiro-Galve et al., 2010a, 2012).

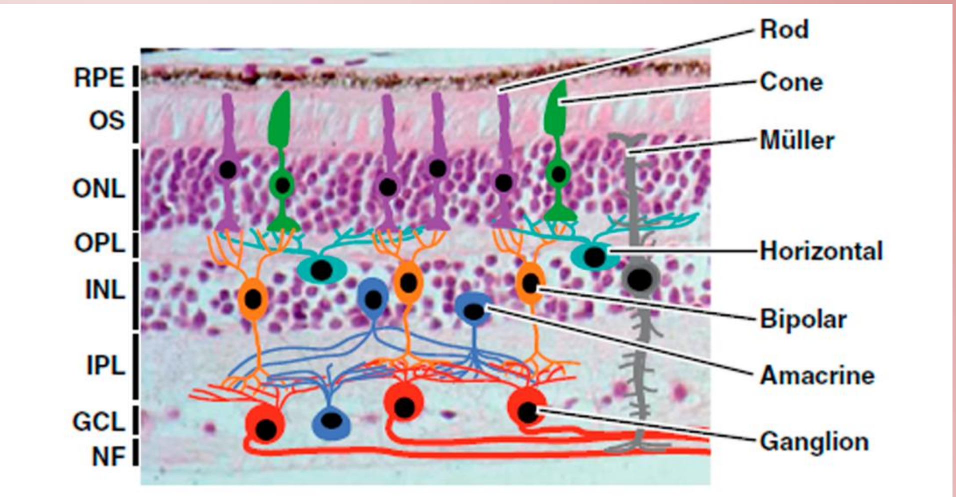
Retinal organization in elasmobranchs is essentially the same as in other vertebrates (**BOX 3**), with some differences regarding its structure and function.

Several studies have analyzed the spatial and temporal pattern of proliferation and differentiation of retinal cells during retinal histogenesis in teleosts (Bejarano-Escobar et al., 2009, 2010; Candal et al., 2005a, b; Kitambi and Malicki, 2008; Raymond et al., 1995), and also (but to a lesser extent) in elasmobranchs (Bejarano-Escobar et al., 2012; Ferreiro-Galve et al., 2010a, b; Harahush et al., 2009). Specifically, some histological descriptions about retinogenesis have been reported in the small shark *S. canicula,* allowing us to better understand the events that occur during retinogenesis. Three periods can be described in the retina of *S. canicula* as regard the growth pattern [**see Chapter 1** and Fig. 1 in Ferreiro-Galve et al. (2010a)]. The *first developmental period* between stages 26 and 29, the *second developmental period* from stage 30 to 32, and the *third developmental period* comprises from stage 33 onwards.

Previous developmental studies have shown some differences with respect to teleosts in the order of cell differentiation (Harahush et al., 2009) and in the development of the GABAergic system (Ferreiro-Galve et al., 2008). The pattern of cell proliferation and its relation with photoreceptor differentiation in the embryonic and postembryonic retina have been also described in detail (Ferreiro-Galve et al., 2010a), as well as the analysis of the differentiation pattern with other neurochemical markers (Bejarano-Escobar et al., 2012; Ferreiro-Galve et al., 2010b, 2012). Studies in different vertebrates have shown that retinal neurogenesis is initiated in the GCL. In the other layers, the order of cell production may vary slightly. Generally, GCs and horizontal cells (HCs) differentiate first, followed-up by cone photoreceptors, amacrine cells (ACs), rod photoreceptors, and BCs in an overlapping way; MGCs differentiated at the end (Bejarano-Escobar et al., 2012; Harahush et al., 2009; Malicki, 2004; Marquardt and Gruss, 2002; Olson et al., 1999; Rapaport et al., 2004).

BOX 3. Retinal organization in elasmobranchs/vertebrates

As in other vertebrates, six major types of neurons and one macroglial cell type are arranged in three neuronal cell layers and two synaptic layers.



Retinal organization in vertebrates. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NF, nerve fiber; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; RPE, retinal pigment epithelium. Taken from Basset and Wallace (2012).

Several physiological and morphological studies have typified the retinal circuitry and cell types in elasmobranchs. Photoreceptor cells represent the first neural connection of the visual system with the environment. Photoreceptor cells establish synapses with BCs and HCs in the OPL, which pass the information to retinal GCs within the IPL. Interestingly, in elasmobranchs photoreceptor cells and retinal GCs have been extensively studied because their variations in number depend on lifestyle and environment. According to the morphology and function, the vertebrate retina possesses two types of photoreceptors: rods and cones. It seems that the majority of sharks possess both rods and cones photoreceptors in the retina (duplex retina; Gruber et al., 1975), although in many species, the proportion of rods is much higher to provide a scotopic vision. The retina of *S. canicula*, a benthic species living in dim light environments, mostly contains rods (rod-cone ratio >100:1), which provides higher visual sensitivity that can be enhanced in dim light by the *tapetum lucidum* (see below) (Bozzano et al., 2001; Collin, 2012; Hart et al., 2006, 2011; Lisney et al., 2012; Litherland et al., 2009). The length of rod outer segments also enhances the retina sensitivity. In addition, its length varies with retinal localization. In *S. canicula*, rods located in central retina are longer than those at the periphery. The rod visual pigments in elasmobranchs appear to be related with the deep habitat (Bedore et al., 2013; Bozzano et al., 2001; Cohen, 1989; Collin, 2012; Gruber, 1977; Hart et al., 2006; Lisney et al., 2012; Litherland et al., 2009; Northcutt, 1990; Schieber et al., 2012; Sillman et al., 1996).

Four types of neurons were found in the INL. The outermost are the HCs. The retina of elasmobranchs is very rich in HCs, which send their processes to the OPL, where they contact with the terminals of rods and cones. HCs are responsible for the lateral spread of visual signal in the OPL that serves to pass the signal input to the inner retinal circuitry, involved in contrast enhancement. Different types of HCs have been described in elasmobranch species (Bejarano-Escobar et al., 2009; Hart e al., 2006; Lisney et al., 2012; Toyoda, 1978). The nuclei of BCs are found internally to the HCL. These neurons transfer the signals from photoreceptors to retinal GCs, generating graded potentials in response to light stimulation (Ashmore and Falk, 1980a, b). In the retina of *S. canicula*, a large number of rods connect to a single bipolar cell in the OPL providing the scotopic sensitivity, but reduce the spatial resolving power, typical of low light habitats. Rod BCs receive input from rods and contact with AII ACs, which synapse with cone BCs and therein retinal GCs. Distinct types of BCs have been described in the retina of different elasmobranch species (Ashmore and Falk, 1980a, b; Hart et al., 2006; Lamb, 2013; Lisney et al., 2012). The nuclei of MGCs are placed approximately in the middle of the INL. MGCs are the major type of glial cells in the retina and their processes span the entire retina in a radial arrangement. MGCs are responsible for supporting the metabolism and function of retinal neurons (Bejarano-Escobar et al., 2012; Newman and Reichenbach, 1996; Reichenbach and Bringmann, 2013). ACs are localized in the innermost part of the INL (INLi), although some displaced somas are found in the GCL. Dendrites of ACs end in the IPL and connect to BCs, so they represent the connection between the rod pathway and the GCs. There are different types of ACs that varied in the size of the soma, dendritic processes and layering (reviewed in Hart, 2006; Lisney et al., 2012). The retina of sharks possesses many rod BCs that converge in a single GC, contributing to increase retinal sensitivity. The somas of GCs occupy the innermost nuclear layer, the GCL, although some of them are displaced to more outer regions. The morphology of GCs is extremely variable and it has been found that different subtypes differ in functional properties. In addition, the size of the soma varies depending on the retinal location and species. The topography of retinal GCs in cartilaginous fishes has been well studied since they represent the final cells in the visual pathway. Each GC possesses a single axon which leaves the retina via the ON, transferring the visual information to visual centers in the brain. The number of retinal GCs is usually associated with the photoreceptor density. There are two main patterns of retinal specialization regarding the topography distribution of retinal GCs: an *area centralis,* that consist in an increase of cell density to a central peak in a particular region (generally found in species inhabiting structurally complex environments or predatory species); and a linear *area horizontalis* or visual streak, where cell density increases in a horizontal band across the retina (typical of the most species of elasmobranchs, useful in scanning the surrounding area with minimal eye movements). In *S. canicula* the visual streak locates in the dorsal retina (Bozzano and Collin, 2000; Collin, 2012; Hart, 2006; Lisney and Collin, 2008; Lisney et al., 2012; Litherland et al., 2009).

4.2. The retina of *S. canicula* as a model

The retina of *S. canicula* offers an exceptional model to approach the study of both embryonic and adult neurogenesis due to several reasons: (1) as in all vertebrates, retinogenesis involves the generation of a layered structure where different types of neurons and a single type of glia become highly organized; (2) as in other fishes, proliferation becomes restricted to the CMZ, which consists of a peripheral ring of PCs that persists throughout the entire life of the animal. The continuous proliferation of cells in the CMZ permits the successive addition of concentric rings of new cells, including neurons and MGCs; (3) as in other fishes, the predominant glial cell type in the healthy retina is the MG, which shows morphological characteristics of RGCs; (5) in contrast to fast-developing teleosts, it presents a protracted period of proliferation in the central retina, and a long-lasting transition zone bordering the CMZ that contain both early differentiated and NECs.

The size of the retina (and particularly that of the CMZ and the transition zone) and the peace with which it grows provide a particularly good spatial and temporal frame to solve some of the main questions raised in the field. First, it may serve to identify molecular changes at the transition from stem to progenitor to differentiated cells. Second, it may serve to precisely define a transition from NECs to RG progenitors during development, which is not obvious in teleost fishes. Third, it can help to understand NSC and/or progenitor heterogeneity within the CMZ and how it relates to their position in the niche. Finally, it can help to solve whether CMZ contribution to adult neurogenesis involves a transition through a RG state. The results obtained in *S. canicula* will be compared with that reported previously in zebrafish and mouse, representative species that have been previously used to study neurogenesis. In this context, data obtained from studies in highly neurogenic environments will be useful to identify methods of stimulation of endogenous neurogenesis in the mammalian brain.

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Abbreviations

AC	amacrine cell
aNSC	adult neural stem cell
BC	bipolar cell
BLBP	brain lipid binding protein
BV	blood vessels
CE	ciliary epithelium
CMZ	ciliary marginal zone
CNS	central nervous system
GC	ganglion cell
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
HC	horizontal cell
IKNM	interkinetic nuclear migration
INL	inner nuclear layer
INLi	inner part of the INL
IPC	intermediate progenitor cell
IPL	inner plexiform layer
MA	mantle
MAP	microtubule associated protein
MG	Müller glia
MGC	Müller glial cell
micG	microglia
MT	microtubule
NEC	neuroepithelial cell
NF	nerve fiber
NSC	neural stem cell
OLM	outer limiting membrane
ON	optic nerve
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	outer segments

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PC	progenitor cell
PS	photoreceptor segments
RG	radial glia
RGC	radial glial cell
RPE	retinal pigment epithelium
SVZ	subventricular zone
VZ	ventricular zone

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RATIONALE AND AIMS

RATIONALE AND AIMS

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Rationale and aims

Neurogenesis is defined as the process by which primary progenitor cells give rise to new neurons and glia. During development, neural stem cells (NSCs) gradually change in potential to generate progenitors (neuroepithelial cells and radial glia) that in turn generate different types of neurons and glial cells at different times and locations. In mammals, postnatal progenitors (adult NSCs) are generated from a population of radial glial cells (RGCs) present in the embryo. These adult NSCs that set aside from those RGCs can remain quiescent until they become reactivated at different ages in the postnatal brain to produce distinct types of neurons and glia. However, RGCs are mostly transient in mammals (they largely differentiate at postnatal stages) and postnatal progenitors appear incapable of endogenous reparation, which is behind the low regenerative capacity of the mammalian brain.

Contrasting with the situation in mammals, RGCs are widely maintained in the adult central nervous system (CNS) of anamniote vertebrates (particularly fishes and salamanders), where they contribute to CNS regeneration. Recent studies have focused on how these vertebrates activate progenitor cells, regenerate particular cell types, and functionally integrate them into the mature CNS. However, cellular hierarchies involving different types of progenitors, i.e., if neuroepithelial cells (NECs) are at the origin of adult RGCs, remains unsolved.

The retina of fishes appears as a good model to approach long-lasting constitutive neurogenesis and regenerative neurogenesis because of the presence of high rates of cell proliferation in the adult (from the ciliary marginal zone and from adult radial glia; RG) and because of the occurrence of reparation processes involving RGCs (Müller cells). One main goal while studying these aspects in fishes and comparing them with the situation in mammals is to be able to differentiate the mandatory (conserved) versus specific (diverged) components of the adult germinal niches, i.e., the order and place where neurons are produced, where they migrate and where they are ultimately incorporated.

Most of the investigations examining the presence of adult neurogenesis have taken place in tetrapod vertebrates (especially in mammals) and teleost fishes. Strikingly, other vertebrate classes, such as cartilaginous fishes (in which adult neurogenesis has been found) are not typically used for this kind of analyses. Cartilaginous fishes represent an ancient radiation of vertebrates, currently considered the sister group of bony fishes, which makes them an essential comparative reference that may provide insight into the extent of shared and/or divergent characteristics of adult neurogenesis among vertebrates. Among cartilaginous fishes, the lesser spotted dogfish, *Scyliorhinus canicula*, offers a great potential as a model species because of its phylogenetic position, and because of the relative size of the retina and the peace with which it

grows, which provide a particularly good spatial and temporal frame to solve some of the main questions raised in the field.

This thesis includes three chapters and one appendix:

Chapters 1 to 3 are aimed to fill the gap of knowledge about **constitutive neurogenesis** in basal vertebrates and to shed light into the ancestral condition of this process. Some general aspects related to proliferation and differentiation patterns in the retina of this species had been previously addressed in the retina of *S. canicula* (see **General introduction**). Since neurogenesis also relates to migration and cell positioning in appropriate layers, we have analyzed the spatio-temporal pattern of doublecortin (DCX) expression, which has been largely considered a marker of migrating neuroblasts in the developing and adult CNS. We additionally aimed to differentiate neuroepithelial progenitors, RG progenitors and mature RGCs during development and in the mature retina, which is not obvious in teleost fishes. The results obtained in *S. canicula* will be compared with that reported previously in zebrafish and mouse, representative species that have been previously used to study neurogenesis.

The **Appendix** is aimed to explore if the mechanisms involved in **retinal regeneration** are the same used during constitutive neurogenesis. This appendix includes a report on preliminary results of the neurogenic process observed after retinal damage in *S. canicula* as well as a brief report of the results obtained during the training stay in the laboratory of the Professor Mike O. Karl, in the Center for Regenerative Therapies TU Dresden (CRTD; Germany).

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The specific aims of this thesis are:

1) To investigate the spatial and temporal distribution of DCX during retinogenesis and in the mature retina of the small shark *S. canicula*. The results of this study are presented in **Chapter 1**, entitled ***Spatiotemporal organization of doublecortin immunoreactivity in the retina of Scyliorhinus canicula***.

2) To test for a relationship between DCX expression and the neurogenic state of DCX-labelled cells by exploring its co-localization with proliferation markers (proliferating cell nuclear antigen; PCNA) and the neuronal differentiation marker HuC/D. To test for a relationship between DCX expression and cell migration, by comparing its expression with that of the glial marker GFAP (glial fibrillary acidic protein). To characterize DCX-expressing cells in mature areas of the retina by double immunocytochemistry using antibodies against Calbindin, a marker for bipolar and horizontal cells in the retina of *S. canicula* and Pax6, which has been proposed as a regulator of cell proliferation, cell differentiation, and neuron diversification in the neural retina of this species. The results of this study are presented in **Chapter 2**, entitled ***Doublecortin and neurogenesis in the developing and mature retina of sharks: an immunohistochemical comparative study with proliferation and differentiation markers***.

3) To identify RG progenitors in the retina of by means of the immunohistochemical detection of glial markers as the GFAP and glutamine synthetase (GS). To explore the relationship between RG and neurogenesis events in the retina by exploring the emergence and localization of GFAP with respect to the proliferation marker PCNA (proliferating cell nuclear antigen) and the neuronal migration marker DCX. The results of this study are presented in **Chapter 3**, entitled ***Identification of radial glial progenitors in the retina of Scyliorhinus canicula***.

Abbreviations

CNS	central nervous system
DCX	doublecortin
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
NEC	neuroepithelial cell
NSC	neural stem cell
PCNA	proliferating cell nuclear antigen
RG	radial glia
RGC	radial glial cell

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MATERIAL AND METHODS

MATERIAL AND METHODS

1. Animals

Many of the embryos of the lesser spotted dogfish (*Scyliorhinus canicula*) were supplied by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France). Additional embryos and juveniles were kindly provided by the Aquaria of Gijón (Asturias, Spain), the Aquaria of O Grove (Pontevedra, Spain), and the Aquaria of Finisterrae (A Coruña, Spain). Adults were provided by a local fisherman and were processed immediately after incoming to the lab. Around sixty-five embryos from stages 24 to 34, twelve juveniles and two adults were used. Upon arrival to the lab, eggs and juveniles were raised in seawater tanks in standard conditions of temperature (15-16 ºC), pH (7.5-8.5), and salinity (35 g/L); in 12:12 hours day/night cycle. They were first introduced into the tanks inside plastic bags to get acclimated. Embryos were easily maintained under lab conditions until hatching, which usually occurs about 170-220 days at 16 °C. In natural conditions, the eggs have tendrils that allow them to attach to a substrate such as corals or seaweed. These

tendrils were used to anchor them to floating rods thus facilitating their development.

All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

2. Staging embryos

The transparency of the egg shells made possible the selection of the required developmental stages by observing their development without removing the embryo from the shell. After removal, embryos were identified by their external features using a stereoscopic microscope, following the descriptions in Ballard et al. (1993). For more information about the correspondence between embryonic stages and body size, gestation period and hatching, see Table 1 in Ferreiro-Galve et al., (2010).

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3. Tissue processing

Embryos up to stage 32 were deeply anesthetized with 0.5 % tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in a small container in seawater. Soon after, they were removed from the yolk and fixed by immersion in 4 % paraformaldehyde (PFA) in elasmobranch phosphate buffer [EPB: 0.1 M phosphate buffer (PB), containing 670 mM

urea, pH 7.4] for 48-72 h, or in Clark's solution for 12-36 h, depending on the developmental stage.

Embryos from stage 32 onwards, juveniles, and adults, were deeply anesthetized in the same way and then perfused intracardially with elasmobranch Ringer's solution (1.7 % NaCl, 0.024 % KCl, 0.031 % $CaCl_2$, 0.044 % $MgCl_2$, 0.113 % Na_2SO_4, 0.049 % $NaCO_3H$, and 2.7 % urea), followed by the appropriate fixatives. Afterward, the eyes of stages 32-34 embryos, juveniles, and adults, were removed and postfixed in the corresponding fixative for 24-48 h at 4°C, depending on the size (around 158 eyes were processed in all). The brains of each specimen were also removed, processed and stored in methanol at -20 °C.

Specimens fixed in 4 % PFA were rinsed in phosphate buffer saline (PBS), cryoprotected with 10 %-20 %-30 % sucrose in PB, embedded in NEG 50™ (Thermo Scientific, Kalamazoo, MI), frozen with liquid nitrogen-cooled isopentane and cut on a cryostat. Parallel series of transverse and sagittal sections with respect to the brain (16-18 µm thick) were mounted on Superfrost Plus slides (Menzel-Glässer®, Madison, WI). Specimens fixed in Clark's solution were dehydrated, embedded in paraffin and cut on a microtome. Parallel series of transverse and sagittal sections with respect to the brain (10 µm thick) were mounted on Superfrost Plus slides (Menzel-Glässer®, Madison, WI).

4. Immunohistochemistry

The main technique used in this thesis was immunohistochemistry (IHQ), which is based on the specific union between an antigen and its specific antibody *in situ* (**Fig. 1**). There are numerous variations of this technique. In this thesis three of these variations were used.

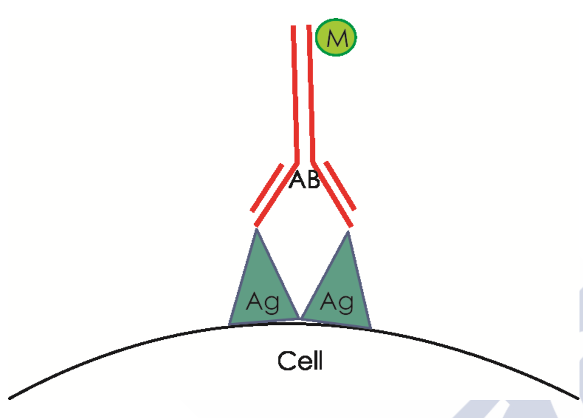

Figure 1. Simplified drawing of direct immunohistochemistry. Antigens (Ag, in blue) are placed in the surface of the cell. The antibody (AB, in red), which is coupled to a marker (M, in green), specifically recognizes the Ag.

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4.1. Simple indirect IHQ

This method aims to bind and label one specific tissue antigen. The indirect method involves an unlabeled primary antibody (first layer) that reacts with tissue antigen, and a secondary antibody (second layer) that reacts with the primary antibody. The secondary antibody can be conjugated with the horseradish peroxidase enzyme (HRP-conjugated antibody). The peroxidase is then developed by the substrate 3, 3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma) to produce a colorimetric (brown) end product (**Fig. 2A**). The secondary antibody can be also conjugated with biotin (biotinylated antibody). Biotin has a very high affinity for avidin, which in turn can be labelled with peroxidase. This complex of avidin-biotin peroxidase thus constitutes a third layer. As above, the peroxidase is developed by DAB (**Fig. 2B**).

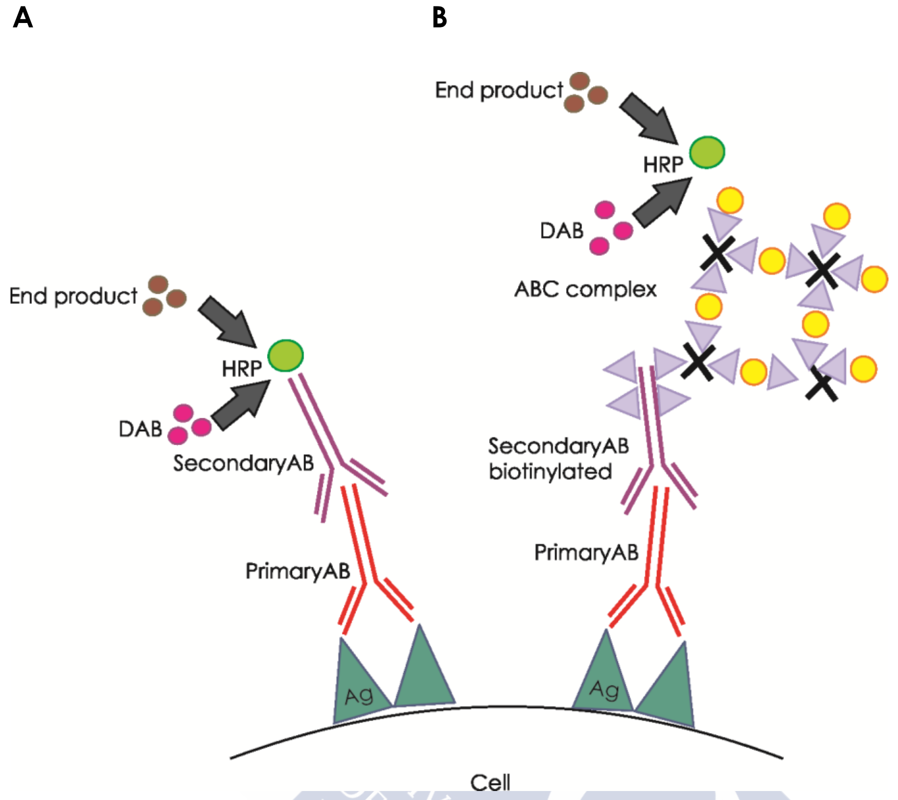

Figure 2. Simplified drawing of simple indirect IHQ. Simple indirect IHQ with the HRP (**A**), and with ABC method (**B**). Both revealed using DAB.

1) Sections for procedures using HRP-conjugated secondary antibodies were pre-treated with 0.01 M citrate buffer, pH 6.0, for 30 min at 90 °C for heat-induced epitope retrieval. After cooling, sections were rinsed twice in Tris buffer saline (TBS; pH 7.4) for 5 min each. Endogenous peroxidase activity is found in many tissues and was removed by pre-treating the tissue section with hydrogen peroxide prior to incubation overnight (ON) in the primary antibody solution. Secondary HRP-conjugated antibodies were subsequently incubated for 1h at room temperature (RT), and finally the immunolabeling was visualized with 0.25 mg/ml DAB (Sigma) in TBS with 0.00075 % H_2O_2, or with SIGMAFAST™ 3, 3'-DAB tablets (Sigma). Finally, sections were dehydrated, mounted, and coverslipped.

2) The avidin-biotin complex (ABC) procedure can enhance other immunoenzyme methods based on the highly affinity between avidin and biotin molecules, an irreversible union. The biotinylated enzyme becomes complexed with avidin in solution. This complex is then introduced to the biotinylated secondary antibody, which binds to the primary antibody-antigen sites (Bratthauer, 2010; Diamandis and Christopoulos, 1991; Hsu et al., 1981). Sections for ABC method were also pre-treated with citrate buffer, cooled for 20-30 min, and rinsed twice in TBS for 5 min each. Endogenous peroxidase activity was removed as above and avidin/biotin activity was removed by incubation with avidin/biotin blocking kit (Vector, Burlingame, CA). Then, sections were incubated with the primary solution ON. Sections were then rinsed in TBS (two 10-min each) and incubated in the appropriate biotinylated secondary antibody for 1 h, rinsed in TBS (two 10-min each), and then incubated with the avidin-biotinylated horseradish peroxidase complex (Vector laboratories) for 30 min. The immunoreaction was finally developed with DAB. Sections were then dehydrated, mounted, and coverslipped. All dilutions of antibodies were made with TBS containing 15 % normal serum from goat (NGS; Millipore, Billerica, MA) or donkey (NDS; Millipore, Billerica, MA), depending on the secondary antibodies used, 0.2 % Triton X-100 (Sigma), and 4 % bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber at RT.

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4.2. Double and triple immunofluorescence

Immunofluorescence is a method which combines antibodies and fluorophores for the detection of specific targets in cells and tissues. It is a technique with multiple variations (Fritschy and Hartig, 1999). In this case, secondary antibodies were conjugated with different fluorophores excited with different wavelengths, which allows identifying up to three different antigens in the same sample (**Fig. 3**).

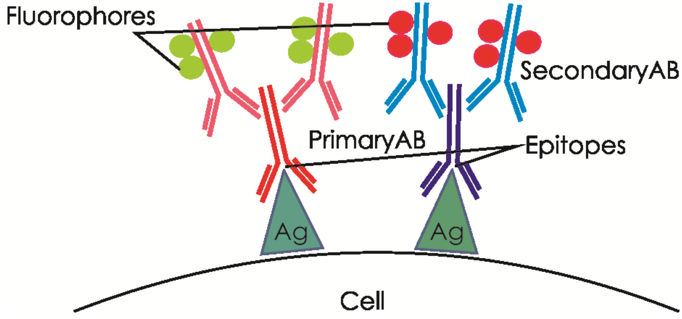

Figure 3. Simplified drawing of double immunofluorescence IHQ.

For heat induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer pH 6.0 for 30 min at 95 °C, and were allowed to cool for 20-30 min at RT. Then sections were rinsed twice in 0.05 M TBS for 5 min each, and incubated ON with cocktails of primary antibodies mixed at optimal dilutions. Sections were rinsed twice in TBS for 5 min each, and incubated in the mixtures of appropriate secondary antibodies for 1 h at RT. All dilutions of antibodies were made with TBS containing 15 % normal serum [from goat (NGS; Millipore, Billerica, MA) or donkey (NDS; Millipore, Billerica, MA), depending on the secondary antibodies used], 0.2 % Triton X-100 (Sigma), and 4 % bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber at RT. Sections were then rinsed in TBS for 30 min and in distilled water (twice for 30 min), they were allowed to dry for 2 h at 37 °C, and were finally mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany).

Note that double immunofluorescence with primary antibodies raised in the same species was performed as described in Tornehave et al. (2000).

5. Antibodies used: controls and specifity

The following antibodies were used in this project: rabbit and goat polyclonal anti-doublecortin (DCX), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA), mouse monoclonal anti-human HuC/HuD (HuC/D), rabbit polyclonal anti-Pax6, rabbit polyclonal anti-calbindin D-28k, rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), and anti-glutamine synthetase (GS).

5.1. Anti-DCX antibodies

In *S. canicula* anti-DCX antibodies have been previously used to label migrating neuroblasts in different regions of the central nervous system (CNS) (Pose-Méndez et al., 2014; Quintana-Urzainqui et al., 2014; Rodríguez-Moldes, 2011). In this thesis two different anti-DCX polyclonal antibodies were used. The rabbit polyclonal anti-DCX antibody (Cell Signaling) was produced by immunizing rabbits with human doublecortin synthetic peptides. According to manufacturers, this antibody was purified by protein A and peptide affinity chromatography. In Western blot analysis of extracts from mouse and rat brain, this antibody recognized one band of approximately 45 kDa. A single band of 45 kDa was also recognized in Westen blot analyses of brain extracts of adult lesser spotted dogfish (Pose-Méndez et al., 2014). The goat polyclonal anti-DCX antibody (Sta. Cruz Biotechnology) was produced by immunized goats with a peptide mapping at the C-terminus of DCX protein of human origin. In Western blot analysis of extracts from mouse embryo, this antibody recognized one band of approximately 45 kDa (manufacturer's information). To confirm the specificity of the anti-DCX antibodies used in this study, we double-labeled sections of retina with the rabbit and goat polyclonal antibodies. The staining pattern of both antibodies was coincident (**see Fig. 1 in Chapter 2**).

5.2. Anti-PCNA antibody

The monoclonal anti-PCNA antibody (Sigma) recognizes a protein of 36 KDa corresponding to the acidic non-histone auxiliary protein of DNA polymerase (also known as polymerase delta accessory protein, according to the manufacturer). PCNA labeling is observed in proliferating cell nuclei in interphase (G1, S and G2 phases of the cell cycle), specifically in germinal centers. In *S. canicula*, this antibody was previously used to label proliferating cells in the brain (Coolen et al., 2009), in the olfactory epithelium (Quintana-Urzainqui et al., 2014), and in the retina (Ferreiro-Galve et al., 2010).

5.3. Anti-HuC/D antibody

The mouse monoclonal anti-HuC/D has been shown to specifically label neuronal cells in zebrafish, birds and humans [manufacturer's information; see also (Ekström and Johansson, 2003; Marusich et al., 1994; Soukkarieh et al., 2007; Vellema et al., 2010)]. The same antibody labeled different types of neurons in the spinal cord (Sueiro et al., 2004) and in the olfactory system (Quintana-Urzainqui et al., 2014) of embryos and juveniles of lesser spotted dogfish. In the chick retina, this antibody has been used as an early marker of neuronal differentiation since it has been shown in amacrine and ganglion cells shortly after the terminal mitosis and in neurons with immature morphology (elongated soma and poorly defined neurites) that are found directly adjacent to the CMZ (Ghai et al., 2008). The HuC/D antibody has been also shown in the vast majority of amacrine cells and cell somas located in the ganglion cell layer of the embryos, in developing and mature rat retina (Ekström and Johansson, 2003).

5.4. Anti-Pax6 antibody

The rabbit polyclonal Pax6 antibody was raised against a peptide derived from the C-terminus of the mouse Pax6 protein and subsequently purified on a Protein A Column. Multiple sequence alignment (Corpet, 1988) of Pax6 (GenBank NP_000271.1), Pax6-5a (GenBank NP_001595.2), and Pax6-ΔPD (GenBank AAL40860) showed that the C-terminus of the protein recognized by the rabbit antibody is identical in the three Pax6 isoforms (Ferreiro-Galve et al., 2012). The specificity of Pax6 antibody in the retina and brain of *S. canicula* was previously tested in our laboratory by preadsorbing the primary antibodies with the antigenic Pax6 peptide (NB100-2913PEP; Novus Biologicals, Littleton,CO) used for generation of the NB100-2913 antiserum. The immunostaining was completely abolished in sections treated with primary antibodies at working dilution and preadsorbed with the blocking peptide (see Ferreiro-Galve et al., 2012).

5.5. Anti-calbindin D-28k antibody

The rabbit anti-Calbindin D-28k was produced against recombinant rat calbindin D-28k. This antibody crossreacts for many species, including human, monkey, rat, mouse, chicken and fish. In immunoblot it recognizes a single band of approximately 27-28 kDa (manufacturer's information). This antibody has been previously found in some bipolar cells and in its Landolt's processes in the developing and in the adult retina of *S. canicula* (Bejarano-Escobar et al., 2012).

5.6. Anti-GFAP antibody

GFAP is the main intermediate filament in mature astrocytes in the CNS, both in normal and pathological conditions. The polyclonal anti-

GFAP antibody (DakoCytomation) is a purified immunoglobulin fraction of rabbit antiserum generated to bovine spinal cord GFAP. This antibody has been previously used as a glial marker in *S. canicula* (Quintana-Urzainqui et al., 2014; Sueiro et al., 2007; Wasowicz et al., 1999).

5.7. Anti-GS antibody

The enzyme GS catalyzes the conversion of ammonia and glutamate to glutamine acts in the detoxification of ammonia in the brain as well as in the metabolic regulation of neurotransmitter glutamate. The monoclonal anti-GS antibody (Merk-Millipore) is a purified immunoglobulin fraction of 45 kDa from sheep brain generated in mouse. This antibody cross-reacts for many species including human, mouse, rat and sheep (manufacturer's information). This antibody has been used as a specific glial cell marker in the retina of fishes, (Bejarano-Escobar et al., 2009, 2010; Lillo et al., 2002; Mack et al., 1998; Thummel et al., 2008). In *S. canicula* this antibody has been previously found in the Müller glia (Bejarano-Escobar et al., 2012).

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The primary and secondary antibodies used in each part of this thesis, together with their working dilution and sources, are reported in the corresponding chapters.

6. Image acquisition

Light field sections were photographed with an Olympus BX51 microscope equipped with an Olympus DP71 color digital camera. Labeled fluorescent sections were studied with spectral confocal laser scanning microscopes TCS-SP2 and SP5 (Leica, Wetzlar, Germany). Some sections were photographed with an epifluorescence photomicroscope Olympus AX70 fitted with an Olympus DP70 color digital camera. Photographs were minimally adjusted for brightness and contrast by using Adobe Photoshop CS5 software (Adobe, San Jose, CA).

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Abbreviations

AB	antibody
ABC	avidin-biotin complex
Ag	antigen
BSA	bovine serum albumin
CNS	central nervous system
DAB	3, 3'- Diaminobenzidine tetrahydrochloride
DCX	doublecortin
EPB	elasmobranch phosphate buffer
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
HRP	horseradish peroxidase
HuC/D	human HuC/HuD
IHQ	immunohistochemistry
M	marker
NDS	normal serum from donkey
NGS	normal serum from goat
ON	overnight
PB	phosphate buffer
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
RT	room temperature
TBS	Tris buffer saline

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CHAPTER 1

Spatiotemporal organization of doublecortin immunoreactivity in the retina of *Scyliorhinus canicula*

SPATIOTEMPORAL ORGANIZATION OF DOUBLECORTIN IMMUNOREACTIVITY IN THE RETINA OF *SCYLIORHINUS CANICULA*

1. Introduction

Neurogenesis consists of a coordinated set of events, including cell proliferation, migration and differentiation, which are necessary to transform progenitor cells into differentiated and functional neurons (McConnell, 1995; Paridaen and Huttner, 2014). Migratory movements of neurons from their birth place to their final location are essential for the establishment of a functional neural circuitry and, consequently, for the proper functioning of the brain (Nadarajah et al., 2001; Tabata and Nakajima, 2003). In fact, abnormal neuronal migration causes abnormal cortical function (Guerrini and Parrini, 2010; Guerrini et al., 2008). During migration, either along radial glia or independently of it (Wichterle et al., 1997; Yokota et al., 2007), cells suffer several morphological changes that require the reorganization of the cytoskeleton (Gleeson et al., 1999; Rivas and Hatten, 1995). Indeed, many proteins seem to be involved in neuronal migration by regulating the reorganization of the neuronal cytoskeleton (reviewed in Feng and Walsh, 2001; Oshima, 2015).

Particularly, microtubule-associated proteins (MAPs) are responsible for regulating microtubule (MT) dynamics during neuronal migration (Horesh et al., 1999; Jin et al., 2010; Oshima, 2015; Schaar et al., 2004; Tanaka et al., 2004b). Thus, deletions and mutations in genes related with cytoskeletal organization cause defects in neuronal migration (Des Portes et al., 1998) and, as consequence, in cortical (laminated) organization (Guerrini and Parrini, 2010). Among these genes, the *doublecortin* gene (*dcx*) has received particular attention because *dcx* mutations are associated with lissencephaly and double cortex syndromes, two X-linked allelic disorders that, in humans, affect the laminar organization of the cortex (Corbo et al., 2002; Kawauchi and Hoshino, 2007; Koizumi et al., 2006; Tanaka et al., 2004a). This gene encodes the 40 kDa MAP doublecortin (DCX) (Des Portes et al., 1998; Gleeson et al., 1998; Sossey-Alaoui et al., 1998) that can bind the MTs of the cytoskeleton (Francis et al., 1999), which in turn is involved in cell mitosis, motility and transport (Guzik and Goldstein, 2004; Kapitein and Hoogenraad, 2015). DCX binds selectively 13 protofilaments of the MTs, stimulating MT polymerization through its interaction with the dynein motor protein complex (Feng and Walsh, 2001; Friocourt et al., 2003; Moores et al., 2004; **see also section 2.2 in General Introduction**). DCX shares many properties with other neuronal MAPs (Cassimeris and Spittle, 2001) but differs in others, such as the structure of the tandem repeat motifs located in the N-terminal part of the protein, which adopts a globular structure with an ubiquitin-like fold (Kim et al., 2003; Reiner et al., 2006).

Concurring with its MAP activity, DCX was shown to be required for cell migration within the human cerebral cortex by regulating MTs stability (Feng and Walsh, 2001; Friocourt et al., 2007; Horesh et al., 1999; Kawauchi and Hoshino, 2007; Moores et al., 2004, 2006; Pramparo et al., 2010; Sapir et al., 2000; Tanaka et al., 2004a; Toriyama et al., 2012), and also for the development and lamination of the mouse hippocampus (Corbo et al., 2002; Kerjan et al., 2009).

Immunohistochemical studies have shown the expression of DCX in the mouse developing cortex, lateral ganglionic eminence, thalamus, midbrain, hindbrain, cerebellum, spinal cord and retina (Gleeson et al., 1999; Lee et al., 2003). Typically, DCX-expressing cells does not express Nestin (a marker for stem cells) and DCX expression decreases with neuronal maturation (Kuhn et al., 1996; Rao and Shetty, 2004). According to these developmental data and its expression in migrating cells (**Fig. 1A-C**), DCX has been extensively used as a marker of migrating neuroblasts in developing mammalian brain (Des Portes et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Kim et al., 2006). Additionally, this protein is usually found in adult brain regions that are known to incorporate new neurons (Brown et al., 2003; Couillard-Despres et al., 2005; Klempin et al., 2011; Paolone et al., 2014; von Bohlen und Halbach, 2007, 2011; Yang et al., 2004), which has endorsed this protein as a general indirect tool to survey adult neurogenesis (Brown et al., 2003; von Bohlen und Halbach, 2011).

However, some exceptions to this general pattern have been reported. DCX was recently described in Nestin-expressing stem cells in the retina of mammals after neurotoxic-induced dedifferentiation of macroglial cells (**Fig. 1A**; Chang et al., 2007). DCX was additionally found in adult regions not associated with adult neurogenesis (**Fig. 1C**). Indeed, DCX has been found in some morphological mature (and non-proliferating) neurons in the suprachiasmatic nuclei, in granular neurons of the dentate gyrus, in granular and periglomerular neurons of the olfactory bulb, and in other areas of the adult rat and mice brain (Geoghegan and Carter, 2008; Kremer et al., 2013; Nacher et al., 2001). Additionally, DCX has been reported in some cells located in regions that do not recruit new neurons in the adult brain of canaries (Balthazart et al., 2008; Boseret et al., 2007; Vellema et al., 2014), in unipolar brush cells in the dorsal cochlear nucleus and vestibulocerebellum of the adult rat (Paolone et al., 2014), and in horizontal cells of the adult rat retina (Wakabayashi et al., 2008). Accordingly, it has been suggested that DCX play a role in neural

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plasticity by means of MT reorganization during the establishment of new synapses (e.g. Friocourt et al., 2003; Nacher et al., 2001; Kremer et al., 2013).

Despite the importance of DCX in neural development and neuronal positioning (and hence, in layering), rare attention has been paid to the expression of DCX in the developing retina, a layered structure that has been used in the last decades as a model for studying neurogenesis, including cell migration to proper locations (Baye and Link, 2008; Centanin and Wittbrodt, 2014; Del Bene et al., 2008; Hatten, 1999; Norden et al., 2009; Spear and Erickson, 2012a; Stenkamp, 2007). As in the cortex, layering in the retina occurs during embryogenesis, when migration cell events took place in a well defined order which finally establish the highly organized cellular architecture typical of this structure (Chow and Lang, 2001). However, DCX expression has only been examined in the developing retina of chick (Kim and Sun, 2012) and rat (Lee et al., 2003), and in the adult retina of teleosts (Tozzini et al., 2012) and rat (Wakabayashi et al., 2008).

The retina of elasmobranchs, and particularly that of *Scyliorhinus canicula*, is becoming a prevailing model for studying various aspects related to neurogenesis (**see General introduction**), because of three main reasons: (1) as in other fishes, it contains retinal stem cells and progenitors within the ciliary marginal zone (CMZ) that gives rise to all cell types (Ferreiro-Galve et al., 2010a), which might become positioned in their corresponding cell layers throughout the entire life of the animal; (2) the large size and the slow development of the embryos compared to that of zebrafish, a fast-developing representative of teleost fishes, make the retina of *S. canicula* a suitable model for unravelling details that might remain unnoticed when analysing other animal models . Indeed, several developmental studies have been carried out in the retina of elasmobranchs (Bejarano-Escobar et al., 2012; Bozzano and Collin, 2000;

Ferreiro-Galve et al., 2008, 2010a, b, 2012,; Harahush et al., 2009), but any of them addressed the possible involvement of DCX in the neurogenic process, including cell positioning to appropriate layers. Accordingly, we have investigated the spatial and temporal distribution of DCX during retinogenesis and in the mature retina of the small shark *S. canicula*.

2. Material and methods

The following embryonic stages were analyzed: stage 19 (1), stage 20 (1), stage 25 (3), stage 26 (2), stage 27 (2), stage 28 (3), stage 29 (6), stage 30 (7), stage 31 (8), stage 32 (8), and prehatching (PH, 8). Additionally, 5 juveniles (107-117 mm) were used.

For more information about experimental animals, tissue preparation, immunohistochemistry, specificity of the antibody used, and image acquisition and analysis, see **Section 3** of this thesis: **Material and methods.**

Primary and secondary antibodies used in this chapter are shown in **Table 1**.

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3. Results

Previous studies of our group have divided the retinal development of *S. canicula* in three developmental periods (Ferreiro-Galve et al., 2010a). The *first developmental period* comprises stages 26-29 and is characterized by the neuroepithelial appearance of the retina and the absence of apparent differentiation or layering. The *second developmental period* includes stages 30-32 and it is recognizable by the progressive formation of layers in the central part of the retina, from vitreal to scleral and from central to marginal. It begins in stage 30 when the inner plexiform layer (IPL) becomes recognizable between the ganglion cell layer (GCL) and the inner nuclear layer (INL), simultaneously whit the appearance of a primordial optic fiber layer (OFL). This period ends at stage 32 with the appearance of the outer plexiform layer (OPL) between the outer nuclear layer (ONL) and the INL. Layering formation allows defining the CMZ as a wedge-shaped non-layered zone adjacent to the ciliary epithelium. The transition zone (TZ) is located between the CMZ and the layered central retina and it is formed by an inner layered part and an outer neuroepithelial part (**Fig. 1D**). In the *third developmental period*, from stage 33 onwards, layering progresses to nearly the entire circumference of the retina following a central-to-peripheral gradient, acquiring the mature organization characteristic of postembryonic stages. All the layers are clearly distinguished except in the optic disc (not shown), in the CMZ and in the outer part of the TZ (see in **Fig. 1D**), which are characterized by a neuroepithelial appearance.

We have analyzed the pattern of DCX immunoreactivity in the retina of *S. canicula* throughout these developmental periods.

3.1. Distribution of DCX in the developing retina of *S. canicula*

Immunoreactivity to DCX was first detected at early stage 27 (**Fig. 2A**). Very weak DCX-immunoreactivity was observed in the neuroepithelial retina (**arrows**), in contrast with other encephalic regions (**arrowhead in Fig. 2A**) by the same developmental period. At stage 28 (**Fig. 2B**), DCX immunoreactivity was mostly located in the inner (basal) part of the neuroblastic layer, where some round-shaped and spindle shaped cell bodies were observed (**arrows in Fig. 2B**). In addition, radial DCX immunoreactive (-ir) processes coursed toward the inner and outer regions of the neuroepithelium (**arrowheads in Fig. 2B**). At stage 29 (**Fig. 2C**), the density of DCX-ir cells increased in the neuroepithelium. DCX immunoreactivity was similar to that observed at stage 28, though it was slightly more intense than in previous developmental stages. Weak DCX immunoreactivity was also evident in some processes and cell bodies in the outer part of the retina (**arrows in Fig. 2C**).

At the beginning of the *second developmental period* (stage 30; **Fig. 2D-F**), the organization of DCX immunoreactivity in the most central (and more mature) part of the retina was different to that observed in the peripheral retina and the retina bordering the recently formed optic nerve (ON) head (**Fig. 2E**). In the central retina (**Fig. 2F**), strong DCX immunoreactivity was observed in a thick band of large round-shaped cells grouped in the innermost region of the retina (prospective GCL; **black arrowhead in Fig. 2F**) and their axons in the OFL (**asterisk in Fig. 2F**). Intense DCX-ir cells were also located in the inner region of the prospective INL (INLi; **white arrowhead in Fig. 2F**). At this stage a primordial IPL first appears, as noted by the presence of DCX-ir processes located between GCL and INLi cells (**arrow in Fig. 2F**). DCX-ir cells and processes were also observed at outer levels throughout the neuroepithelium, but their density decreased in either the intermediate neuroblastic layer (prospective outer part of the INL, the INLo) or in the outer neuroblastic

retina (prospective ONL). Similar DCX-ir cell populations were observed in the retina of stage 31 embryos (not shown), including DCX-ir cells in the GCL and INLi, and their axons and processes in the OFL and IPL, respectively. Some weakly DCX-ir spindle and round-shaped cell bodies were found in the intermediate neuroblastic layer and in the outer retina. In early 32 stage embryos (**Fig. 2G, H**), DCX-ir cells in the central retina become clearly arranged in the GCL, in several cell layers in the INLi, and also in a loose band at the primordial horizontal cell layer (HCL, **arrows in Fig. 2G**). Intense DCX-ir axons and processes were observed in the OFL and IPL, respectively. At the end of the *second developmental period* (late stage 32; **Fig. 2I**), DCX immunoreactivity becomes increased in cell bodies in the GCL, INLi and HCL (**Fig. 2I-2I'''**), and also in cell processes in the OFL and IPL (**asterisk in Fig. 2I**). During this period, DCX immunoreactivity increased in cells and processes following a centre-to-periphery gradient, except in the most peripheral non-layered retina that presented very faint levels of DCX immunoreactivity (**asterisk in Fig. 2J**). Adjacent to this zone, a non-layered area of the retina presented DCX-ir cells and processes in a similar pattern to that observed in the central retina at stage 28-29. In the TZ, where the IPL (but not the OPL) becomes apparent, the pattern of DCX immunoreactivity was similar to that found in the central retina at stage 30, i.e. DCX immunoreactivity was found in axons and cell processes in the OFL and IPL, respectively, in cells in the GCL and INLi, and in cells and processes in the outer non-layered part of the retina (**arrowhead in Fig. 2J**). DCX immunoreactivity was also found in the ON (**Fig. 2K**).

3.2. DCX immunoreactivity in the mature retina

During the *third developmental period*, the retina acquires the organization characteristic of postembryonic stages (juveniles) (**Fig. 3**). At PH stages (**Fig. 3A-C**), DCX immunoreactivity was observed in the central

retina in different postmitotic areas. Intensely stained DCX-ir processes were observed in the IPL, analogous to that observed at previous stages. Immunoreactivity in the INL was decreased, but some DCX-ir cells with small rounded somas were observed in this layer (**arrows in Fig. 3A**). These less intensely labeled cells in the innermost row of the INLi may represent amacrine cells. DCX labelling was also observed in different cell types in the INLo (**arrowhead in Fig. 3A**) and their processes extending towards the IPL, the OPL or the outer limiting membrane. Some of these cells, bipolar in shape, showed a thin process (Landolt's club, **asterisks in Fig. 3A, B**) directed to the outer limiting membrane (**Fig. 3B**). Some cells showed an ovoid or round soma, an axon oriented radially towards the IPL and highly branched dendrites into the OPL (**Fig. 3B'**). Another DCX-ir cell type was located in the HCL or below its vitreal surface. The morphology of these cells, with the soma localized in the outer border of the INLo and their processes extending horizontally forming a network into the OPL, suggested that they were horizontal cells (**arrow in Fig. 3B''**). The peripheral retina can be clearly divided into three different regions in the basis of DCX immunoreactivity. Very faint DCX immunoreactivity was found in the most peripheral CMZ (CMZ peripheral; CMZp), which showed no signs of layering. Bordering this region, another peripheral non-layered region (CMZ middle; CMZm) was distinguished that contained intense DCX immunoreactivity in a pattern that resembled the central retina at stage 28-29 (**Fig. 3C**). Adjacent to it, the thick TZ consisted on a layered inner part where the GCL (**arrow**) and IPL were clearly visible, and an outer non-layered part. Arrowheads point to amacrine cells in the INLi. At the TZ, the organization of DCX cells resembled that observed in the central retina at stage 30.

In juveniles the thickness of the INL decreased (**Fig. 3D-F**). The pattern of DCX immunoreactivity was very similar to that observed during the *third developmental period*, both in the central and in the peripheral retina. A few faintly DCX-ir cells were observed in the GCL (**arrowhead in**

Fig. 3D). Strongly DCX-ir processes of ganglion, amacrine and bipolar cells were also found in the IPL. Weakly stained DCX-ir cell bodies, apparently amacrine cells, were observed in the innermost row of the INLi (**arrows in Fig. 3D**, detail in **Fig. 3E**), whereas some strongly stained DCX-ir bipolar cells were observed in the scleral-most part of the INLo (**curved arrows in Fig. 3D**, details in **Fig. 3E'**). Note that in some of these bipolar cells a thin Landolt's club was directed to the outer limiting membrane (**asterisk in Fig. 3D, E'**). DCX immunoreactivity in HCL persists long after maturation and positioning of horizontal cells occurred (**open arrowheads in Fig. 3D**). In the peripheral retina the pattern of DCX immunoreactivity was similar to that described at previous developmental stages (**Fig. 3F**).

4. Discussion

This study shows for the first time the distribution of DCX in the retina throughout the lifespan of a fish by using immunohistochemistry.

4.1. Neuroepithelial, non-layered retina

We have shown weak DCX immunoreactivity initially in neuroepithelial cells close to the basal and apical surfaces at stage 27, when retina is still a proliferative neuroepithelium (see Ferreiro-Galve et al., 2010a). Interestingly, the presence of low DCX levels in neuroepithelial cells was not reported before, even in other brain regions by using immunohistochemistry. Pseudostratified neuroepithelia are highly proliferative tissues where elongated cells are arranged in a single layer, though their nuclei locate at distinct positions, which gives the neuroepithelium a stratified aspect (e.g. Baye and Link, 2008). As cells

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

progress through the cell cycle, the nuclei of neuroepithelial cells oscillate from the apical to the basal surface, a process which is known as interkinetic nuclear migration (IKNM; Sauer, 1935; Spear and Erickson, 2012a, 2012b; Taverna and Huttner, 2010). Proliferative neuroepithelial cells have elongated ovoid or teardrop-shaped nuclei during interkinetic nuclear migration and round nuclei when the nucleus stops at the apical surface for division (Das et al., 2003). In the mouse cerebral cortex, MTs and regulators of the dynein motor protein (such as dynactin, Lis1and NudC) are involved in apical nuclear migration (see Hu et al., 2013; Spear and Erickson, 2012b). Although the role of the microtubule binding-protein DCX in IKNM has not been studied so far, the fact that DCX coimmunoprecipitate with Lis1 in brain lysates from mouse embryos (Caspi et al., 2000), and the fact that DCX interacts with Lis1 and dynein to aid in the nuclear translocation of cerebellar granule neurons (Tanaka et al., 2004a), lead us to consider the possibility that DCX may also be involved in IKNM in the retina of fishes (see **Chapter 2**). Interestingly, very faint DCX immunoreactivity has been found in the CMZp, which is comprised of neuroepithelial cells, both in embryos and juveniles.

DCX intensity progressively increased during the *first developmental period* in the innermost part of the non-layered retina. At stage 29, the distribution of DCX was similar to that found in the rat retina (Lee et al., 2003), which was firstly observed at E15 in a few neuroblasts in the innermost part of the non-layered retina. A similar pattern was described in the mouse retina at E14 (Gleeson et al., 1999), and in the developing chick retina (Kim and Sun, 2012). Though the increase in DCX immunoreactivity in the innermost retina at stage 29 fairly coincides with the time and location where first postmitotic cells are observed in the retina of *S. canicula* (see Ferreiro-Galve et al., 2010a), co-localization studies would be necessary to ascertain if DCX is expressed in proliferating or early postmitotic cells (see **Chapter 2**). In addition, we also observed faint DCX immunoreactivity in non-layered areas that were previously

described to contain proliferating cells (Ferreiro-Galve et al., 2010a), including the outer non-layered retina of embryos up to stage 31, the CMZm and outer non-layered part of the TZ throughout development. DCX immunoreactivity bordering the CMZ has been also identified in the adult retina of the teleost *Nothobranchius furzeri* (Tozzini et al., 2012). Since layering is not completed in these areas (i.e., cells are not definitely settled in their definitive cell layers), our results suggest that DCX is present in neuroblasts as they radially migrate to their final locations, as has been described in other regions in developing mammalian brain (Des Portes et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Kim et al., 2006).

4.2. Progressive layering of the retina. Synaptogenesis

The sequence of appearance of DCX in different cell populations may be important to elucidate its possible roles during retinogenesis. In *S. canicula* retinal ganglion cells are born first (Bejarano-Escobar et al., 2012; Ferreiro-Galve et al., 2010a) and migrate to the most basal retinal layer, followed by amacrine cells that begin to differentiate at stage 31. At this stage, intense DCX immunoreactivity was clearly observed simultaneously in cells in the GCL and INLi, in cell processes in the IPL and axons from ganglion cells in the OFL. The same distribution pattern was described in rat as development progresses (Lee et al., 2003). Since DCX is found in cells while they differentiate and set new connections, our results support the idea that DCX is important for the differentiation and organization of the inner retina and are in agreement with previous hypothesis suggesting a role for DCX in synaptogenesis (Horesh et al., 1999; Lee et al., 2003; Nacher et al., 2001). Indeed, coinciding with retinal layering in the outer retina, DCX-ir cells become clearly positioned in the outermost part of the INLo and extended their processes in the OPL and towards the IPL.

4.3. Mature organization

At PH stages, some DCX-ir cells in the GCL were identified as ganglion cells because of their localization and large size. We also observed strong DCX-ir processes in the IPL. DCX-ir cells in the INLi were less intensely immunorreactive compared to previous stages. Some of these DCX-ir cells on the basis of their morphology and localization in the innermost row of the INLi may correspond to amacrine cells. This immunorreactive pattern in the inner retina of *S. canicula* at PH stages is slightly different from that reported in rat. After P1, DCX immunoreactivity decreased in the inner retina, not only in cell somas, as in sharks, but also in the IPL, wherein DCX immunoreactivity was hardly distinguished although it persisted until P15 (Lee et al., 2003). At this stage, DCX-ir cells in the inner retina were located in their definitive places and they did not show any migrating features. Therefore, in the retina of fishes, as in mammals, DCX is still present in cells after migration/cell positioning has ended. Despite the presence of DCX in adult tissues has been mostly related to adult neurogenesis (see **Introduction**), these cells in *S. canicula* correspond to mature neurons since they are located in areas far away from the neurogenic niches of the retina (see Ferreiro-Galve et al., 2010a). This suggests that DCX might have other roles in the adult different from neurogenesis. In this context, it would be useful to compare its expression with proliferation and differentiation markers in order to ascertain if DCX is exclusively found in neuroblasts or if it is present in mature cells (see **Chapter 2**). In addition, we have also shown DCX-ir cells in bipolar and horizontal cells in the INLo at PH stages and juvenile stages. DCX was also reported in adult horizontal cells in rat (Wakabayashi et al., 2008), but not in teleosts (Tozzini et al., 2012). Dendrite arborization and remodeling is a usual feature of mature horizontal cells in mammals (see Wakabayashi et al., 2008 and references therein), which could explain the presence of DCX in the retina of mammals. The presence of DCX in mature cells has been also described in other brain regions in mammals (see **Introduction**).

This fact could be related to MT reorganization that occurs in dynamic neuronal plasticity, axonal outgrowth or synaptogenesis (Geoghegan and Carter, 2008; Nacher et al., 2001; Vellema et al., 2014; Wakabayashi et al., 2008).

4.4. DCX in the CMZ

As discussed above, different levels of DCX were found in the peripheral retina, which allowed subdividing the CMZ in a peripheral region (CMZp) containing weakly DCX-ir cells and an adjacent region (CMZm) where DCX immunoreactivity resembles that described for the central retina of stage 29 embryos. These regions could correspond with the peripheral and middle CMZ defined in zebrafish (Raymond et al., 2006) where cell arrangement reflects the temporal sequence of retinogenesis (i.e., the CMZ-peripheral contain multipotent progenitors while the CMZ-middle contain proliferating but fate-restricted progenitors, see General Introduction).

5. Conclusions

In summary, the present work shows the distribution pattern of DCX protein in the developing and mature retina of an elasmobranch and provides information to extend our knowledge about the possible roles of DCX during neurogenesis in the CNS, using a laminated structure different from the cortex. Our results show DCX immunoreactivity in the neuroepithelial non-layered retina and in the CMZ throughout the lifespan of *S. canicula*, indicating that it is present in regions where new cells are continually added. DCX immunoreactivity is also present in cells and cell processes in the IPL and OPL at the time of new connections are established. DCX expression persisted in juveniles, and also in cells after migration has finished. This is the case of horizontal cells and bipolar cells,

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA USC

suggesting that DCX might have other roles different from neurogenesis, possibly related with neuronal plasticity and synaptogenesis in differentiated cells. Co-localization studies with proliferation and differentiation markers are necessary to address the possible involvement of DCX in the neurogenic process.

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USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

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Abbreviations

CE	ciliary epithelium
CMZ	ciliary marginal zone
CMZm	CMZ middle
CMZp	CMZ peripheral
DCX	doublecortin protein
dcx	*doublecortin gene*
GCL	ganglion cell layer
HCL	horizontal cell layer
IKNM	interkinetic nuclear migration
-ir	immunoreactive
INL	inner nuclear layer
INLi	inner region of the INL
INLo	outer region of the INL
IPL	inner plexiform layer
MAP	microtubule-associated protein
MT	microtubule
OFL	optic fiber layer
ONL	outer nuclear layer
OPL	outer plexiform layer
PH	prehatching
TZ	transition zone

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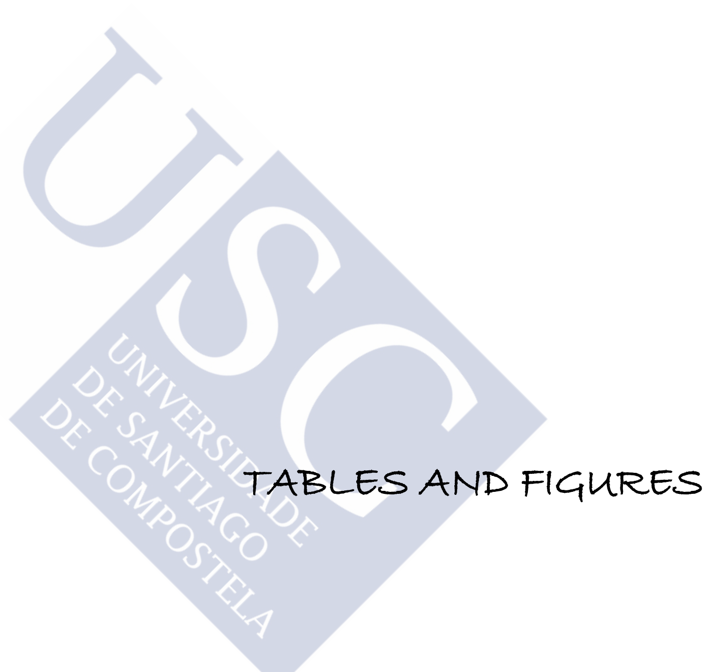

TABLES AND FIGURES

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Table 1. Primary and secondary antibodies used.

Primary antibody	Source	Working dilution
DCX	Polyclonal rabbit anti-DCX Cell Signaling Technology Catalog number: 4604S, Lot: 3	1:300

Secondary antibody	Source	Working dilution
GARb	Biotinilated goat anti-rabbit Dako Glostrup, Denmark Catalog number: E 0432	1:500
GAR-HRP	Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase Conjugate Bio-Rad Catalog number: 172-1019	1:200

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Figure 1. Eschematic representation of DCX-expressing cells according to cell differentiation sequence (A-C) and haematoxylin-eosin staining of a vertical section along the dorsoventral axis of the peripheral retina of a prehatching embryo of *S. canicula* (D). Representation of DCX immunoreactivity levels (red bar) in stem cells (Nestin-expressing cells; **A**), in migrating neuroblasts (Nestin-non-expressing cells; **B**), and in differentiated cells (**C**). (**D**) The CMZ is the most peripheral neuroepithelial region, adjacent to the ciliary marginal epithelium (CE). The transition zone (TZ) consists of an inner part wherein the IPL separates the GCL and the INLi, and an outer neuroepithelial part wherein the OPL is not observed. nl represents the neuroblastic non-layered area in the outer part of the TZ. Scale bar: 25 µm.

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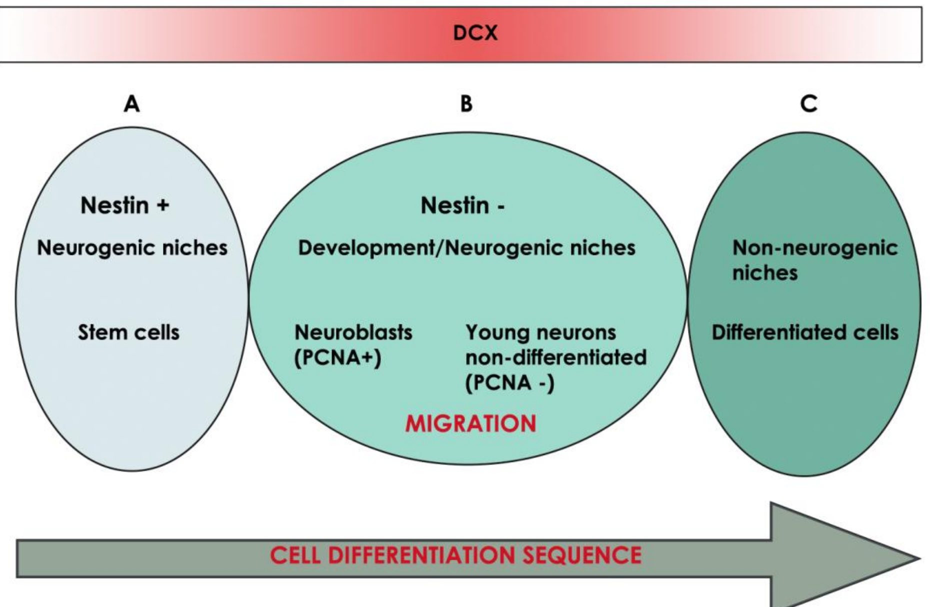

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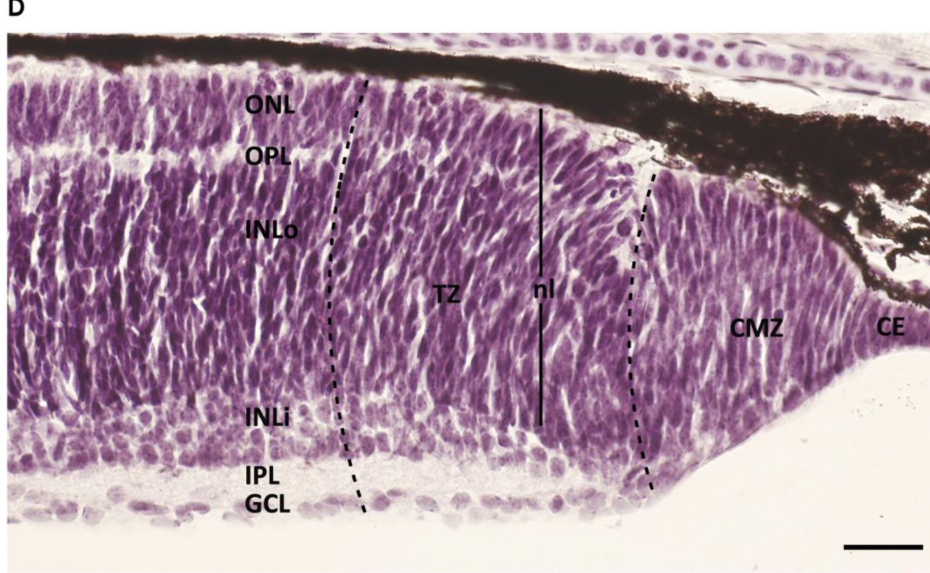

Figure 1

Figure 2. DCX immunoreactivity pattern in the retina of *S. canicula* during the *first* (A-C) and *second* (D-K) *developmental periods*. (A-C) Vertical (transversal) sections along the dorsoventral axis of the retina of *S. canicula* embryos from stages 27 to 29. (**A**) Stage 27 embryo showing the first detection of DCX immunoreactivity in the neuroepithelial retina in both vitreal and scleral surfaces. The **arrowhead** points to intense DCX immunoreactivity within encephalic walls. (**B**) DCX immunoreactivity is found in cells in the basal surface (**arrows**), and also in cell processes (**arrowheads**). (**C**) At stage 29, DCX immunoreactivity increases with respect to previous stages. **Arrows** point to DCX-ir cell bodies located at different levels of the neuroepithelium. (**D-F**) Vertical sections along the dorsoventral axis of the retina of stage 30 embryos. (**D**) Strong DCX-ir cells are observed in the innermost region of the central retina. (**E**) Strong DCX immunoreactivity is also observed in the ON head. (**F**) Detail of the central retina to show round-shaped DCX-ir cells in the prospective GCL (**black arrowhead**), and DCX-ir cells in the prospective INLi (**white arrowhead**), and axons and processes in the OFL (**asterisk**) and IPL. DCX-ir cell bodies are also observed in the outer non-layered retina. (**G-K**) Vertical sections along the dorsoventral axis of stage 32 embryos. (**G**) DCX-ir cells are arranged in a band at the primordial HCL (**arrows**). (**H**) Note that DCX immunoreactivity is fairly absent in the most peripheral non layered retina. (**I**) The density of DCX-ir processes notably increases in the IPL (asterisk). (**I'-I'''**) Round-shaped DCX-ir somas are shown in the INLi (**arrows, I'**), in the HCL (**I''**) and in the GCL (**arrow, I'''**). (**J**) Faint DCX immunoreactivity is observed in the most peripheral non layered retina at late stage 32 (**asterisk**), but some DCX-ir cells and processes are found in the adjacent non-layered retina and in the TZ (**arrowhead**). (**K**) Strong DCX immunoreactivity is observed in the ON. Scale bars: 25 µm in B, C, E, F, I', I'', I''', J; 50 µm in G, H, I; 100 µm in A, D, K.

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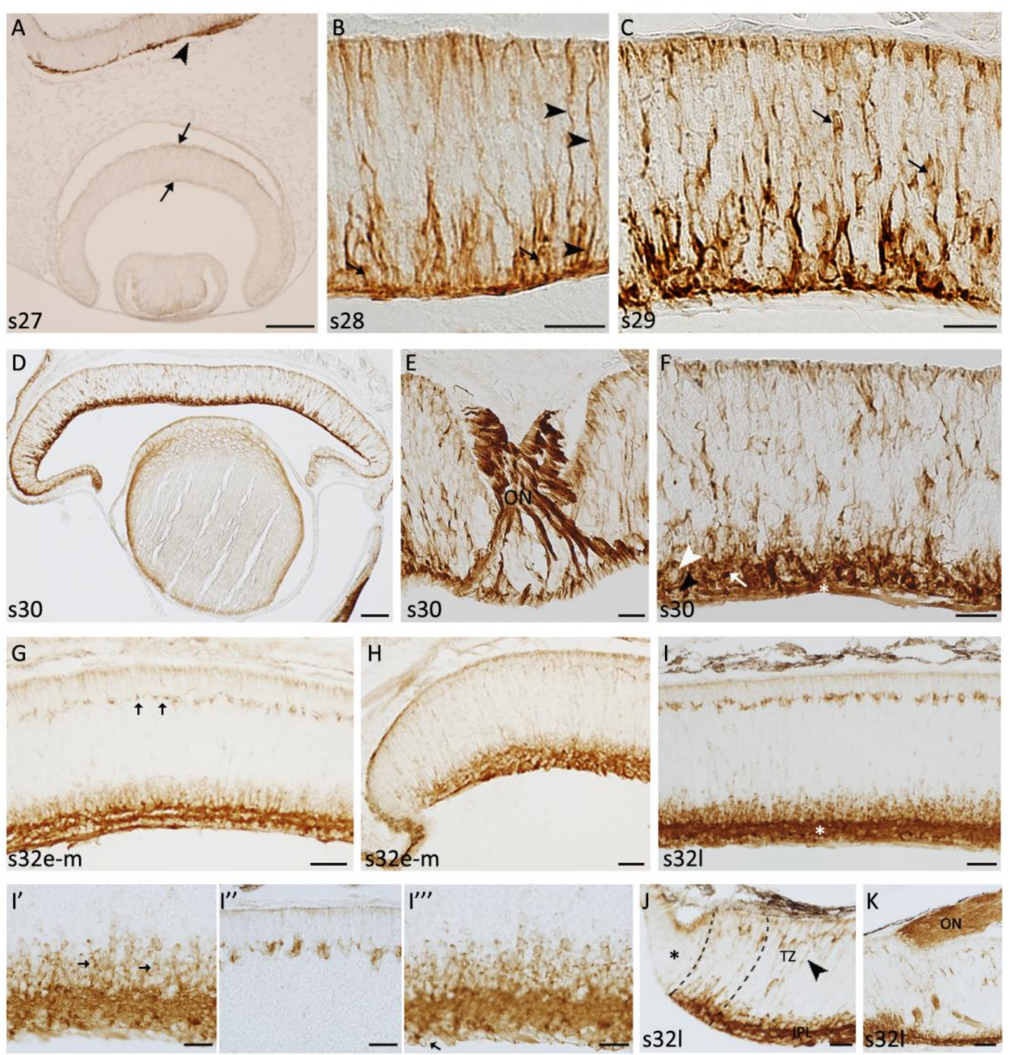

Figure 2

Figure 3. Spatiotemporal pattern of DCX immunoreactivity in the mature retina of *S. canicula* of PH embryos (A-C) and juveniles (D-F). (A-C) Vertical sections along the dorsoventral axis of the retina of PH embryos. (**A**) Strong DCX immunoreactivity is observed in processes in the IPL and in amacrine cells in the INLi (**arrows**). DCX immunoreactivity is found also found in cell somas and processes in the INLo. Asterisk indicates a Landolt's club from a bipolar cell. The arrowhead indicates cell processes directed towards the IPL. (**B**) Detail of a DCX-ir bipolar cell showing a thin process (Landolt's club) towards the outer limiting membrane (**asterisk**). (**B'**) Detail of a DCX-ir bipolar cell placed in the INLo, with horizontal dendrites extending into the OPL. (**B''**) Detail of DCX-ir cell placed in the HCL (**arrow**). (**C**) DCX immunoreactivity is fairly absent in the most peripheral CMZ (CMZp). Adjacent to it, DCX immunoreactivity is similar that shown in the central retina at previous developmental stages (see text for details). (**D-F**) Vertical sections along the dorsoventral axis of the retina of juveniles. (**D**) Central retina. Several types of DCX-ir cells are observed: large and rounded ganglion cells in the GCL (**arrowhead**), amacrine cells in the INLi (**arrows**) and bipolar cells and processes in the INLo (**curved arrows**). Some of bipolar cells show a Landolt's club (**asterisks**). DCX-ir horizontal cells and processes are seen in the HCL (**open arrowheads**). (**E**) Detail of an amacrine cell showing strong DCX immunoreactivity (**arrow**). Note the strong DCX-ir processes in the IPL. (**E'**) Detail of a DCX-ir bipolar cell showing a Landolt's club (**asterisk**) and a process directed towards the IPL (**curved arrow**). (**F**) In the TZ the organization of DCX-ir cells and processes is similar to that of the central retina at earlier stages. Arrowheads point to amacrine cells in the INLi. Scale bar: 10 µm in E; 25 µm in A, C, D, E'; 50 µm in B, B', B'', F.

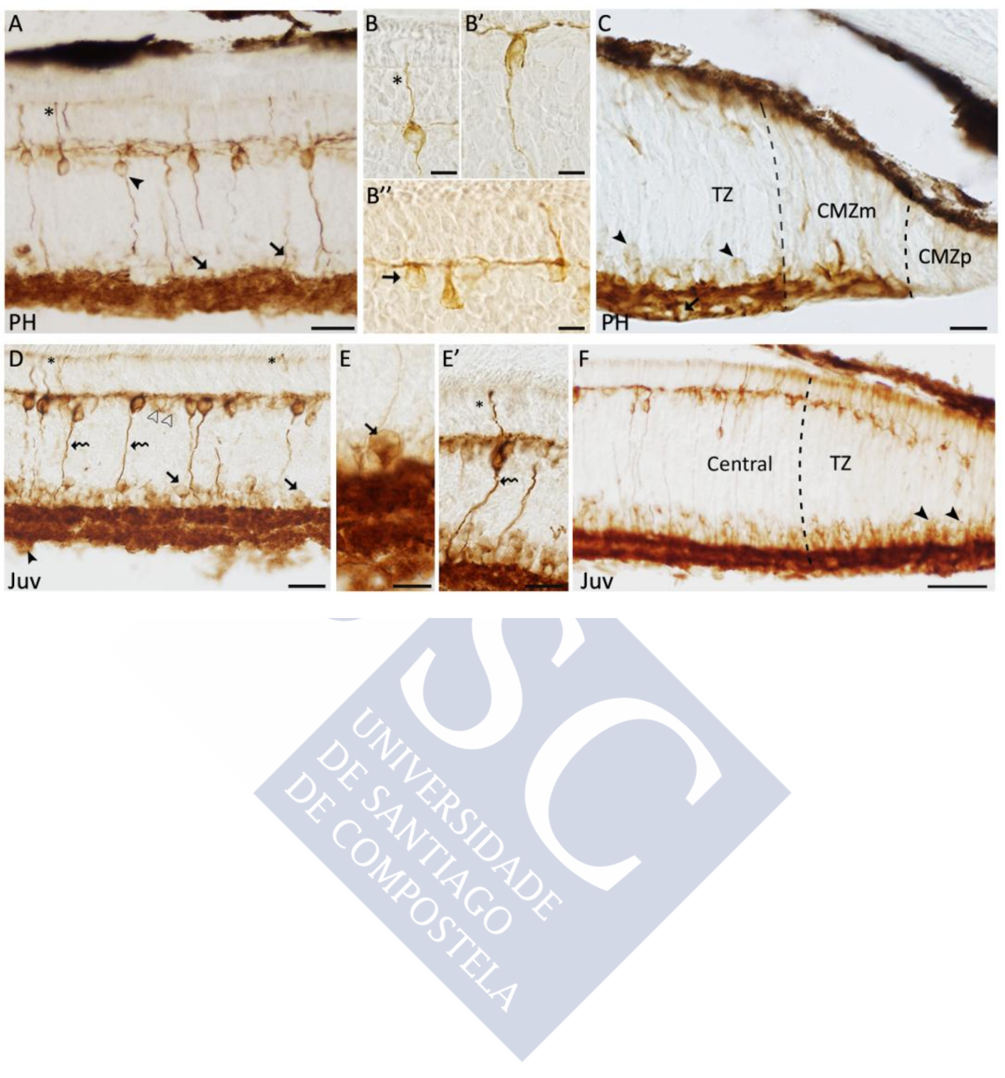

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Figure 3

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CHAPTER 2

Doublecortin and neurogenesis in the developing and mature retina of sharks: an immunohistochemical comparative study with proliferation and differentiation markers

Results of the present work are published in Sánchez-Farías, N., Candal, E., 2015. Doublecortin is widely expressed in the developing and adult retina of sharks. *Exp. Eye. Res.* 134, 90-100.

DOUBLECORTIN AND NEUROGENESIS IN THE DEVELOPING AND MATURE RETINA OF SHARKS: AN IMMUNOHISTOCHEMICAL COMPARATIVE STUDY WITH PROLIFERATION AND DIFFERENTIATION MARKERS

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1. Introduction

Doublecortin (*dcx*) is a nervous system-specific gene first described in 1998 (Gleeson et al., 1998) that is mutated in human X-linked lissencephaly and double cortex syndrome, two neurodevelopmental disorders associated with the abnormal migration of cerebral cortical neurons from the ventricular zone to the cortical plate (Feng and Walsh, 2001; Gleeson et al., 1998; Sossey-Alaoui et al., 1998). The *dcx* gene encodes a 40 kDa protein DCX, currently known to belong to a super-family of microtubule associated proteins (MAPs) defined by sequence homology and the presence of a conserved microtubule-binding domain (Francis et al., 1999; Gleeson et al., 1998; Horesh et al., 1999; Reiner et al., 2006).

DCX was shown to regulate the migration of multiple classes of cortical neurons by nucleation and stabilization of microtubules at the distal ends of neuronal processes (Friocourt et al., 2003, 2007; Geoghegan

and Carter, 2008; Moores et al., 2004, 2006; Reiner et al., 2004; Weimer and Anton, 2006) and thus it is frequently used as a marker of neuronal migration (Brown et al., 2003; Couillard-Despres et al., 2005; Des Portes et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Paolone et al., 2014; von Bohlen und Halbach, 2007, 2011).

DCX has been shown to be also required to maintain the progenitor pool during neurogenesis (Cameron and McKay, 2001; Pramparo et al., 2010; Rao and Shetty, 2004). Few immunohistochemical studies have shown the expression of DCX in various regions of the developing nervous system of mouse (e.g. Gleeson et al., 1999; Lee et al., 2003) and in the chick retina (Kim and Sun, 2012). During development, DCX is not present in Nestin-expressing neural stem cells in normal tissues, but it is widely expressed in neuronal-determined progenitors and young neuronal cells (Horesh et al., 1999; Saaltink et al., 2012; von Bohlen Und Halbach, 2007) and becomes down-regulated during neuronal maturation, concurring with the decrease of neurogenesis (Brown et al., 2003; Couillard-Despres et al., 2005). Indeed, in the juvenile and adult brain of mammals, the levels of DCX expression become extremely reduced compared to embryos (Brown et al., 2003; Couillard-Despres et al., 2005; Kuhn et al., 1996; Rao and Shetty, 2004) and DCX become mostly restricted to neurogenic niches where new neurons are continuously generated (Brown et al., 2003; Geoghegan and Carter, 2008; Nacher et al., 2001), which has endorsed this protein as a general indirect tool to explore adult neurogenesis (Brown et al., 2003; von Bohlen und Halbach, 2011).

However, several exceptions were recently reported as DCX has been found in Nestin-expressing stem cells in the retina of mammals after neurotoxic-induced dedifferentiation of astrocytes and Müller glial cells (Chang et al., 2007), in some morphologically mature (non-proliferating) neurons in the adult brain of rodents and canaries (Geoghegan and

Carter, 2008; Nacher et al., 2001)and in horizontal cells of the adult rat retina (Wakabayashi et al., 2008). In mature cells, DCX has been suggested to play a role in microtubule reorganization and synaptogenesis (e.g. Nacher et al., 2001).

The retina of fishes offers an exceptional model to extend our knowledge on the possible role of DCX in promoting neurogenesis, migration or synaptogenesis due to several reasons: (1) it has an active neurogenic niche, the ciliary marginal zone (CMZ), which consists of a peripheral ring of proliferating cells and persist throughout the entire life of the animal; (2) retinogenesis involves the generation of a layered structure that contains a limited number of cell types located in three nuclear layers, with synapses occurring in two plexiform layers; (3) retinogenesis occurs under the control of factors that coordinate cell cycle, cell differentiation and migration to layers in a highly orchestrated way. In particular, the retina of elasmobranch fishes offers several advantages to study neurogenesis (Bejarano-Escobar et al., 2012; Coolen et al., 2009; Ferreiro-Galve et al., 2008, 2010a, 2010b), because of the existence of a protracted period of scattered cell proliferation in mature areas and owing to the continuous presence of a transition zone with high proliferative activity neighboring the CMZ. To our knowledge, DCX expression has been only explored in the developing neural retina of rat (Lee et al., 2003) and chick (Kim and Sun, 2012), in the adult neural retina of rat (Wakabayashi et al., 2008) and the teleost *Nothobranchius furzeri* (Terzibasi Tozzini et al., 2012), and in rat retinal pigment epithelium (RPE) cells in vitro (Engelhardt et al., 2005). However, the expression and localization of DCX in the developing retina of fishes has not been reported so far. We showed previously the distribution pattern of DCX protein throughout retinal development of the small shark *Scyliorhinus canicula,* a representative of the largest order of elasmobranches, from the early stage 27 to juveniles by using simple indirect immunohistochemistry (**Chapter 1**). In this chapter, we additionally

investigated DCX expression pattern in the adult retina of *S. canicula*. We also tested for a relationship between DCX expression and the neurogenic state of DCX-labeled cells by exploring its co-localization with proliferation markers (proliferating cell nuclear antigen; PCNA) and the neuronal differentiation marker HuC/D. The relationship between DCX expression and cell migration was explored by comparing its expression with that of the glial marker GFAP (glial fibrillary acidic protein), as radially migrating neurons use radial glial fibers as substrate (Marín et al., 2010). We additionally characterized DCX-expressing cells in mature areas of the retina by double immunocytochemistry using antibodies against Calbindin (CB), a marker for bipolar and horizontal cells in the retina of *S. canicula* (Bejarano-Escobar, 2012) and Pax6, which has been proposed as a regulator of cell proliferation, cell differentiation, and neuron diversification in the neural retina of this species (Ferreiro-Galve et al., 2012).

2. Material and methods

The following embryonic stages were analyzed: stage 19 (1), stage 20 (1), stage 25 (3), stage 26 (2), stage 27 (2), stage 28 (3), stage 29 (6), stage 30 (7), stage 31 (8), stage 32 (8), and PH (8). Moreover, 5 juveniles (107-117 mm) and one adult (about 400 mm in total length), were also used.

For more information about experimental animals, tissue preparation, immunohistochemistry, specificity of the antibodies used, and image acquisition and analysis, **see Section 3** of this thesis: **Material and methods.**

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Primary and secondary antibodies used were summarized in **Tables 1** and **2** respectively.

To confirm the specificity of the anti-DCX antibodies used in this study, we double-labeled sections of retina with the rabbit and goat polyclonal antibodies. The staining pattern of both antibodies was coincident (**see Fig. 1**).

3. Results

We have previously described three developmental periods as regards the growth pattern of the retina of *S. canicula* (Ferreiro-Galve et al., 2010a; **see also Chapter 1**), which have served as a useful framework for comparison with the retinal development in other vertebrates.

We have analyzed the pattern of DCX immunoreactivity in the retina of *S. canicula* throughout these developmental periods. The two polyclonal anti-DCX antibodies (raised in rabbit and goat; **see Material and methods**) revealed a similar pattern of distribution of DCX-immunorreactive (-ir) cells within the retina of *S. canicula* (**Fig. 1**) and, therefore, the following description applies to both.

3.1. *First developmental period* (stages 26 to 29). Neuroepithelial non-layered retina

During the *first developmental period* (stages 26-29), the retina showed a neuroepithelial appearance characterized by the arrangement of cells in radial columns and by the absence of any regional differentiation or layering (**Fig. 2A**), though mitosis were restricted

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to the apical (ventricular) surface (**arrowheads in Fig. 2A**). During early stage 27, neuroepithelial cells (NECs) display apico-basal polarity, with apical and basal processes that span the neuroepithelium. Very weak DCX-immunoreactivity was observed in the cytoplasm of NECs, including their apical (ventricular) and basal (vitreal) end feet (**arrowheads in Fig. 2B, C, C'**). Of note, DCX-immunoreactivity in retinal neuroblasts was, by far, much less intense than that observed in other encephalic regions during the same developmental period (**arrow in Fig. 2B, C, C'**). At this stage, interphase NECs in the retina were immunoreactive for the proliferation marker PCNA (**Fig. 2B, C, C''**). Of note, co-localization between both markers was not evident since PCNA is a nuclear marker and DCX is usually found within the cytoplasm. At the end of this period (stage 29) the thickness of the neuroepithelium had notably increased, though regional differentiation or layering in the retina were still not apparent in haematoxylin-stained sections (**Fig. 2D**). However, in the central-most part of the retina, differences were observed in the vitro-scleral axis, as DCX immunoreactivity was mostly located in the innermost (basal) part of the neuroblastic layer, where some round-shaped and spindle-shaped cell bodies were observed (**arrows in Fig. 2E, E'**). In addition, faint DCX-ir somas were observed in outward positions (**open arrows in Fig. 2E, E'**). Radial DCX-ir processes were also observed coursing toward the inner and outer regions of the neuroepithelium (**arrowheads in Fig. 2E, E'**). Intense DCX-ir cells, both in the inner retina and outwards in the neuroepithelium, were immunonegative to the proliferation marker PCNA (**arrows in Fig. 2E, E''**). Some of these postmitotic DCX-ir cells were immunoreactive to the neuron marker HuC/D (**arrows in Fig. 2F-F''**). In the peripheral retina, the pattern of DCX immunoreactivity was similar to that observed throughout the retina at previous developmental stages (**Fig. 2G, H**).

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3.2. *Second developmental period*. Progressive layering of the retina

The *second developmental period* (stages 30-32) is recognizable by the progressive organization of cell and plexiform layers in the central part of the retina, by the organization of a wedge-shaped neuroepithelial zone adjacent to the ciliary epithelium (the ciliary marginal zone; CMZ) and by the presence of a TZ between the not layered CMZ and the layered central retina. Of note, the CMZ can be subdivided in a peripheral region (peripheral CMZ; CMZp) containing weakly DCX-ir cells and an adjacent region (middle CMZ; CMZm) where DCX immunoreactivity resembles that described for the central retina of stage 29 embryos (**see Chapter 1**). At stage 30, the inner part of the central retina becomes layered by the presence of the optic fiber layer (OFL) and the inner plexiform layer (IPL). The OFL contains axons from ganglion cells and the IPL separates the ganglion cell layer (GCL, mainly formed by the nuclei of ganglion cells) from the inner part of the inner nuclear layer (INLi, which mostly contains the nuclei of amacrine cells). In the most central part of the retina, DCX-ir ganglion cell axons were seen in the OFL (**arrowheads in Fig. 3A**) and DCX-ir processes from amacrine and ganglion cells became organized within the developing IPL (**arrow in Fig. 3A**). DCX-ir cell bodies were found in the GCL and in the INLi. A layered organization was hardly recognizable in the outer part of the retina, where DCX-ir cell bodies and their radial processes (**open arrowheads in Fig. 3A, A'**) were observed spanning the neuroepithelium. However, most of DCX-ir cell bodies began to appear aligned in the outer third of the retina (compare with **Fig. 2E**). As in stage 29 embryos, DCX-ir cells were postmitotic (PCNA-negative; **Fig. 3A, A''**). DCX immunoreactivity extended to the peripheral retina and bordered the CMZp (**Fig. 3B, B'**). A similar organization was observed in the retina bordering the optic nerve head (**Fig. 3C**). At stage 31 (**Fig. 3D, D'**), in the inner part of the central retina, DCX-ir axons and processes were observed in the OFL and IPL, respectively. Faint round-shaped DCX-ir cells were observed in the GCL, while faint DCX-ir ovoid

perikarya were found in INLi. Intense DCX-ir somas were also found in the outer non-layered retina, which became clearly arranged in a band in the outer one-third. Of note, radial DCX-ir processes from these cells became scarce (**open arrowhead in Fig. 3D**) and DCX-ir processes mostly become horizontally oriented. The organization of DCX in the TZ (**Fig. 3E**) was the same found in the central retina at stage 30 (i.e., DCX-ir cells were found in the GCL and INLi, and DCX-ir cells with radial processes spanned the outer non-layered retina; compare with **Fig. 3A**). In early 32 stage embryos, retinal layering was completed in the central retina with the appearance of a conspicuous OPL between the outer part of the INL (INLo) and the outer nuclear layer (ONL), which showed very reduced proliferation (**open arrow in Fig. 3F**). DCX-ir cells were found in the GCL, INLi and in a single layer of cells in the INLo bordering the OPL. These cells were either round-shaped (**arrows in Fig. 3F**) or triangular in shape (**arrowheads in Fig. 3F**), with processes mostly directed to the OPL. Towards the periphery, the OPL became less conspicuous and the prospective ONL still presented high numbers of proliferating cells (**open arrows in Fig. 3G**). DCX-ir cell bodies with radial processes were additionally found at intermediate positions (**small arrows in Fig. 3G, H**). The presence of these cells in the TZ fairly coincided with the region where the outer retina was not yet layered (**Fig. 3G, H**).

3.3. *Third developmental period*. Layering extended from central to peripheral retina

During the *third developmental period*, layering progresses to almost the entire circumference of the retina and displays the mature organization characteristic of postembryonic stages (**Fig. 4**). In the inner part of the central retina the pattern of DCX immunoreactivity was similar to that found at stage 32. In the outer retina, different types of DCX-ir cells were found in the vicinity of the OPL. Round or ovoid horizontal cells with

cell processes horizontally oriented within the OPL were located in the outermost part of the INLo (**open arrowheads in Fig. 4A, A'**). Ovoid DCX-ir bipolar cells presenting an axon oriented radially towards the IPL and highly branched dendrites into the OPL were also observed (**arrows in Fig. 4A, 4A''**). DCX immunoreactivity was also observed in spindle-shaped bipolar cells that showed their axon oriented to the inner retina and a thick process (known as Landolt's club) directed to the outer limiting membrane (**asterisks in Fig. 4A, A'''**). Radial DCX-ir cell processes from bipolar cells were closely associated with GFAP-ir glial cell processes (**Fig. 4A**). DCX-ir cells in the GCL, INLi and INLo were postmitotic (PCNA-negative) both in the central (**Fig. 4A**) and the peripheral (**Fig. 4B**) retina. The same pattern was maintained in juvenile specimens (**Fig. 4C-E**). Most DCX-ir cells in the GCL and INLi were also immunoreactive to HuC/D (**Fig. 4C, C'**) and to Pax6 (**Fig. 4D**), while most bipolar cells in the INLo were also immunoreactive to CB (**Fig. 4E, E'**). Of note, DCX immunoreactivity in these cells showed a perinuclear arrangement that highly reminds a microtubule cage (see **section 4.3**). Strong DCX immunoreactivity was also found in amacrine, bipolar and horizontal cells in the adult retina (**Fig. 4F**).

3.4. Retinal pigment epithelium

The RPE in the retina of *S. canicula*, consists in a monolayer of non-pigmented cells (**arrowheads in Figs. 5A, B**), located at the interface between the photoreceptor outer segments (**PS in Figs. 5A, B**) and the vascular meshwork of the choroidal layer (**arrow in Fig. 5B**). From vitreal to scleral, the eye is also composed of a tapetal layer (**T in Fig. 5B**), a layer of migratory pigment cells with processes extended among the tapetal plates (**white arrowhead in Fig. 5B**) and the choroid (**Ch in Fig. 5A**). In the marginal retina, the photoreceptor outer segments are short and the retinal pigment cells lie close to the ONL (**arrowheads in Fig. 5C**).

Interestingly, a small number of DCX-ir cells were observed in the retinal pigment epithelium of late embryos (**arrowheads in Fig. 5D, E**) and juveniles (**arrowhead in Fig. 5F**). Most of these cells showed epithelial morphology (**Fig. 5D, F**), though round-shaped cells (**Fig. 5E**) were also observed in this cell layer.

4. Discussion

In spite of the essential role of DCX in neurogenesis and cell migration in the developing and adult nervous system the role of DCX in retinogenesis has received little attention (see **Introduction**). We have investigated the temporal and spatial distribution of DCX-labelled cells in the developing and juvenile retina of *S. canicula* by means of immunohistochemistry (see **Fig. 6**).

4.1. DCX was first detected in the end feet of proliferating cells of the neuroblastic retina

In *S. canicula*, faint DCX immunoreactivity was found surrounding the nuclei of NECs at a stage when virtually all cells in the retina were proliferating (PCNA-ir; **green cells in Fig. 6A**; see also Ferreiro-Galve et al., 2010a). The same pattern was observed in the CMZp, which is maintained as a proliferating pseudostratified epithelium throughout life (see for example, **Fig. 3E**). Therefore, DCX is present in proliferating NECs in the retina, a fact that was never described before. Though DCX expression has been usually reported to commence just following the final mitosis (see **Introduction**), it is not unexpected that low levels of DCX can be found in proliferating cells. In fact, DCX-positive cell populations, isolated

by flow cytometry from developing and adult brains of mouse, contain not only highly DCX-expressing cells restricted to the neuronal lineage but also a sizeable population of low DCX-expressing precursors that retain their multipotentiality (Walker et al., 2007). Interestingly, these two types of cells could be differentiated from each other on the basis of DCX expression levels, regardless of developmental stage or brain region (Walker et al., 2007).

In *S. canicula*, interphase elongated nuclei span the neuroepithelium while round mitotic nuclei were only found at the apical surface. This fact that was first described in the neural tube by Sauer (1935), who proposed that pseudostratification was enabled by apical–basal nuclear movements in phase with the cell cycle, a process that was termed interkinetic nuclear migration (IKNM). It has been reported that nuclei travel within a microtubule 'cage' via microtubule associated motor proteins (see Lee and Norden, 2013), but the role of the microtubule binding-protein DCX in IKNM has not been studied so far. An oriented microtubule cage has been also described surrounding the nuclei of retinal NECs in the retina of zebrafish (Norden et al., 2009), but comparison of IKNM in various epithelia from different species reveals that cytoskeletal components underlying IKNM vary depending on model organisms or tissues (nicely reviewed in Lee and Norden, 2013). In this context, further studies are needed to decipher the significance of DCX expression in the pseudostratified CMZp of sharks.

4.2. DCX immunoreactivity during cell cycle withdrawal

In *S. canicula*, DCX immunoreactivity increased at stage 29, before the occurrence of any regional differentiation or layering. The spatial pattern of DCX-immunoreactivity from stage 29 onwards was highly coincident with that of cell cycle withdrawal (Ferreiro-Galve et al., 2010a)

since strong DCX immunoreactivity was observed in early postmitotic (PCNA-negative) cells. It is well known that cell processes retract as the soma reaches the appropriate laminar position and cell differentiates (Baye and Link, 2008), which could account for the short processes observed in cells that co-labelled with the young neuronal marker HuC/D in the inner retina of *S. canicula* (see **Fig. 2F** and **6B**). The spatiotemporal pattern of DCX immunoreactivity was similar to that described in the developing retina of chick (Kim and Sun, 2012), where DCX was first described across most cells in the non-layered retina. In chick, DCX immunoreactivity was also described in the prospective GCL of the developing retina. A similar spatiotemporal pattern of DCX immunoreactivity has been reported in the developing retina of rat (Lee et al., 2003), where DCX-immunoreactivity was first described at E15 in a few neuroblasts in the innermost part of the neuroblastic (non-layered) retina. DCX in these cells also co-labeled with anti-b-tubulin, a marker of neuronal cells that is present in migrating and differentiating neurons.

In *S. canicula*, DCX-ir cells with spanning processes were additionally observed in the CMZm throughout the entire life of the animal (spatial axis in **Fig. 6B**), in a region where cells become postmitotic and begin their differentiation. In fishes, the CMZ corresponds to a neurogenic region arranged in concentric rings of gradually increased commitment, i.e., NSCs are located in the most peripheral zone (CMZp), nearby the CE, while progressively more fate-restricted progenitors are successively located toward the central (differentiated) retina (see **General Introduction**). DCX expression has been also described in a row of peripheral cells bordering the CMZ in adults of the teleost *Nothobranchius furzeri*, a short-lived annual fish (Terzibasi Tozzini et al., 2012).

Therefore, the increase of DCX expression was coincident with early stages of differentiation of a subpopulation of neurons, which

indicates that DCX in the neuroblastic retina is a marker for developing neuronal precursors.

It is noteworthy that, once the retina begins layering, DCX immunoreactivity was still observed in cells in the outer non-layered retina of the TZ (**yellow cells in Fig. 6C**), which indicates that DCX immunoreactivity is localized in neuron precursors before they become definitely positioned. The same pattern was described in rat (Lee et al., 2003) where DCX immunoreactivity was observed first in cells that formed a single row in the outer one-third of the retina with bipolar processes that spanned the neuroblastic layer, and then in multipolar cells with three to four branched processes located at the outer part of the INLo. DCX become then localized in round- or triangular-shaped cells with processes mostly directed to the OPL (see **section 4.3**). Because these cells expressed CB (a good marker of horizontal cells in rat), it has been suggested that DCX might be involved in migration of horizontal cell precursors (Lee et al., 2003). Of note, radially-migrating DCX-expressing cells have been previously found in the developing and adult brain of mouse (Des Portes et al., 1998; Gleeson et al., 1999; Nacher et al., 2001).

Taken together, our results are compatible with previous findings suggesting that DCX labels neuronal-determined and/or young migrating neurons (Lee et al., 2003).

4.3. DCX immunoreactivity in mature cells

In the mature retina, the OFL and IPL are patent and DCX-immunoreactivity was clearly observed in cells in the GCL, INLi and INLo (**orange cells in Fig. 6C**). Based on their morphology and location, round-shaped cells in the GCL were interpreted as ganglion cells while oval-shaped cells in the INLi that projected to the IPL were identified as

amacrine cells. Round or ovoid cells with cell processes horizontally oriented within the OPL were interpreted as horizontal cells while cells that colocalize with CB and showed one axon oriented to the inner retina and one or two dendrites directed either to the OPL or to the outer limiting membrane were interpreted as bipolar cells. DCX immunoreactivity appears to be present in these cells after nuclear/cell positioning processes have ceased, i.e, after these cells become positioned within their corresponding layer and acquired a mature morphology.

A similar pattern has been described in the inner retina of the developing retina of chick (Kim and Sun, 2012) and mammals (Gleeson et al., 1999; Lee et al., 2003). Interestingly, differences are observed in regard to the life-span of DCX-expression. In rat, DCX is transiently expressed in the inner retina and becomes progressively downregulated from P1 to P15 (Lee et al., 2003), with residual expression at P21, while in *S. canicula*, this immunoreactivity pattern remained until juveniles and adult stages (**Fig. 6D, E**). Despite the presence of DCX in adult tissues has been mostly related to adult neurogenesis (**see Introduction**), these cells in *S. canicula* correspond to mature neurons since they are located in areas far away from the neurogenic niches of the retina (see Ferreiro-Galve et al., 2010a) and they colocalize with HuC/D and Pax6 (which is located in postmitotic cells in the GCL and INLi of juvenile specimens; Ferreiro-Galve et al., 2012). It has been previously suggested that DCX expression in differentiated neurons could be related to microtubule reorganization linked to dynamic neuronal plasticity, axonal outgrowth or synaptogenesis (Geoghegan and Carter, 2008; Nacher et al., 2001; Vellema et al., 2014; Wakabayashi et al., 2008). Indeed, in the retina of mouse, DCX appears to be independent from migration and has been related to cell differentiation and microtubule remodeling during neurite formation and synaptogenesis in the IPL (Lee et al., 2003). As the retina of fishes expand actively throughout the life of the animal by addition of new cells, the connectional pattern among cells must change accordingly to accommodate new neurons.

Consequently, the possibility should be considered that DCX-mediated processes of synaptogenesis or synapse remodeling are required through life.

DCX has also been reported in horizontal cells in the developing retina of chick (Kim and Sun, 2012) and rat (Lee et al., 2003). Interestingly, DCX-immunoreactivity was also found in horizontal cells at juvenile and adult stages of *S. canicula* (present results) and in mature horizontal cells in the adult retina of rat (Wakabayashi et al., 2008), but not in the teleost *Nothobranchius furzeri* (Terzibasi Tozzini et al., 2012). It has been previously shown that dendrite arborization and remodeling is a usual feature of mature horizontal cells in mammals (see Wakabayashi et al., 2008 and references therein) and that phosphorylation of DCX facilitates neurite outgrowth (reviewed in Reiner et al., 2004). This could explain why DCX is present not only in the continuously growing retina of sharks (present results), but also in the retina of mammals.

On the other side, in *S. canicula*, most bipolar cells retract their apical process during maturation, but some mature bipolar cells maintain a thick cell process oriented to the outer limiting membrane known as Landolt's club (present work), which has previously been described in some CB-expressing bipolar cells in the retina of *S. canicula* (Bejarano-Escobar et al., 2012). In the retina of chick, retraction of apical processes seems to occur only after the dendritic arbors form, which had led to suggest that apical processes are important for the process of synaptogenesis (see Morgan et al., 2006). This could be the case in *S. canicula*, as synapse remodeling is likely to be required through life in the retina of sharks (see above). Alternatively, DCX could be involved in nuclei stabilization, as the cellular location of DCX-immunoreactivity in these cells is strongly reminiscent of the perinuclear microtubule cage described during nuclear migration and stabilization processes (e.g. Tanaka et al., 2004; Koizume and Gleeson, 2009). The possible significance

of DCX expression in these cells deserves further investigation. Of note, DCX-ir bipolar cells have not been described in the mature retina of teleosts, chick or rat (Kim and Sun, 2012; Terzibasi Tozzini et al., 2012; Wakabayashi et al., 2008), which suggest that mechanisms regulating synaptogenesis or nuclei stabilization may vary between species.

4.4. DCX in the RPE

The RPE is a highly specialized monolayered epithelium placed at the interface between the photoreceptors of the neural retina and the choroid layer, which shares its origin with the neural retina as an anterior neural plate derivative (e.g. Martínez-Morales et al., 2004). The embryonic RPE is capable of proliferation and transdifferentiation into a neural retina in many vertebrate species. Indeed, adult urodeles and post-hatched birds retain this capability in adult stages (Martínez-Morales et al., 2004; Wohl et al., 2012 and references therein). Interestingly, DCX has been also found in cell explants of adult rat RPE (Engelhardt et al., 2005), in cells that had neuronal morphology and expressed other markers of neuronal identity. It has been largely believed that regenerative potential in the adult retina of fishes was restricted to the CMZ and Müller cells, and that the RPE of fishes was unable to transdifferentiate into neurons (e.g. Wohl et al., 2012). In a previous work from our group we showed the presence of proliferating cells in the RPE of juvenile specimens of *S. canicula* (Ferreiro-Galve et al., 2010a). In this study we show that some cells in the RPE, which showed epithelial or round morphologies, were immunoreactive to DCX. While we cannot discard that round cells in the RPE correspond to displaced migratory pigment cells, recent studies that used neural progenitor culture conditions described morphological changes of RPE-derived cells from epithelial to spherical or spindle-like, a shape that is reminiscent of that of neural stem or progenitor cells (Haruta et al., 2004; Engelhardt et al., 2005 and references therein). Taken

together, these observations suggest that RPE cells in elasmobranch fishes have certain neuronal differentiation potential. Future studies are necessary to explore the ability of RPE cells as neuronal progenitors in the retina of elasmobranch fishes.

5. Conclusions

DCX immunoreactivity in the developing retina of elasmobranch fishes highly increases coinciding with the time that cells appeared to shift from proliferation to early differentiation. The pattern of expression of DCX in mature ganglion, amacrine, bipolar and horizontal cells of *S. canicula* indicates that DCX is not a specific marker for adult neurogenesis, and is compatible with previous suggested roles for DCX in nuclear stabilization or continuous neurite remodeling through late development and adulthood. Moreover DCX expression in adults could reflect a degree of neuronal plasticity that was not found in the adult retina of teleosts by means of DCX immunohistochemistry. DCX was also found in RPE cells, which suggests neuronal differentiation potential for RPE cells in elasmobranch fishes. The retina of fishes appears as a valuable model to further investigate the significance of DCX in promoting neurogenesis, migration or synaptogenesis.

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Abbreviations

CB	calbindin
Ch	choroid
CMZ	ciliary marginal zone
CMZm	middle CMZ
CMZp	peripheral CMZ
DCX	doublecortin
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
IKNM	interkinetic nuclear migration
INL	inner nuclear layer
INLi	inner part of the INL
INLo	outer part of the INL
IPL	inner plexiform layer
-ir	immunorreactive
MAPs	microtubule associated proteins
NECs	neuroepithelial cells
OFL	optic fiber layer
ONL	outer nuclear layer
PCNA	proliferating cell nuclear antigen
PH	prehatching
PS	photoreceptor outer segments
RPE	retinal pigment epithelium
T	tapetal layer
TZ	transition zone

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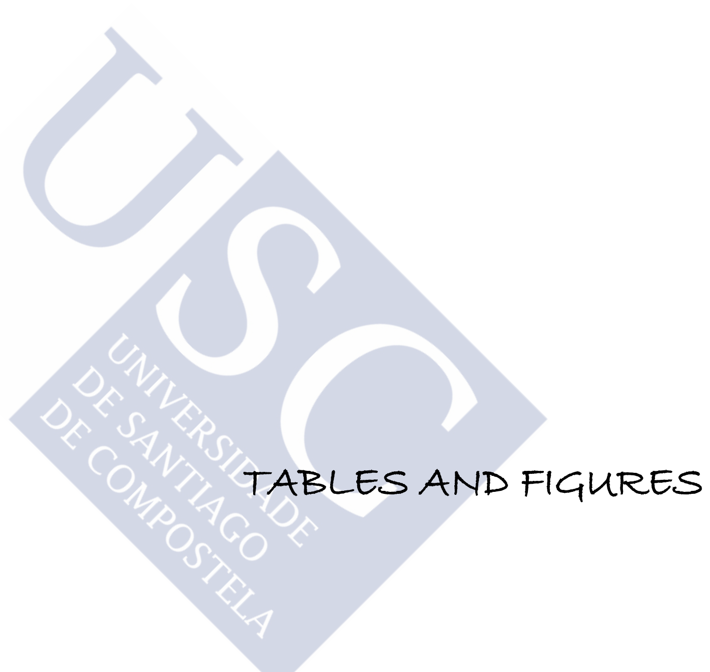

TABLES AND FIGURES

Table 1. Primary antibodies.

Primary antibody	Source	Working dilution
DCX	Polyclonal rabbit anti-DCX Cell Signaling Technology Catalog number: 4604S, Lot: 3	1:300
DCX	Polyclonal goat anti-DCX Santa Cruz Biotechnology Catalog number: sc-8066, Lot: C2513	1:100
PCNA	Monoclonal mouse anti-PCNA Sigma-Aldrich Catalog number: P8825, Lot: 082M4844	1:800
HuC/D	Monoclonal mouse anti-HuC/D Invitrogen, Molecular Probes Catalog number: A-21271, Lot: 833294	1:800
Pax6	Polyclonal rabbit anti-Pax6 Covance Catalog number: PRB-278P-100, Lot: E12CF00567	1:200
CB	Polyclonal rabbit anti-CB D-28k Swant Catalog number: CB-38a, Lot: 9.03	1:800
GFAP	Polyclonal rabbit anti-GFAP DakoCytomation Catalog number: Z 0334, Lot: 00082268	1:500

Table 2. Secondary antibodies.

Primary antibody	Source	Working dilution
546-conjugated donkey anti-rabbit (DAR^{546})	Molecular probes Catalog number: A10040	1:100
546-conjugated donkey anti-mouse (DAM^{546})	Molecular probes Catalog number: A10036	1:100
488-conjugated donkey anti-rabbit (DAR^{488})	Molecular probes Catalog number: A21206	1:100
488-conjugated donkey anti-goat (DAG^{488})	Molecular probes Catalog number: A11055	1:100
633-conjugated donkey anti-mouse (DAM^{633})	Molecular probes Catalog number: A31571	1:100

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Figure 1. Double immunofluorescence with two different anti-DCX polyclonal antibodies. Merged immunofluorescence image (**A**) of rabbit polyclonal anti-doublecortin (**B**) and goat polyclonal anti-doublecortin antibody immunofluorescence (**C**), to show co-localization of both antibodies in retinal cells of a juvenile specimen.

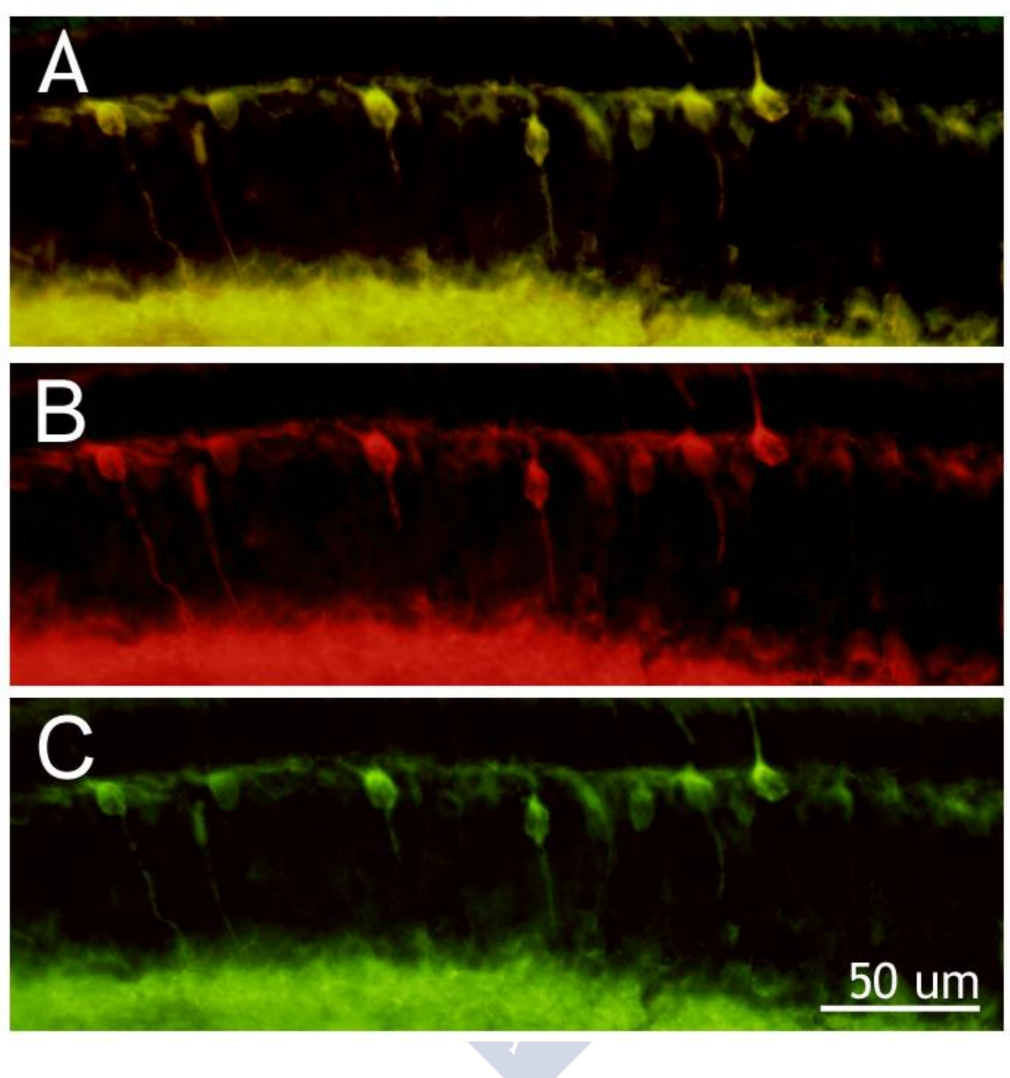

Figure 1

Figure 2. Comparison of DCX, PCNA and HuC/D immunoreactivities in neuroepithelial non-layered retina from stages 27 to 29. (**A-C**) Vertical (transverse) sections along the dorsoventral axis of the retina of stage 27 embryos. (**A**) Hematoxylin-eosin staining showing the neuroepithelial retina at stage 27. Arrows indicate round and mitotic cells in the outer retina. (**B**) Double DCX/PCNA immunofluorescence to show that most neuroepithelial cells are PCNA immunoreactive and show very weak DCX-immunoreactivity in their cytoplasm (**arrowheads**). The arrow points to intense DCX-ir processes in the brain. (**C-C''**) Detail of the region squared in B. (**D-H**) Vertical sections along the dorsoventral axis of a stage 29 retina. (**D**) Hematoxylin-eosin staining to show the increased thickness of the neuroepithelial retina. (**E-E''**) Double DCX/PCNA immunolabeling in sections of the central retina reveals strongly DCX-ir cells in the basal neuroblastic layer (NBL) that do not colocalize with PCNA (**arrows**). Weakly DCX-ir cells are found in the outer region (**open arrows**). Arrowheads indicate DCX-ir processes. (**F-F''**) Double DCX/HuC/D immunolabeling to show that some DCX-ir cells also express HuC/D (**arrows**). (**G, H**) Double DCX/PCNA (**G**) and DCX/HuC/D (**H**) to show that immunolabeling in peripheral sections of the retina is similar to that found in the central retina at previous developmental stages. Scale bars: 25µm in D, E; 50 µm in A, C, F-H; 100 µm in B.

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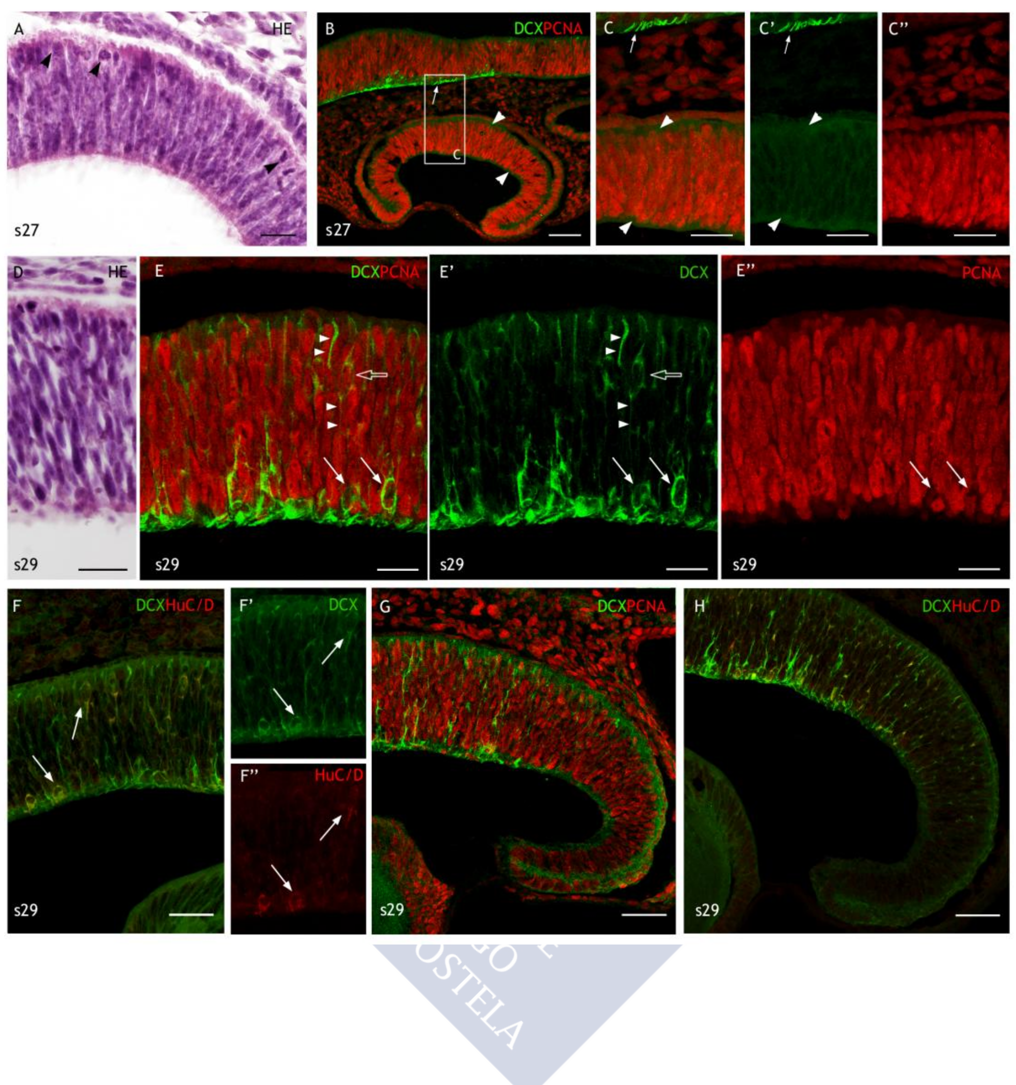

Figure 2

Figure 3. Patterns of DCX- and PCNA-immunoreactivity in the retina from stages 30 to 32. (**A-C**) Vertical sections along the dorsoventral axis of the retina of stage 30 embryos. (**A-A''**) Section through the central retina to show that DCX-ir cells are observed in PCNA-negative cells. DCX was observed in cell axons in the OFL (**arrowheads in A**), cell processes the IPL (**arrow in A**), and cell somas in the INLi and GCL. Open arrowheads in (**A, A'**) point to DCX-ir radial processes. (**B, B'**) Section through the peripheral retina to show that DCX immunoreactivity extended to the retina bordering the CMZp close to the ciliary epithelium (CE). (**C**) A similar organization is found in the retina bordering the optic nerve head. (**D-E**) Vertical sections along the dorsoventral axis of a stage 31 embryo. (**D, D'**) In the inner retina, DCX-ir cells are observed in the GCL and weakly DCX-ir cells are observed in the INLi. DCX-ir axons and processes are also observed in the OFL and IPL. In the outer retina, DCX-ir somas were mostly arranged in a single layer. Scarce radial DCX-ir processes were found (**open arrowhead**). (**E**) Detail of the peripheral retina (TZ, CMZm and CMZp), where the DCX immunoreactivity is reminiscent to that found in the central retina at previous stages. Open arrowhead points to radial processes while arrows point to the presence of weak DCX immunoreactivity in proliferating cells in the CMZp (**compare to Fig. 2 B, C**). (**F-H**) Vertical sections from the most central (**F**) to the most peripheral (**H**) retina of embryos at stage 32. Arrows in (**F**) point to DCX-ir round-shaped cells. Arrowheads in (**F**) point to DCX-ir triangular-shaped cells. Open arrows in (**F, G**) point to PCNA-ir cells while arrows in (**G**) point to DCX-ir radial cells and processes. Scale bars: 50 µm in A, C-H; 100 µm in B. For abbreviations, see list.

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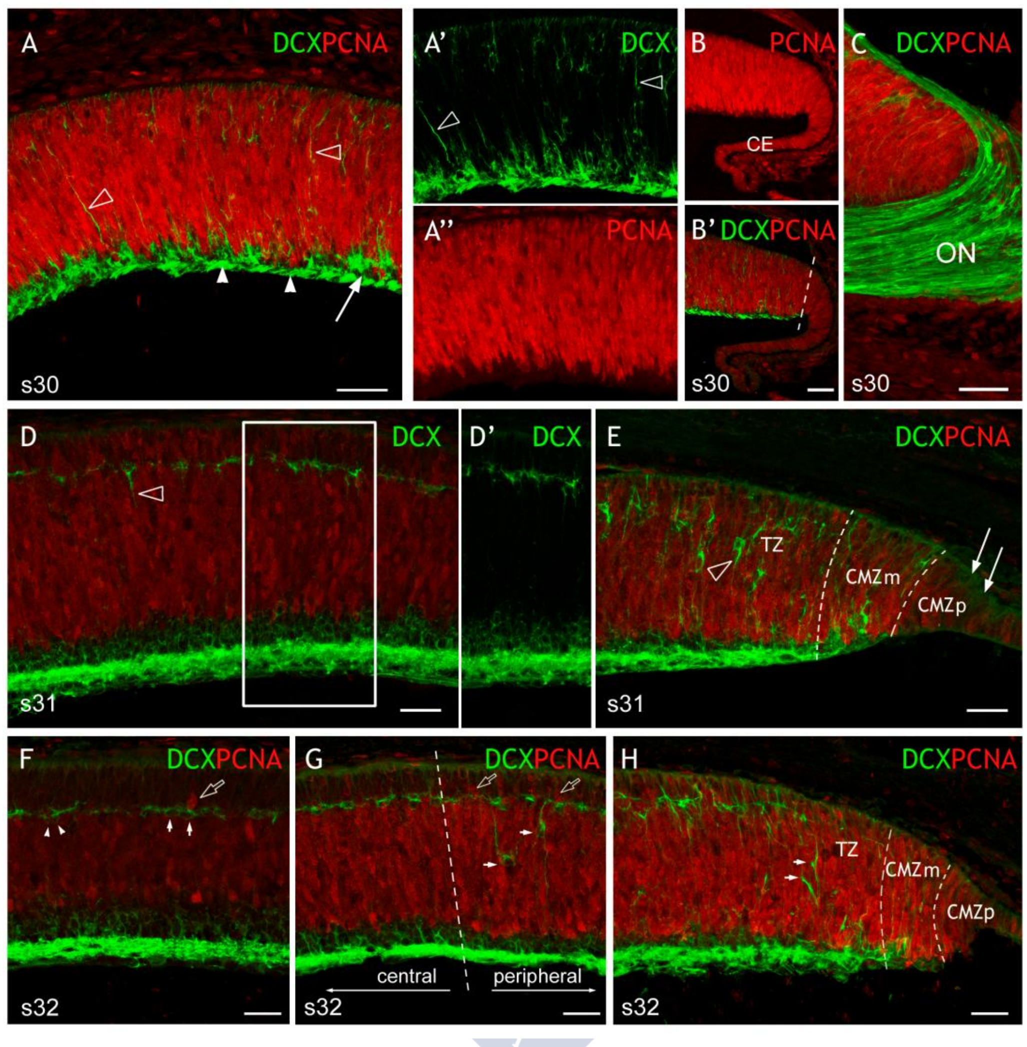

Figure 3

Figure 4. Comparison of spatiotemporal patterns of DCX, PCNA, GFAP, HuC/D, Pax6 and CB in the mature retina. (**A, B**) Vertical sections along the dorsoventral axis of the retina of prehatching embryos. (**A**) DCX immunoreactivity is present in the central retina in PCNA-negative areas. DCX-ir cells were observed in the GCL, INLi and INLo. Open arrowhead in (**A, A'**) points to round or ovoid horizontal DCX-ir cells with processes horizontally arranged within the OPL. Arrows in (**A, A''**) point to bipolar cells and their processes. Asterisk in (**A, A'''**) indicates a bipolar cell that had an apical directed process (Landolt's club). Note that DCX-ir is observed in cell processes closely associated along the radial glia (GFAP-ir). (**B**) Section to show the organization in the peripheral retina. DCX-ir cells are also postmitotic (PCNA-negative cells). (**B'**) The inset shows GFAP-ir processes. (**C-E**) Vertical sections along the dorsoventral axis of the retina of juveniles and adults. Arrows in (**C, C'**) indicate DCX-ir cells in the inner retina that were also HuC/D-ir. Arrows in (**D**) indicate strongly DCX-ir in the INLi that were also Pax6-ir. Arrows in (**E, E'**) indicate strongly DCX-ir bipolar and multipolar cells in the INLo that were also CB-ir. (**F**) Vertical section along the dorsoventral axis of the retina of an adult showing the important decrease in thickness of the retina. Note that strongly DCX immunoreactivity was also found in amacrine (**white arrowhead**), bipolar (**asterisk**) and horizontal (**open arrowhead**) cells in the retina of adult specimens. Scale bars: 25 µm in A, D, E; 50 µm in C, F; 100 µm in B.

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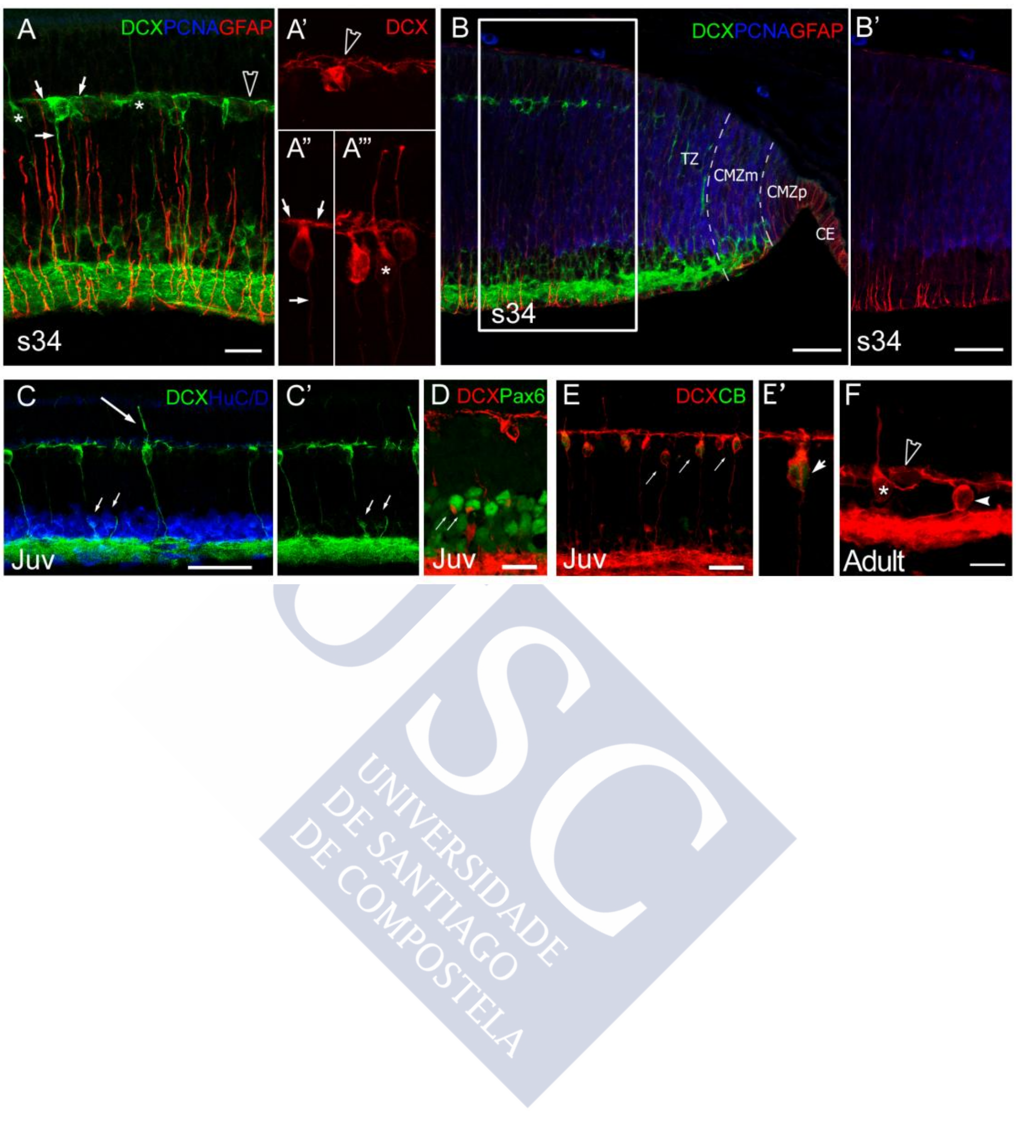

Figure 4

Figure 5. Structure and pattern of DCX immunoreactivity in the RPE at late development. (**A-C**) Hematoxylin-eosin staining of vertical sections along the dorsoventral axis of the central (**A, B**) and peripheral (**C**) retina of juvenile elasmobranch fishes to show the RPE. (**B**) Detail of the region squared in (**A**). Black arrowheads in (**A-C**) indicate cells with epithelial morphology. Arrow in (**B**) indicates an endothelial cell of the vascular meshwork. Note that the photoreceptor outer segments in peripheral sections (**C**) are shorter than in the central retina (**B**). (**D, E**) DCX immunoreactivity is present in a few cells in the RPE of prehatching embryos (**arrowheads**). Arrow in (**E**) indicates the Landolt's club of a bipolar cell. (**F**) DCX immunoreactivity in a juvenile specimen. Arrowhead point to DCX-ir cells in the RPE. Arrow in (**F**) indicates the Landolt's club of a bipolar cell. For abbreviations, see list. Scale bars: 25 µm in F; 50 µm in A; 100 µm in D, E.

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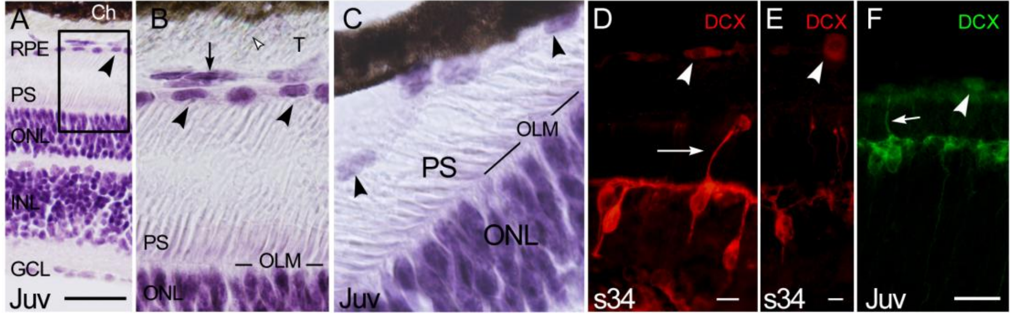

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Figure 5

Figure 6. Sequence of DCX immunoreactivity during the maturation of the retina. Neuroepithelial cells (NECs) are represented in green, postmitotic immature cells are represented in yellow and mature cells are indicated in orange. (**A**) DCX is faintly expressed in cells in the proliferating pseudostratified neuroepithelium at stage 27, as well as in the CMZp through life. (**B**) Following cell cycle exit, DCX is strongly expressed in prospective GCL cells and in elongated cells and their processes in the outer neuroblastic layer at stage 29; the same pattern is found in the peripheral retina bordering the CMZp (CMZm) through life. (**C**) As development progresses (stage 30) the IPL becomes clearly identifiable and DCX is found in ganglion and amacrine cells and their dendritic arborizations in the inner retina, a pattern that is maintained in the TZ up to adulthood. DCX is also found in cells than progressively shorten their processes and become localized in a single layer in the outer retina. (**D**) As the retina maturates (stage 32 onwards) DCX-ir cells in the outer retina extend their processes towards the IPL and the OPL. (**E**) DCX was maintained in mature ganglion, amacrine, bipolar and horizontal retinal cells in late embryos, juveniles and adults. DCX was also observed in a few cells in the RPE. Note that the peripheral-to-central pattern of DCX expression in the mature retina (up-line, in the schema) recapitulates the temporal sequence of DCX expression in the central retina through retinal maturation (botton-line, in the schema). For abbreviations, see list.

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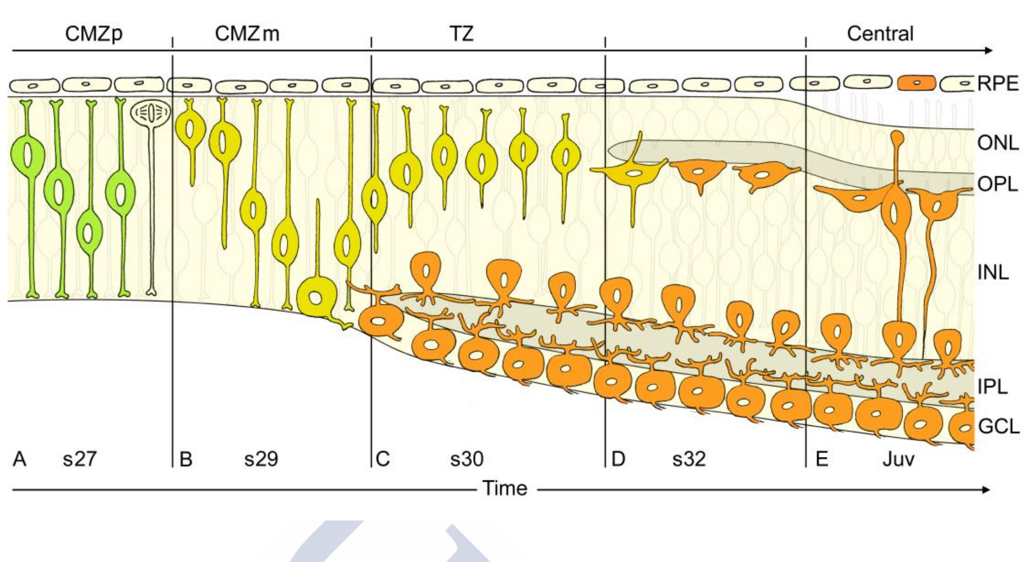

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Figure 6

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CHAPTER 3

Identification of radial glial progenitors in the retina of *Scyliorhinus canicula*

IDENTIFICATION OF RADIAL GLIAL PROGENITORS IN THE RETINA OF *SCYLIORHINUS CANICULA*

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1. Introduction

In the developing brain of mammals, neural stem cells (NSCs) give rise to progenitors termed neuroepithelial cells (NECS), which in their turn produce radial glial (RG) progenitors (**see General introduction**). The finding that radial glial cells (RGCs) serve as progenitor cells in development (Malatesta et al., 2000) changed radically the prevailing notion about their function. While these cells had been first considered as guiding cables for migrating neurons towards their final destinations, they are now accepted to be the major source of all the main lineages (neurons, astrocytes, oligodendrocytes, ependymocytes and even adult neural stem cells) in the central nervous system (CNS; see Malatesta and Götz, 2013; Malatesta et al., 2008) both under physiological and regenerative conditions. Importantly, this fact has led to the misperception that all RGCs are stem cells, even if only a minority of them

self-renew an indefinite number of times and persist for the entire life of the organism (reviewed in Götz et al., 2015).

Therefore, the precise contribution of the RG to adult neurogenesis has been matter of intense research (Borrell and Götz, 2014; De Juan Romero and Borrell, 2015; Ever and Gaiano, 2005; Kriegstein and Götz, 2003; Ninkovivc and Götz, 2015; Paridaen and Huttner, 2014; Pilz et al., 2013). The retina of teleost fishes has been increasingly used to approach this question because of the presence of a single type of RG, even in the adult stage, the Müller glia. Müller glial cells have processes that span the entire thickness of all retinal layers, supporting significantly the retinal function and maintaining visual attainment in the growing eye (Bringmann et al., 2006; Gallina et al., 2014; Jadhav et al., 2009; Mack et al., 1998). In the retina of fishes, Müller cells also exhibit neurogenic properties (Wohl et al., 2012) and participates in sustained proliferation in the adult, since they can occasionally re-enter the cell cycle to generate a population of rod photoreceptor progenitors that give rise to rods throughout the entire life of the fish (Bernardos et al., 2007; Stenkamp, 2011). Müller cells are mostly quiescent, but after injury, they re-enter the cell cycle (Raymond et al., 2006), de-differentiate, and can serve as multipotent retinal stem cells that generate not only photoreceptor progenitors but also all other retinal cell types *in vivo* (Bernardos et al., 2007; Fischer and Reh, 2003; Lenkowski and Raymond, 2014; Raymond et al., 2006; Wohl et al., 2012; Wu et al., 2001).

Despite the ability of Müller cells to produce photoreceptors in physiological conditions, a long-lasting neurogenic pool of NECs persists in the marginal zone of the retina, the so called ciliary marginal zone (CMZ), which apparently is able to generate all retinal cell types without an obligatory transition trough the RG state (reviewed in Than-Tong and Bally-Cuif, 2015). In this context, a deep characterization of the NEC to RGC transition during development should help to determine if this process also

occurs in the mature retina, i.e., whether RG highly contributes to adult neurogenesis.

The retina of *Scyliorhinus canicula* offers an exceptional model to deeply analyze the NEC to RG transition, and the subsequent RG differentiation from development to the adulthood because of the presence of a CMZ containing retinal progenitors, the presence of a large and long-lasting transition zone (TZ) bordering the CMZ that contain both proliferating neuroblasts and early differentiated cells (**see Chapter 2**), and a protracted period of proliferation in the central retina (where differentiated neurons and glia are arranged). Previously, we defined a CMZ subdivided in a peripheral region (peripheral CMZ; CMZp) containing weakly DCX-ir cells and an adjacent region (middle CMZ; CMZm) where DCX immunoreactivity resembles that described for the central retina of stage 29 embryos (**see Chapter 1**).

Several markers are currently used to label RGCs in the brain of mammals, some of them shared with other progenitor cells (such as NECs or adult progenitor cells) and others shared with mature astrocytes and ependymal cells (**see General introduction and Fig. 3 therein**). Among them, the glial fibrillary acidic protein (GFAP), an intermediate filament protein typically found in mature astrocytes, has been increasingly used to label RG progenitors within the CNS (Middeldorp and Hol, 2011). Several studies have described the expression of GFAP in Nestin-expressing RG progenitors during development (reviewed in Kriegstein and Alvarez-Buylla, 2009; Than-Trong and Bally-Cuif, 2015), and also within neurogenic niches of the adult mammalian brain such as the subventricular zone of the lateral ventricles of the telencephalon (Doetsch et al., 1999; Garcia et al., 2004; Imura et al., 2003; Kriegstein and Alvarez-Buylla, 2009) and the subgranular zone of the hippocampus (Seri et al., 2001; Steiner et al., 2006). The presence of GFAP has been additionally proved through *in vivo* assays in progenitors that give rise to proliferative neural precursors in

the postnatal olfactory bulb, hippocampus, and cerebral cortex (Ganat et al., 2006). GFAP has been also observed in Nestin-expressing progenitors in neurospheres derived from the adult rat ciliary epithelium (CE; Das et al., 2006).

In the retina, most descriptions of GFAP expression have been focused on Müller glia identification in adult stages. To date, these studies have addressed GFAP expression in the adult retina of human (Kumpulainen et al., 1983), rabbit (Schnitzer, 1985) and mouse (Björklund and Dahl, 1985; Sarthy et al., 1991), in the adult retina of different species of reptiles (Todd et al., 2015), and in the mature retina of various species of bony (flounders, pipefish, seahorse, killifish, goldfish and zebrafish) and cartilaginous fishes (stingray) (Linser et al., 1985). In the adult retina of these fishes, GFAP has been described in young and mature Müller glia, but not in NECs in the CMZ or in de-differentiated Müller glia after retinal damage (for a review see Than-Trong and Bally-Cuif, 2015). Despite the presence of GFAP in RG progenitors in the CNS (**see above**), only a few studies have described GFAP expression during retinal development in mammals (Ling and Stone, 1988), and very few studies have addressed the expression pattern of GFAP in the neural retina throughout lifespan apart from Sarthy et al. (1991) in mouse and Arenzana et al. (2011) in zebrafish.

We delved into the relationship between RG and neurogenesis events in the retina by exploring the emergence and localization of GFAP expression with respect to the proliferation marker PCNA (proliferating cell nuclear antigen) that allow to detect cells in interphase, and the neuronal migration marker doublecortin (DCX). In addition, we have used the anti-glutamine synthetase (GS) antibody, which has been extensively reported in mature Müller cells (but not in RG progenitors) in both the developing and adult retina of fishes, including *S. canicula* (Bejarano-Escobar et al., 2012; Mack et al., 1998; Peterson et al., 2001; Thummel et al., 2008).

2. Material and methods

The following embryos were analysed: stage 25 (1), stage 26 (1), stage 27 (2), stage 28 (1), stage 29 (3), stage 30 (6), stage 31 (3), stage 32 (4), and prehatching (4). Moreover, 3 juveniles (about 107 mm) and one adult (about 33 cm in total length), were also used.

For more information about experimental animals, tissue preparation, immunofluorescence technique, specificity of the antibodies, and image acquisition and analysis; see **Section 3** in this thesis: **Material and methods**.

Primary and secondary antibodies used were summarized in **Tables 1** and **2** respectively.

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3. Results

We have analyzed the patterns of GFAP and GS immunoreactivity in the retina of *S. canicula* according to the three developmental periods previously described (see Ferreiro-Galve et al., 2010 and **Chapter 1**), which have served as a useful framework for comparison with retinal development in other vertebrates.

3.1. Stages 26-29

During the *first developmental* period, between stages 26 and 29 (**Fig. 1**), the retina showed a neuroepithelial pseudostratified appearance, where cells were arranged in radial columns. The first cells immunoreactive (-ir) to GFAP in the retina were recognized at the end of the stage 26. Weak GFAP immunoreactivity was restricted to the

innermost third of most cell processes, which ended in the innermost part of the neuroepithelium, i.e., in the basal (vitreal) surface (**arrows in Fig. 1A, A'**). Note that GFAP-immunoreactivity in retinal NECs was slightly less intense to that observed in other encephalic regions at the same developmental period (**arrowheads in Fig. 1A, A'**). GFAP-ir cells were also found in lens fibers (**asterisk in Fig. 1A**). At this stage, cells in the retina were immunonegative to DCX, although DCX immunoreactivity was clearly identified in other encephalic regions (**arrowheads in Fig. 1A, A''**). At this stage, interphase nuclei (PCNA-ir) were located at different positions giving the neuroepithelium a stratified aspect (**Figure 1B, C**) and mitosis are only found in the outer (ventricular) margin (**see Chapter 2**), which matches the definition of interkinetic nuclear migration (IKNM). Due to PCNA is a nuclear marker and GFAP is a cytoskeletal protein located in the cytoplasm, co-localization among both markers was not apparent (**arrows in Fig. 1C-C''**).

At the end of this period, stage 29, the thickness of the still proliferating neuroepithelium has increased (**Fig. 1D-G''**). Although the retina did not show any apparent regional differentiation or layering (i.e., cell differentiation has not started and plexiform layers are therefore not organized), several spatial differences in the distribution of GFAP-ir, PCNA-ir and DCX-ir cells were noted at this stage. In the central part of the retina, weakly-GFAP immunoreactivity was observed in some isolated processes in the neuroblastic layer extending toward the outer retina (**curved arrows in Fig. 1D, D', E**). GFAP-ir pyramidal endfeet were mainly aligned in the innermost part of the retina (**arrows in Fig. 1D, D'**), where the intensity of PCNA immunoreactivity decreased in most cells (**Fig. 1D''**). Some cells at this location were also DCX-ir and appear to be associated to GFAP-ir endfeet (**open arrowheads in Fig. 1E, E', E''**). Immunoreactivity for both GFAP and DCX was observed in the emergent optic fiber layer (OFL) though co-localization between these markers was not evident.

In the peripheral retina (**Fig. 1F-G''**), the immunoreactivity pattern of these markers was similar to that observed in central regions at the beginning of this period, i.e., GFAP immunoreactivity was less intense and restricted to cells endfeet (**Fig. 1F-G'**), PCNA immunoreactivity was widely found in interphase cells (**Fig. 1F, F''**) and DCX immunoreactivity was faint (**Fig. 1G, G''; see also Chapter 2**), which clearly allows to discern the prospective CMZp from rest of the retina even before the differentiation of first postmitotic cells in the inner retina (see Ferreiro-Galve et al., 2010 and **Chapter 2**). Interestingly, faint GFAP immunoreactivity was also observed in cells of the CE, especially in their basal domain (**Fig.1F; arrow in Fig. 1G**).

3.2. Stages 30-32

The *second developmental* period (**Fig. 2**) is defined by the progressive layering of the central part of the retina, which in turn allows clearly defining the extension of the CMZ (non-layered) and the TZ, which separates the CMZ from the central (layered) retina. As indicated above, the distribution of DCX in the peripheral retina allowed us to subdivide the CMZ in a CMZp containing weakly DCX-ir cells and an adjacent CMZm where DCX immunoreactivity resembles that described for the central retina of stage 29 embryos (**see Chapters 1, 2**). At stage 30, the central part of the inner retina becomes layered by the gradual formation of the inner plexiform layer (IPL), which separates the ganglion cell layer (GCL) from the inner part of the inner nuclear layer (INLi; **see Chapter 1**). The distribution of GFAP immunoreactivity was similar to that observed earlier at stage 29 (**Fig. 2A, A'**). Intense GFAP immunoreactivity was observed in some processes in the middle and outer neuroblastic region (**curved arrows in Fig. 2A, A'**) and in cells processes at the inner third region of the central retina, which roughly coincided with the area where PCNA-immunonegative nuclei where located (**arrow in Fig. 2A''**). Intense GFAP immunoreactivity was also observed in thick cell endfeet and cell

processes located in the OFL (**arrowheads in Fig. 2A, A'**), where DCX-ir axons from ganglion cells were also observed (**open arrows in Fig. 2B, B''**). The distribution of GFAP in the peripheral retina resembled that found at previous stages (**Fig. 2C, D; compare with Fig. 1F, G**). Very strong GFAP-immunoreactivity was also found in the lens (**asterisk in Fig. 2C**), in the CE (**Fig. 2C**) and in the optic nerve (ON) head (**Fig. 2E, F**). These processes were organized over the surface of DCX-ir ganglion cell axon bundles as they coursed into the optic nerve head (**Fig. 2F**). GFAP was also observed in the ON at later developmental stages.

By the late stage 31, the appearance of GFAP immunoreactivity in the central retina changed considerably (**Fig. 2G-H**). While in previous developmental stages GFAP-ir filaments were disheveled, at this stage GFAP immunoreactivity increased and was neatly arranged along cell processes and endfeet. Again, the strongest GFAP immunoreactivity was restricted to the inner third (**arrowhead in Fig. 2G'**), while less intense GFAP immunoreactivity was found in processes extending through the IPL towards the outer retina (**open arrow in Fig. 2G'**). Of note, GFAP-ir processes were absent from the OFL (which eased identifying GFAP immunoreactivity in distinct endfeet). In the central retina, some GFAP-ir apical-directed processes (**open arrows in Fig. 2H**) reach an emerging horizontal cell layer (HCL) occupied by DCX-ir cells (**arrow in Fig. 2H**) while in peripheral regions the portion of apical processes showing GFAP immunoreactivity was always shorter (about one-third of the cell) (**open arrows in Fig. 2I**). Intense GFAP-ir and DCX-ir outward-directed processes were observed in the peripheral retina except in the CMZp, which was occupied by strong GFAP-ir and very faint DCX-ir processes (**Fig. 2J**). As noted above, copious GFAP immunoreactivity was found in cells of the CE (**open arrowhead in Fig. 2K**). Interestingly, PCNA immunoreactivity was more intense in the peripheral retina than in the CE, the latter containing a high number of faintly PCNA-ir cells.

At stage 32 the retinal layering was well-defined at central regions with the presence of a noticeable outer plexiform layer (OPL) and the prospective outer nuclear layer (ONL). PCNA immunoreactivity has considerably decreased (**Fig. 2L, L'**). The retina of these embryos showed GFAP immunoreactivity in the inner half of cell processes and in the now better defined endfeet of Müller cells, close to the vitreal region (**arrowheads in Fig. 2L''**). GFAP immunoreactivity increased in the apical-directed processes and some GFAP-ir processes reached the apical surface (**open arrows in Fig. 2L''**). In the peripheral retina (**Fig. 2M**), the morphology of GFAP-ir cells resembled that observed in the central retina at previous developmental stages and the CE showed a strong GFAP immunoreactivity.

3.3. Stages 33-34

In the course of the *third developmental* period, layering progressed to almost the entire circumference of the retina, which acquired the mature organization typical of postembryonic stages (**Fig. 3**). Since there were no significant differences in GFAP-immunoreactivity between stages 33 and 34, we consider both as prehatching (PH) embryos.

The central retina contained only a few PCNA-ir cells (**Fig. 3A**). In these embryos, GFAP immunoreactivity was restricted to radially oriented Müller cell processes, especially to their two inner thirds (**curved arrows in Fig. 3A, B, B'**), and also to their endfeet. However, Müller cell bodies, mainly located in the INL, were immunonegative to GFAP. Most DCX-immunoreactivity was found in ganglion, amacrine, bipolar and horizontal cells (**see Chapter 2**), and also in some DCX-ir cell bodies identified at intermediate positions closely associated with GFAP-ir RG processes (**open arrowheads in Fig. 3B, B'' and arrowhead in B, respectively**). Note that

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GFAP-ir processes gradually increased in length from the peripheral to the central retina (**compare Fig. 3C-E**).

With the aim to ascertain if different intensities of GFAP immunoreactivity in cell processes and/or the location of GFAP-ir filaments along cell processes could be related to Müller cell maturation we double-labeled the retina for GFAP and GS which has been previously described in mature Müller cells in the retina of *S. canicula* (**see General introduction** and Bejarano-Escobar et al., 2012). In the most central retina, GS-ir Müller cell bodies were found in the outer part of the INLi (**open arrowheads in Fig. 3C, C''**). Müller cell bodies were immunonegative to GFAP (**Fig. 3C'**) and DCX (**Fig. 3C'''**). However, radial processes from Müller cells were both GS- and GFAP-ir (**curved arrows in Fig. 3C, C', C''**), being immunoreactivity to GFAP much more intense in inner cell processes. GS immunoreactivity gradually decreased in peripheral regions (**open arrows in Fig. 3D'**), where intense GFAP immunoreactivity was still present though restricted to the inner third of cell processes and endfeet (**arrowheads in Fig. 3D'**), which resembled the distribution of GFAP in the central retina at earlier stages (**compare with Fig. 2G**). No GS-ir cells or processes were found in the most peripheral region (**Fig. 3E**), where faint GFAP-ir processes appeared disheveled (**arrowheads in Fig. 3E'; compare with Fig. 1D**). In the non-layered part of the TZ (**Fig. 3F**), faint GFAP-ir processes were observed co-localizing with PCNA-ir nuclei. In the TZ close to the CMZ, some DCX-ir neuroblasts were also found at intermediate positions (**Fig. 3F**). The inner (layered) part of the TZ was comprised of PCNA-negative and DCX-ir cells in both the GCL and INLi, and DCX-ir processes in the IPL and axons in the OFL. In this region, strongly GFAP-ir processes were observed through the GCL, IPL and INLi, and in endfeet in the OFL. In the CMZp the pattern of GFAP immunoreactivity was similar to that found in the central retina at stage 29. In turn, the CMZp was characterized by GFAP-ir cell processes spanning the non-layered neuroepithelium and very faint DCX immunoreactivity.

3.4. Juveniles and adults

Very strong GFAP immunoreactivity was observed in Müller cells in juveniles (**Fig. 4A**). GFAP-ir processes course radially going through the entire thickness of the central retina from the inner limiting membrane (ILM) to the outer limiting membrane (OLM). The pattern of GFAP immunoreactivity in the peripheral retina was similar that observed in the same region in PH embryos (**Fig. 4B, C**). Changes in cell morphology and, accordingly, GFAP immunoreactivity observed throughout development are recapitulated from the CMZp to the central retina (**see Fig. 4A, B**). The cell bodies of Müller cells in the central retina were GS-ir (**Fig. 4D, open arrowheads in D'**). DCX immunoreactivity extended to the retina bordering the CMZp (**asterisk in Fig. 4E**). As in PH embryos, neither GS-ir cell bodies nor processes were found in the TZ (**Fig. 4E, E'**). GS-ir Müller cell bodies were observed in the central retina of juveniles (**open arrowheads in Fig. 4F, F', F'''**). Co-localization between both glial cell markers, GS and GFAP, was found in the radial processes of Müller cells (**curved arrows in Fig. 4F-F'''**). The distribution of GFAP and GS in the peripheral retina was the same found in the peripheral retina in PH embryos (**Fig. 4G, G', G''**). Again, the organization of GFAP in the peripheral retinas was the same found in the central retina in early stages. In adults, the thickness of the retina has considerably decreased (**Fig. 4H**). GFAP immunoreactivity in the central retina was maintained in Müller cell processes as in juveniles. GFAP immunoreactivity was also observed in the periphery, including the CMZ, and in the CE (**Fig. 4H'**).

4. Discussion

Nowadays, it is known that the RG constitute the major source of both neurons and glia in several regions of the CNS. However, the mechanisms underlying the transition from NSC to RGC, as well as the RG differentiation from development to the adult, are not completely understood. The retina of *S. canicula* offered as an excellent model to deeply analyze these processes, particularly because of the great advantage that suppose the presence of a large CMZ and a TZ (**see Chapter 2**).

GFAP has been extensively used in astrocyte identification in the CNS (Eng et al., 2000), and also as an early cellular marker for CNS injury, including Müller cell gliosis in the retina (Bringmann and Reichenbach, 2001; Bringmann et al., 2006, 2009; Gallina et al., 2014; García and Vecino, 2003). Interestingly, GFAP immunoreactivity been also reported in progenitor cells within the CNS (**see General introduction**). In mammals, the transition from immature to mature glial forms is correlated with a change in the expression of GFAP (Bovolenta et al., 1984; Elmquist et al., 1994; Marcus and Easter, 1995). However, the expression and the possible roles of GFAP during retinogenesis are not well understood and to our knowledge, developmental studies have not been carried out so far. The present study describes the GFAP expression pattern in the retina of the small shark *S. canicula*. It is well known that cartilaginous fishes occupy a key phylogenetic position that makes them suitable to access the ancestral condition of vertebrate CNS organization (Coolen et al., 2009). Since GFAP is well conserved over the vertebrate evolution, comparative studies across species, especially in non-mammalian vertebrates could help to understand its possible relevance in specific cell populations.

We aimed to analyze the distribution of GFAP immunoreactivity in the retina during the lifespan of the small shark *S. canicula* by means of immunohistochemistry, since studies focused on the distribution of GFAP in

the retina were very scarce and they not covered all the entire retinal development.

4.1. GFAP as a marker for astrocytes

GFAP is a ~50 KDa protein that represents the main intermediate filament (IF) protein found in mature astrocytes. This 8-9 nm IF was first isolated from a large plaque present in the brain of patients affected by multiple sclerosis (Eng et al., 1971). These plaques were characterized by having demyelinated axons, strong reactive gliosis and because of the formation of a scar, which consisted in an assembly of axons surrounded by astrocytes filled with IFs. GFAP expression in astrocytes is well conserved over the vertebrate evolution and analogous filaments have been observed in glial cells of some invertebrates (Cardone and Roots, 1990; Dahl et al., 1985; Dos Santos et al., 2005; Mencarelli et al., 1993; Sarthy et al., 1991). Most of the literature describing the expression of GFAP in vertebrates refers to the first isoform identified but, to date, seven different splice variants have been additionally found that are specifically expressed in different subpopulations of astrocytes reviewed in (Middeldorp and Hol, 2011).

In *S. canicula*, we observed intense GFAP immunoreactivity in the OFL at stages 29 and 30, which co-distributed with DCX-ir axons of ganglion cells. GFAP immunoreactivity was also observed in the ON at stage 30 and later stages, apparently neighboring DCX-ir axons. This expression coincides with the moment when ganglion cells begin to differentiate and ganglion cell axons bundle to form the primordial ON (Ferreiro-Galve et al., 2008). However, GFAP immunoreactivity in the ON seems to vary within species. In the retina of mammals, GFAP immunoreactivity has been observed in astrocytes (Björklund and Dahl, 1985; Kumpulainen et al., 1983; Sarthy et al., 1991). Astrocyte cell bodies

and processes are almost entirely restricted to the OFL of the retina. In immunocytochemical staining, astrocytes clearly exhibit processes aligned along the ganglion cell axons coursing through the OFL (Höllander et al., 1991; Schnitzer, 1998; Triviño et al., 1996). Thus astrocytes are arranged over the surface of the ganglion cell axon bundles as they course into the ON head forming a tube through which the axons run (Höllander et al., 1991; Schnitzer, 1998; Triviño et al., 1996). In addition, GFAP immunoreactivity was observed in the mature chicken ON head (Gerhardt el al., 2000), and also in astrocytes in the embryonic chicken ON (from onwards E19) (Schuck et al., 2000). In zebrafish, a transient expression of GFAP has been shown in the ON at the end of the embryonic life, but not in the adult (Arenzana et al., 2011). In other teleosts such as goldfish, GFAP immunoreactivity was present in other structures in the visual pathway, in the tectum and tract, but not in the ON (Nona et al. 1989). More recently, a detailed study in the tench ON head has described a particular adaptation of Müller cell processes in the optic disc. This work has shown GFAP immunoreactivity and other proteins related to the glial cytoskeleton, such as cytokeratins, in the ON head (Lillo et al., 2002).

Despite the extensive number of studies that used GFAP as a marker for astrocytes both *in vitro* and *in vivo*, its precise function in these cells is not clear. Several studies in mice have suggested different roles in providing mechanical force and maintaining the shape of the cells, regulating cell migration by providing structural stability to astrocytic processes, contributing to functioning and integrity of the blood brain barrier, mediating signal transduction pathways and intervening in neuron-glia interactions (Bignami et al., 1972; Eng et al., 1971, 2000; Gomi et al., 1995; Liedtke et al., 1996; McCall et al., 1996; Middeldorp and Hol, 2011; Pekny et al., 1995).

While GFAP was firstly thought to be specifically present in mature astrocytes in the CNS (Bignami et al., 1972; Eng, 1985; Eng et al., 1971), GFAP expression has also been found in other glial cell types. In fishes and amphibians, GFAP has been found in young and mature Müller glia (Bignami, 1984), the major glial cell type in the retina (see below). GFAP has been also found in the Müller glia in reptiles and birds (Todd et al., 2015). Many immunohistochemical studies have also reported GFAP-related molecules in the peripheral glia, including Schwann cells (Berg et al., 2013; Björklund and Dahl, 1985; Jessen et al., 1984; Keller et al., 2009) and enteric glial cells (Kato et al., 1998), and in many other cell types and regions outside the CNS (Björklund and Dahl, 1985; Eng et al., 2000; Hatfield et al., 1984; Jessen and Mirsky, 1980; Jessen et al., 1984; Keller et al., 2009; Yen and Fields, 1981).

4.2. GFAP in neuroepithelial (pre-neurogenic) retina

In the present work we reported that GFAP immunoreactivity appeared very early during retinal development in *S. canicula* (stage 26; **see Fig. 1A**). GFAP immunoreactivity was first observed in the vitreal endfeet of cells that we interpret as NECs because of two main reasons. First, at this stage all cells were proliferating, with nuclei located at different positions giving the retina a pseudostratified appearance, which, as stated above, matches the definition of IKNM characteristic of NECs. Indeed, IKNM has been previously reported in NECs in an extensive variety of regions in the CNS (Baye and Link, 2007; Del Bene, 2011; Kosodo, 2012; Reiner et al., 2012; Spear and Erickson, 2012). Second, the neurogenic process has not begun since DCX (a marker of the neuronal lineage; **see Chapter 2**) is not expressed at this stage. The neuroepithelial appearance of the retina at early stages was very similar to that observed in other pseudostratified epitheliums along the CNS before the neurogenesis

process begins (Götz and Huttner, 2005; Paridaen and Huttner, 2014). A similar pattern was observed in the CMZp of the mature retina (**see Fig. 5**).

Therefore, our observations in the pre-layered undifferentiated retina suggest that retinal GFAP-ir cells in *S. canicula* shared some phenotypic properties with NECs elsewhere in the CNS. Interestingly, the presence of GFAP in NECs in the retina has not been reported so far in any other species. NECs does not show GFAP immunoreactivity until stage 26, which suggest the time of appearance of the first retinal GFAP immunoreactivity in the retina of *S. canicula* may just precede the transition from NECs to early RGCs, which in turn will give rise to neuroblasts (DCX-ir) and mature RG (**see below**).

4.3. GFAP immunoreactivity in early RGCs

At stage 29 the first signs of regional differentiation can be observed in the basis of PCNA and DCX immunoreactivity (**see Fig. 1D, E**), what indicates that neurogenesis has commenced. The same pattern is observed in the CMZm of the mature retina (**Fig. 5**). According with previous works concerning embryonic neurogenesis (Alvarez-Buylla et al., 2001; Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009; Sild and Ruthazer, 2011), NECs elongate and transform into RGCs when progenitors change from a symmetric to an asymmetric mode of cell division that coincides with the beginning of neurogenesis (Kriegstein and Götz, 2003; Lenkowski and Raymond, 2014; Rakic, 2003). Therefore, pale GFAP-ir processes of RGCs observed at this stage could correspond to ***early RGCs*** according with the nomenclature used in the cortex by (Kriegstein and Alvarez-Buylla, 2009). GFAP has been already described in RGCs in the ventricular zone at the cerebral cortex during development (nicely reviewed in Kriegstein and Alvarez-Buylla, 2009), but to our knowledge, it has not been described in early RGCs in the developing retina before.

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One of the most recent advances in neurogenesis was the fact that the RG had properties of progenitor cells even in the developing brain (Doetsch, 2003; Feng et al., 1994; Hartfuss et al., 2001), therefore GFAP could be a marker for progenitor cells in the early neuroepithelial retina of sharks. Further studies would be necessary to actually demonstrate this hypothesis.

4.4. GFAP during the layering process. Transition from RG to Müller glia

At stage 30, differentiation begins in the innermost part of the central retina which was occupied by PCNA-negative cells, some of them being also DCX-ir. GFAP immunoreactivity was more intense in the inner third of cells processes, (**Fig. 2**) in the area occupied by PCNA-negative nuclei. This pattern was also observed in the TZ of the mature retina (**see Fig. 5**). Because of the increase in GFAP expression in this region and the disorganized aspect of GFAP-ir filaments, we will refer these cells as ***late RGCs***.

Between stages 31 and 32, some significant differences in the appearance of GFAP immunoreactivity in cell processes take place, so that GFAP-ir filaments are now neatly arranged in their thick basal-directed apical processes. These cells thus acquire the typical morphology characteristic of ***young Müller cells*** coinciding with the maturation of the central retina (**see Fig. 5**): ganglion and amacrine cells differentiate and are clearly separated in their respective layers by the presence of a conspicuous IPL. Our results would be in line with the ultrastuctural study in the brown banded bamboo shark, where Müller cells have been also identified at early stages (Harahush et al., 2009), simultaneously with ganglion cell differentiation, and are compatible with previously reported roles of Müller cells in the histological organization of the developing retina, in the correct establishment of the neuronal circuits

and as a scaffold for young migrating neurons (Bringmann et al., 2006; Willbold et al., 1997). Indeed, we also observed some radial DCX-ir bipolar cell processes closely associated with these GFAP-ir glial cell processes.

Of note, Bejarano-Escobar et al. (2012) did not observed the typical morphology of Müller cells in the retina of *S. canicula* before the stage late 32 by using GS immunohistochemistry. However, these could correspond to ***mature Müller cells*** in the basis of observations in the retina of other species. Indeed, while structural analyses suggest that Müller cells appear early in development of the retina of zebrafish, birth-dating analyses and the appearance of biochemical markers of maturation of various glial markers including GS, suggest that mature Müller cells appear late in development, after the retina is morphologically established (Peterson et al., 2001).

At stage 32, the central retina reaches its mature organization. GFAP immunoreactivity was intense in the inner two thirds of Müller cells, while GS-ir processes in the same cells spanned throughout the entire retina (**Fig. 3, 5**). Both GFAP and GS immunoreactivity was maintained in these cells from this stage until the adulthood. While only weakly GFAP-ir Müller processes have been observed in the mature retina of the stingray (Linser et al., 1985), the presence of GFAP immunoreactivity in radial processes of Müller cells has been found in the mature retina of a few teleosts such as the goldfish (Bignami, 1984; Nona et al. 1989), zebrafish (Arenzana et al., 2011; Yazulla and Studholme, 2001), and pipefish (Linser et al., 1985). Since the retina in fishes grows during the entire life of the animal, the presence of GFAP-ir in these cells and their capacity to proliferate and act as late retinal progenitor cells has led to the proposal that Müller cells in the adult retina can serve the same role as postnatal RG (late progenitor cells) found in neurogenic niches in the adult CNS of mammals. In fishes, Müller cells give rise to late-stage retinal progenitors of the rod photoreceptor lineage. Müller cells that contribute to adult

neurogenesis have been also reported in mammals. In the adult mammalian retina, where constitutive proliferation is limited, GFAP was found in astrocytes but Müller cells do not express GFAP or contain only low amounts of this protein (Sarthy et al., 1991). While GFAP immunoreactivity was very weak or even non-existent (Björklund and Dahl, 1985; Sarthy et al., 1991) and GFAP has been classically used as a tool to differentiate astrocytes (GFAP+) from Müller cells (GFAP-) (Gariano et al., 1996; Lemmon and Rieser, 1983), following injury or in response to the loss of retinal neurons, Müller cells become reactive , showing an upregulation in the expression of GFAP (Bargagna-Mohan et al., 2010; Björklund and Dahl, 1985; Bringmann et al., 2009; Eisenfeld et al., 1984; Erickson et al., 1987). GFAP has been also reported in the Müller glia in reptiles, but their possible role as progenitor cells have not been reported so far.

Of note, GFAP immunoreactivity was also found in horizontal cells in several teleost fishes but not in cartilaginous fishes (Linser et al., 1985) and present results). While horizontal cells in elasmobranchs also contain true neurofilaments (Linser et al., 1985), this difference support the suggestion that the presence of GFAP immunoreactivity in horizontal cells is not evolutionary conserved.

4.5. GFAP in the CMZ and CE

Though Müller cells can be neurogenic under certain conditions, they are not considered the main progenitor cells for retina in the adult because of their late appearance during the neurogenic period (**see General introduction**). The other neurogenic niche in the retina of fishes, amphibians and turtles is the CMZ, which persists in the adulthood and contain progenitor cells that proliferate and add new cells to the peripheral retina (Centanin and Wittbrodt, 2014; Fisher et al., 2013; Perron and Harris, 2000; Than-Tong and Bally-Cuif; 2015; Todd et al., 2015). A CMZ

that persists into the adulthood was also found in the avian retina (Fischer and Reh, 2000; Fischer and Bongini, 2010; Kubota et al., 2002) though the neurogenic capacity of its progenitors is limited. In mammals, non-pigmented cells at the peripheral edge of the retina have been considered to serve as a CMZ-like zone, since they show stem cell characteristics when they are cultured in vitro. However, it has been reported that they do not represent a significant source of regeneration (Ahmad et al., 2000; Fischer and Bongini, 2010; Fischer and Reh, 2003; Kubota et al., 2002; Perron and Harris, 2000; Todd et al., 2015; Tropete, 2000). The fact that Müller glial cells become rapidly quiescent after they are generated from the CMZ and, after constitutive activation, they give rise only to photoreceptors, has led to the hypothesis that the chief contribution of the CMZ (non-glial) cells to generate all types of adult-born neurons does not implies an obligatory transition through the RG state. In teleost fishes, neuroepithelial progenitors in the CMZ express both NSC and progenitor markers (Nestin, BLBP, Sox2) but not mature glial markers (as GFAP). Curiously, glial markers (vimentin) have been recently reported in the marginal region of the neural retina (Bhatia et al., 2009). In *S. canicula* GFAP immunoreactivity was found in NECs in the CMZ that are strongly PCNA-ir. Since they express GFAP-ir and have radial morphology, they can represent progenitor RG, which implies that CMZ contribution to adult neurogenesis indeed implies a transition through the RG state.

On the other hand, whether CMZ cells in the adult retina completely fulfill the definition of a NSC (self-renewal and multipotency at the single cell level) is today a matter of debate. A shocking trait of the NECs in the CMZ is its high proliferating rate, a feature that does not match with the long-lasting maintenance of stem cells. Various hypotheses have been proposed on this regard (**see General introduction**), including the existence of a small number of quiescent cells within the pool, of quiescence phases within each individual cell in the pool, or of a continuous renewal of the pool from a different quiescent

source (see Than-Trong and Bally-Cuif, 2015). Interestingly, strong GFAP immunoreactivity was also observed in the CE, where scarce PCNA-ir cells where observed, which raises the hypothesis that cells in the CE could serve as a quiescent source of stem cells for retinal development and growth.

4.6. GFAP in Müller cells increases with age

Several studies have demonstrated that GFAP expression increases progressively with age in rat and human brain (Morgan et al., 1999; Nichols et al., 1993; Rozovsky et al., 2005). In the mammalian brain, GFAP expression normally increases in astrocytes during normal aging (Morgan et al., 1999; Nichols et al., 1993; Rozovsky et al., 2005), probably in response to the changes that take place in some brain regions after maturation (Finch, 1993; Masliah et al., 1993; O'Callaghan and Miller, 1991). In contrast to the brain, where the two main types of macroglial cells are present, in the retina, oligodendrocytes are totally absent, and astrocytes are present only in mammals, specifically in the OFL. In accordance with our results, in the mature retina of *S. canicula*, GFAP is a marker for Müller cell processes. In addition, we have also observed an increase in GFAP-ir processes with age. Note that in adult specimens this increase is considerably higher with respect to juveniles, and in juveniles with respect to earlier stages. It is well known that Müller cells are able to provide the functions characteristic of astrocytes or oligodendrocytes in other regions of the CNS (Newman and Reichenbach, 1996). Consequently, it is not surprising to observed an increase in GFAP-ir in these cells, due to its importance in the metabolism of retinal neurons and its important role in normal retinal function (Bringmann et al., 2006; Reichenbach and Bringmann, 2013; Reichenbach et al., 1993 Tsacopoulos and Magistretti, 1996).

4.7. GFAP in other structures related with the eye

GFAP immunoreactivity has also been detected in other structures in the eye of *S. canicula,* apart from neural retina. It is not surprising, since GFAP is not a one individual polypeptide but a group of closely related polypeptides with a similar molecular weight located in different cell types (Björklund and Dahl, 1985; Jessen et al., 1984). We have observed GFAP-ir fibers in the lens from the early stage 27 from the mature eye. At early stages, this immunoreactivity was mainly shown in lens primarily fibers. As development progresses, GFAP immunoreactivity was shown in lens epithelial cells and in the soma. To our knowledge, the presence of GFAP-like was described in several tissues in the rodent eye (Björklund and Dahl, 1985). This work demonstrates the presence of GFAP in iris fibers and in lens epithelial cells, apart from Müller cells and retinal astrocytes.

5. Conclusions

Summarizing, we have shown that GFAP immunoreactivity appears very early during retinogenesis, in the endfeet of NECs, suggesting that those cells shared some phenotypic properties with NECs elsewhere in the CNS. We have found GFAP immunoreactivity in *early* and *late RGCs* and in *young Müller cells* as development progresses; GFAP is also present in processes in *mature Müller glia*, even in the adult. Therefore, this glial cell marker has allowed us identify RGCs from NECs to mature Müller glial cells, which has not been noticed in teleosts. Additionally, based on GFAP and GS immunoreactivity patterns and in the peculiarities of the CMZ and the TZ in *S. canicula* over other teleosts such as zebrafish, we were able to distinguish a transition from NECs trough the RG state also in adult neurogenesis. Of note, we have found GFAP-ir cells

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in the CE, which pointed the possibility that cells of the CE could serve as quiescent source of stem cells for the CMZ. The retina of *S. canicula* could serve as a helpful model for monitoring RG in the CNS, both during development and in the adult.

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Abreviations

CE	ciliary epithelium
CMZ	ciliary marginal zone
CMZm	middle CMZ
CMZp	peripheral CMZ
CNS	central nervous system
DCX	doublecortin
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
HCL	horizontal cell layer
IF	intermediate filament
IKNM	interkinetic nuclear migration
ILM	inner limiting membrane
INLi	inner part of the INL
INLo	outer part of the INL
IPL	inner plexiform layer
-ir	immunoreactive
NEC	neuroepithelial cell
NSC	neural stem cell
OFL	optic fiber layer
OLM	outer limiting membrane
ON	optic nerve
ONL	outer nuclear layer
OPL	outer plexiform layer
PCNA	proliferating cell nuclear antigen
PH	prehatching
RG	radial glia
RGC	radial glial cell
TZ	transition zone

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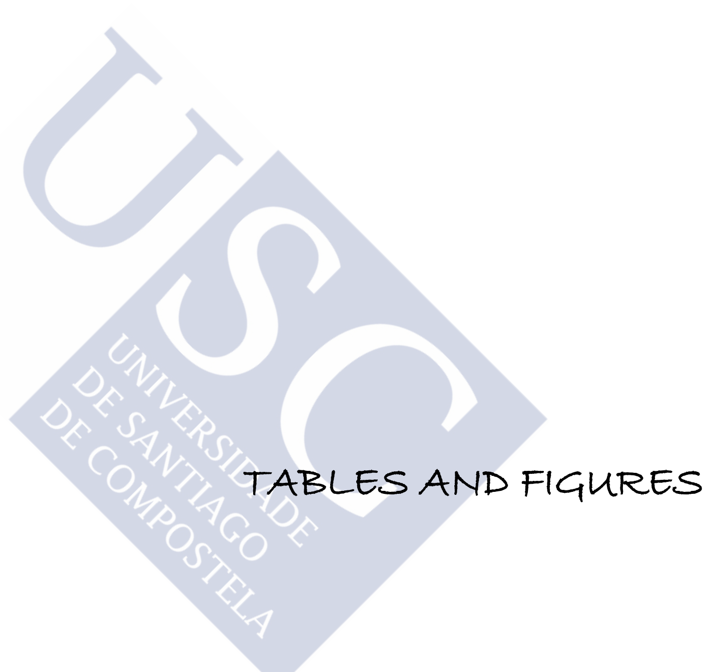

TABLES AND FIGURES

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Table 1. Primary antibodies.

Primary antibody	Source	Working dilution
GFAP	Polyclonal rabbit anti-GFAP DakoCytomation Catalg number: Z 0334, Lot: 00082268	1500
GS	Monoclonal mouse anti-GS Merk-Millipore Catalog number: MAB302, Lot: 2090111	1:100
DCX	Polyclonal rabbit anti-DCX Cell Signaling Technology Catalog number: 4604S, Lot: 3	1:300
DCX	Polyclonal goat anti-DCX Santa Cruz Biotechnology Catalog number: sc-8066, Lot: C2513	1:100
PCNA	Monoclonal mouse anti-PCNA Sigma-Aldrich Catalog number: P8825, Lot: 082M4844	1:800

Table 2. Secondary antibodies.

Primary antibody	Source	Working dilution
546-conjugated donkey anti-rabbit (DAR^{546})	Molecular probes Catalog number: A10040	1:100
546-conjugated donkey anti-mouse (DAM^{546})	Molecular probes Catalog number: A10036	1:100
488-conjugated donkey anti-rabbit (DAR^{488})	Molecular probes Catalog number: A21206	1:100
488-conjugated donkey anti-goat (DAG^{488})	Molecular probes Catalog number: A11055	1:100
488-conjugated donkey anti-mouse (DAM^{488})	Molecular probes Catalog number: A21202	1:100
633-conjugated donkey anti-mouse (DAM^{633})	Molecular probes Catalog number: A31571	1:100

Figure 1. Comparison of GFAP, PCNA and DCX immunoreactivities in neuroepithelial non-layered retina from stages 26 to 29. (**A-C''**) Vertical (transverse) sections along the dorsoventral axis of the retina of stage 26-27 embryos. (**A**) Vertical section of the central retina showing GFAP immunoreactivity for the first time at stage 26, in DCX-negative cells (**A', A''**). (**B**) Vertical section of the retina at stage 27 showing that most NECs are proliferating cells (PCNA-ir). (**C**) Detail of the region squared in B showing that GFAP immunoreactivity is less intense than that observed in the brain. Note that GFAP and PCNA do not co-localize within the cell (**arrows in C, C', C''**). (**D-G''**) Vertical sections along the dorsoventral axis of a stage 29 retina. (**D**) Double GFAP/PCNA immunolabeling in sections of the central retina reveals GFAP-ir pyramidal endfeet (**arrows in D, D'**) that do not co-localize with weakly PCNA-ir cells (**D**''). Curved arrows indicate isolated faintly GFAP-ir processes (**D, D', E**). Some DCX-ir cells appear to be associated with GFAP-ir endfeet (**open arrowheads in E, E', E''**). GFAP and DCX are observed in the emergent OFL, albeit they do not co-localize. In the peripheral retina (**F-G''**) the pattern of these markers is similar to that found in the central retina at previous developmental stages, i.e., GFAP immunoreactivity is less intense and restricted to the endfeet. GFAP immunoreactivity is present in cells of the CE (**F, arrow in G**). Scale bars: 25 µm in D-D'', F-F''; 50 µm in C, E-E''; 75 µm in A-A'', B, G-G''.

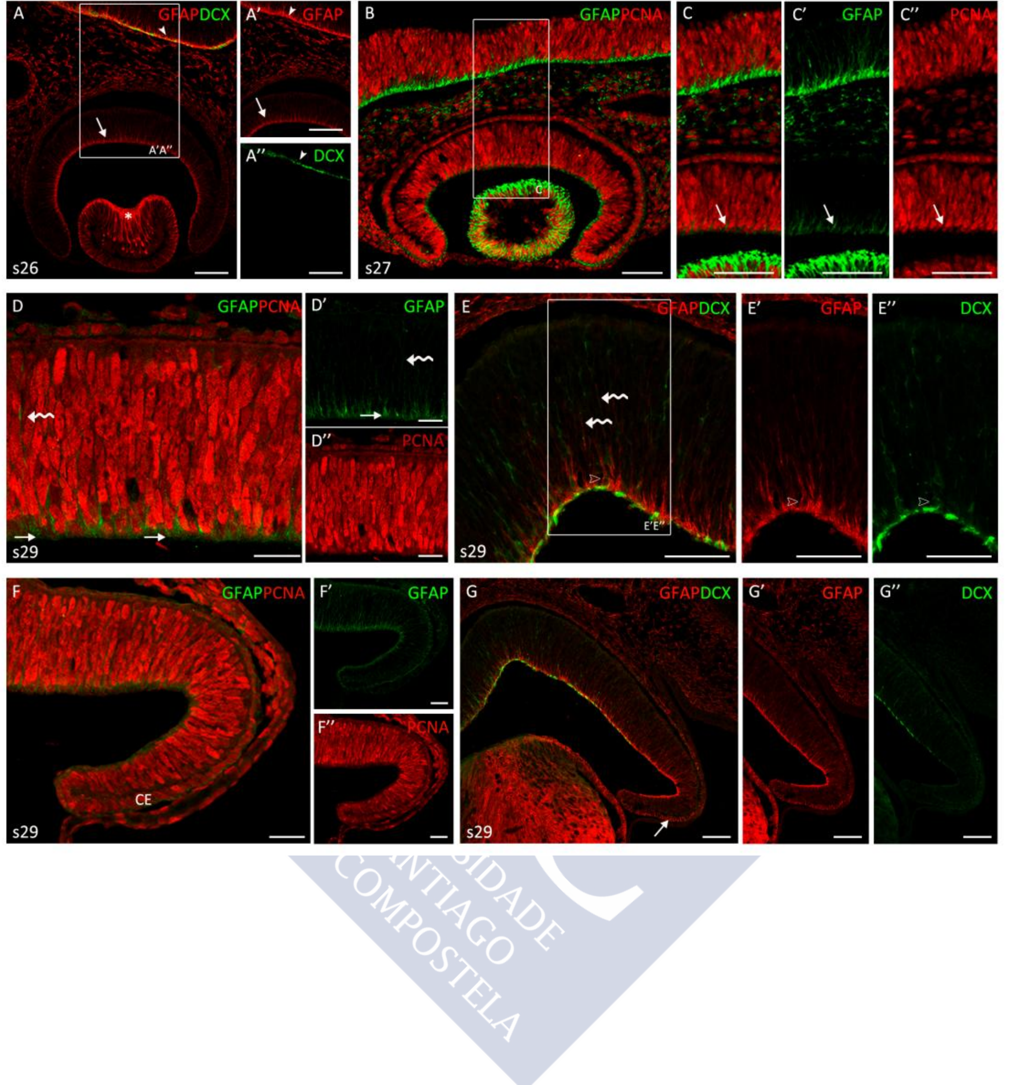

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Figure 1

Figure 2. GFAP, PCNA and DCX immunoreactivity patterns in the retina of *S. canicula* during the layering process (A-M). Vertical (transversal) sections along the dorsoventral axis of the retina. (**A-F**) Vertical sections of the retina at stage 30. (**A**) Intense GFAP immunoreactivity is observed in some processes in the middle and outer neuroblastic regions (**curved arrows in A, A'**). GFAP-ir cell processes are observed in the inner third region, where pale PCNA-ir nuclei are placed (**arrow in A''**). Arrowheads point to GFAP-ir cell processes in the OFL (**A, A'**), where DCX-ir axons from rounded-ganglion cells are observed. The arrow in A'' indicates PCNA-negative cells in the inner retina. (**B-B''**) Detail of the central retina to show DCX and GFAP immunoeactivities in the OFL (open arrows). (**C, D**) Details of the peripheral retina, where the GFAP immunoreactivity is reminiscent of that found in the central retina at previous stages. Strong GFAP immunoreactivity is found in the lens (**asterisk in C**), in the CE (**C**), and in the ON head (**E, F**). (**G-K**) Vertical sections of the retina at stage 31l-32e. GFAP immunoreactivity increases in the central retina along cell processes (**open arrows in G', H)** and in endfeet (**arrowhead in G'**). Some GFAP-ir-apical directed processes reach an emerging HCL occupied by DCX-ir cells (**arrow in H**). (**I**) In peripheral regions the portion of GFAP-ir apical processes is shorter (**open arrow**). (**J**) The CMZp is occupied by strong GFAP immunoreactivity and very faint DCX immunoreactivity. GFAP immunoreactivity is observed in the CE (**open arrowhead in K**). (**L-M**) Vertical sections of the retina at stage 32l. GFAP immunoreactivity is found in the inner half of cell processes and in the better defined endfeet of Müller cells, close to the vitreal region (**L, arrowheads in L''**). GFAP immunoreactivity increases in the apical-directed processes and some of them reache the apical surface (**open arrows in L''**). (**M**) In the peripheral retina, GFAP and DCX immunoreactivity patterns are similar to those found at previous stages in the central retina. Note the high intensity of GFAP-ir cells in the CE. Scale bars: 25 µm in B, B'', G-I, L-M; 75 µm in A-A'', B, C-F, J, K.

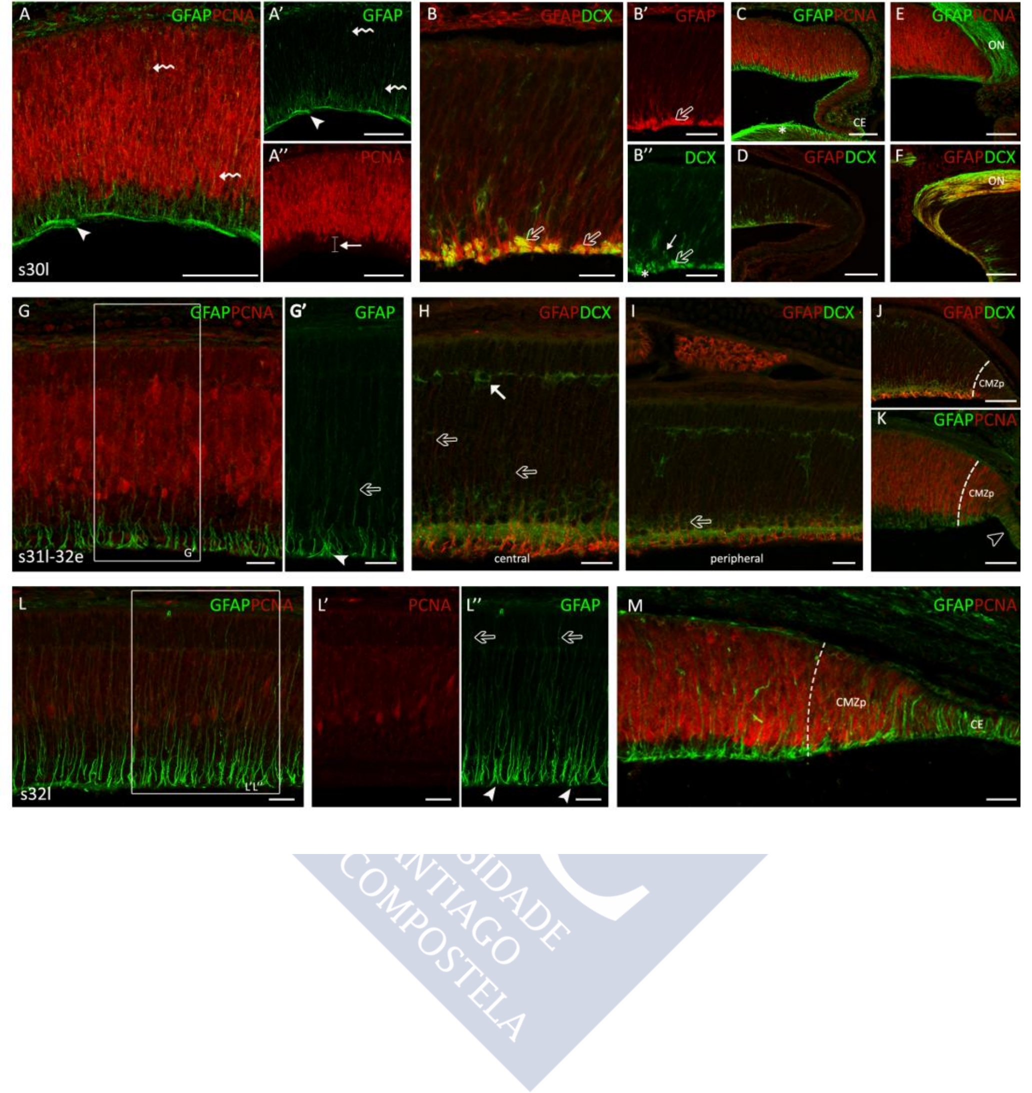

Figure 2

Figure 3. GFAP, GS, PCNA and DCX immunoreactivity patterns in the retina of *S. canicula* at prehatching stages. (**A-F**) Vertical sections along the dorsoventral axis of the retina of PH embryos. Strong GFAP immunoreactivity in radially oriented Müller cell processes (**curved arrows in A, B, B'**) and in their endfeet. DCX-ir cells are placed at intermediate positions associated with GFAP-ir radial Müller cell processes (**open arrowhead in B, B''**). DCX-ir cells are located in the central retina in PCNA-negative areas, in the GCL, INLi, INLo and HCL (**B''**). GFAP-ir processes gradually increase in length from peripheral to the central retina (**compare C-E**). In the most central part, GS-ir Müller cell bodies are placed in the INLi (**open arrowheads in C, C''**). Müller cell bodies are GFAP- and DCX-immunonegative (**C and C''', respectively**). Radial Müller cell processes are GS- and GFAP-ir (**curved arrows in C, C', C''**). Detail of DCX-ir cells (**C'''**). In peripheral regions (**D-F**), GS immunoreactivity gradually decreases (**open arrows in D'**). Intense GFAP immunoreactivity is restricted to the inner third of cell processes and endfeet (**arrowhead in D'**). No GS-ir cells or processes are observed in the most peripheral region (**E, E'**). (**F**) The layered part of the TZ includes PCNA-negative and DCX-ir cells. Intense GFAP-ir processes are observed in the endfeet in the OFL, in the CMZp and in the CE. Arrowheads in (**E', F**) show changes in GFAP immunoreactivity in cells in the peripheral retina (CMZp, CMZm ant TZ). Scale bars: 25 µm in A-C''', F; 50 µm in D-E'.

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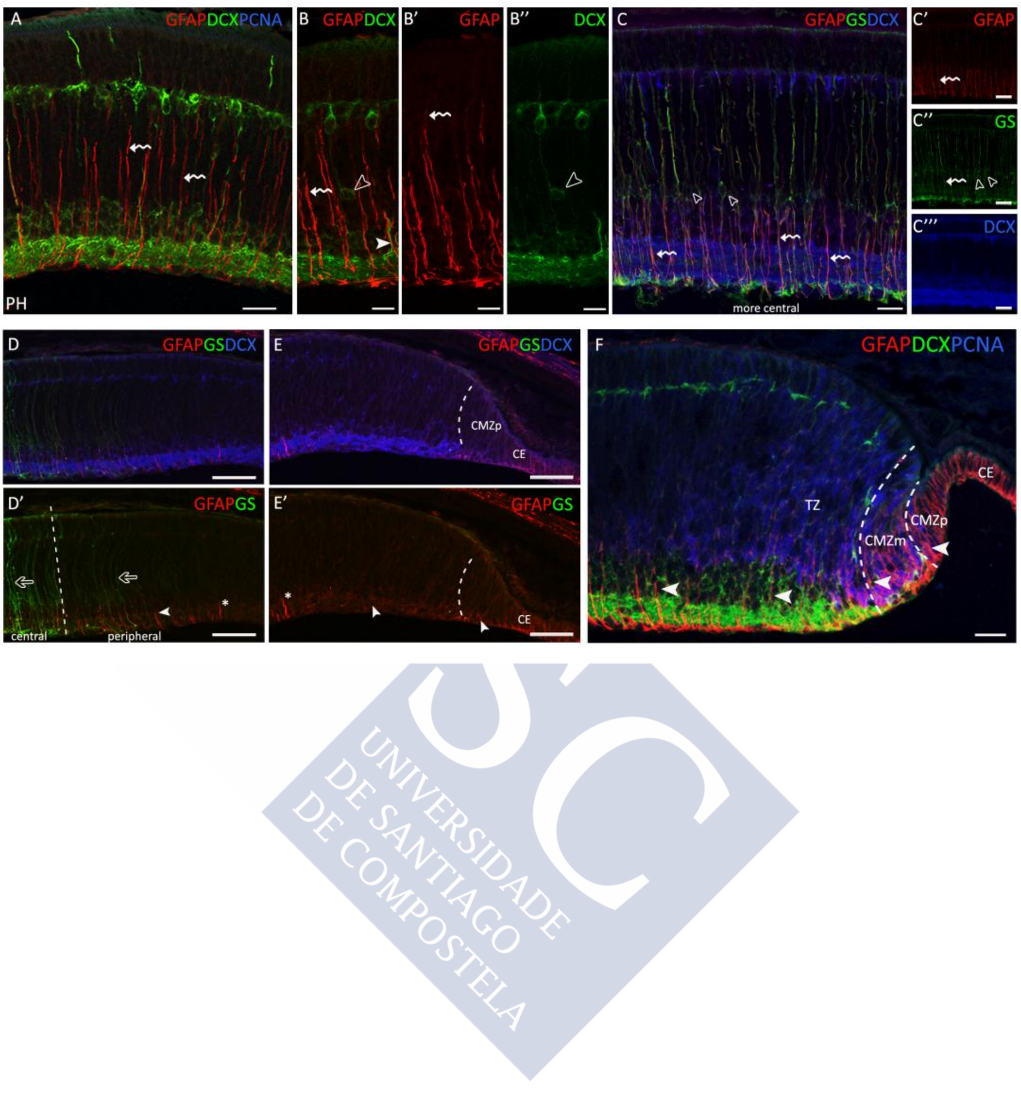

Figure 3

Figure 4. Comparison of spatiotemporal patterns of GFAP, GS, PCNA and DCX in the juvenile and adult retina of *S. canicula*. (**A-G''**) Vertical sections along the dorsoventral axis of the retina of juveniles. (**A**)Very strong GFAP-ir Müller cell processes are observed in the central retina. (**B**) GFAP immunoreactivity is observed in the CMZp, as in PH embryos. (**C, C', C''**) Details of GFAP and DCX labeling in the CMZ. Arrowheads in C' point to GFAP immunoreactivity in the CE and in the CMZ. The open arrowhead in C'' points to intense DCX immunoreactivity bordering the CMZp. (**D**) GS-ir Müller cell bodies in the central retina. Open arrowheads in D' point to GS-ir Müller cell bodies. (**E**) DCX immunoreactivity is faint in the CMZp (asterisk). (**E'**) GS immunoreactivity is absent in cells and processes in the TZ, CMZm and CMZp (arrowhead). (**F-F'''**) Detail of the central retina. Open arrowheads indicate GS-ir cell bodies. Curved arrows indicate GS-ir or GFAP-ir cell procesees. (**G-G''**)GFAP and GS immunoreactivities in the peripheral retina are similar to that found in PH embryos. (**H-H'**) Vertical sections along the dorsoventral axis of the retina in adults. Strong GFAP-ir Müller cell processes in the central retina (**H**). GFAP immunoreactivity is also observed in the CMZ and in the CE (**H'**). Scale bars: 25 µm in D-D'', H; 50 µm in A-C'', E-G''; 100 µm in H'.

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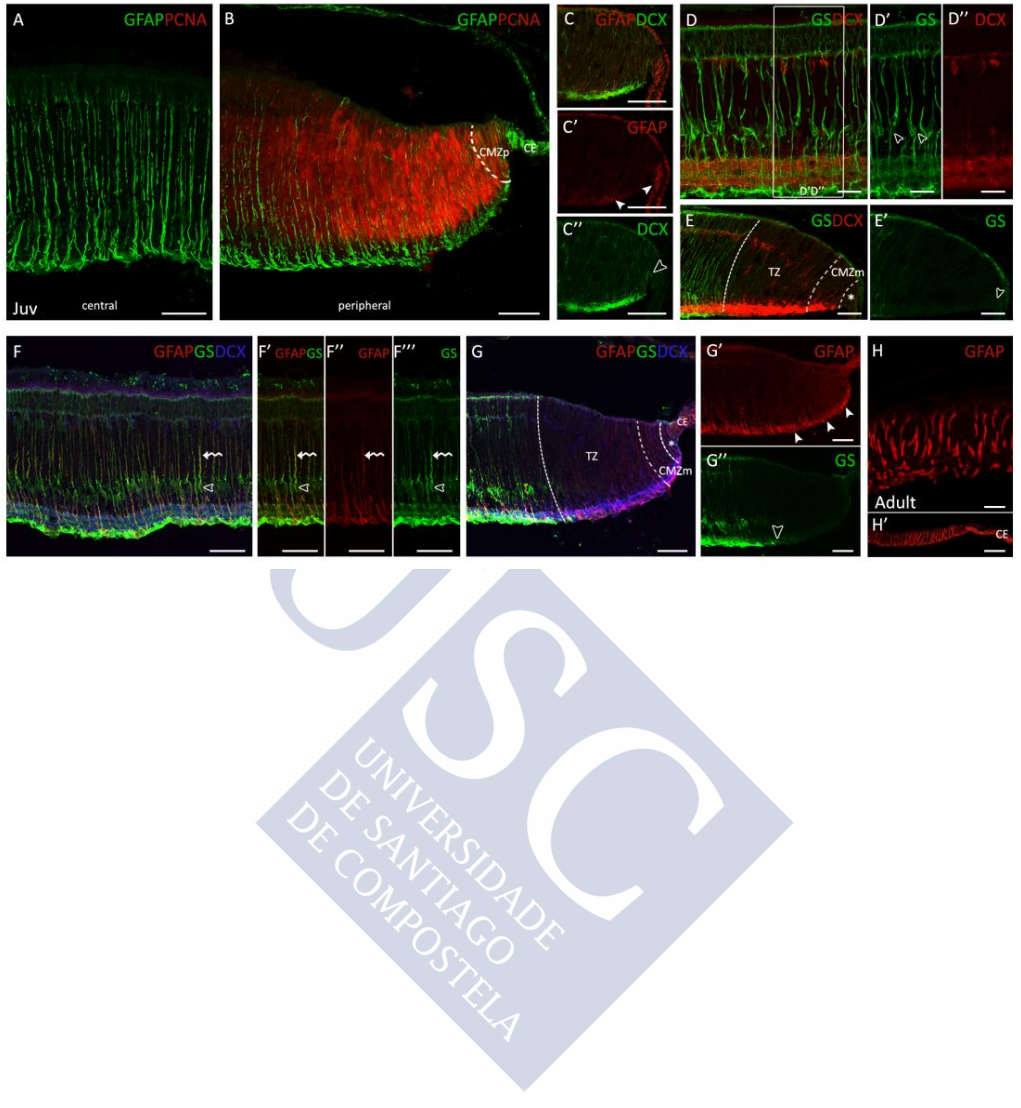

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Figure 4

Figure 5. Sequence of DCX, GFAP and GS immunoreactivity during maturation of the retina in *S. canicula*. DCX-ir cells are represented in green, GFAP-ir cells are represented in pink and GS-ir cells are represented in purple. Different shades of the same color represent different states of cell maturation.

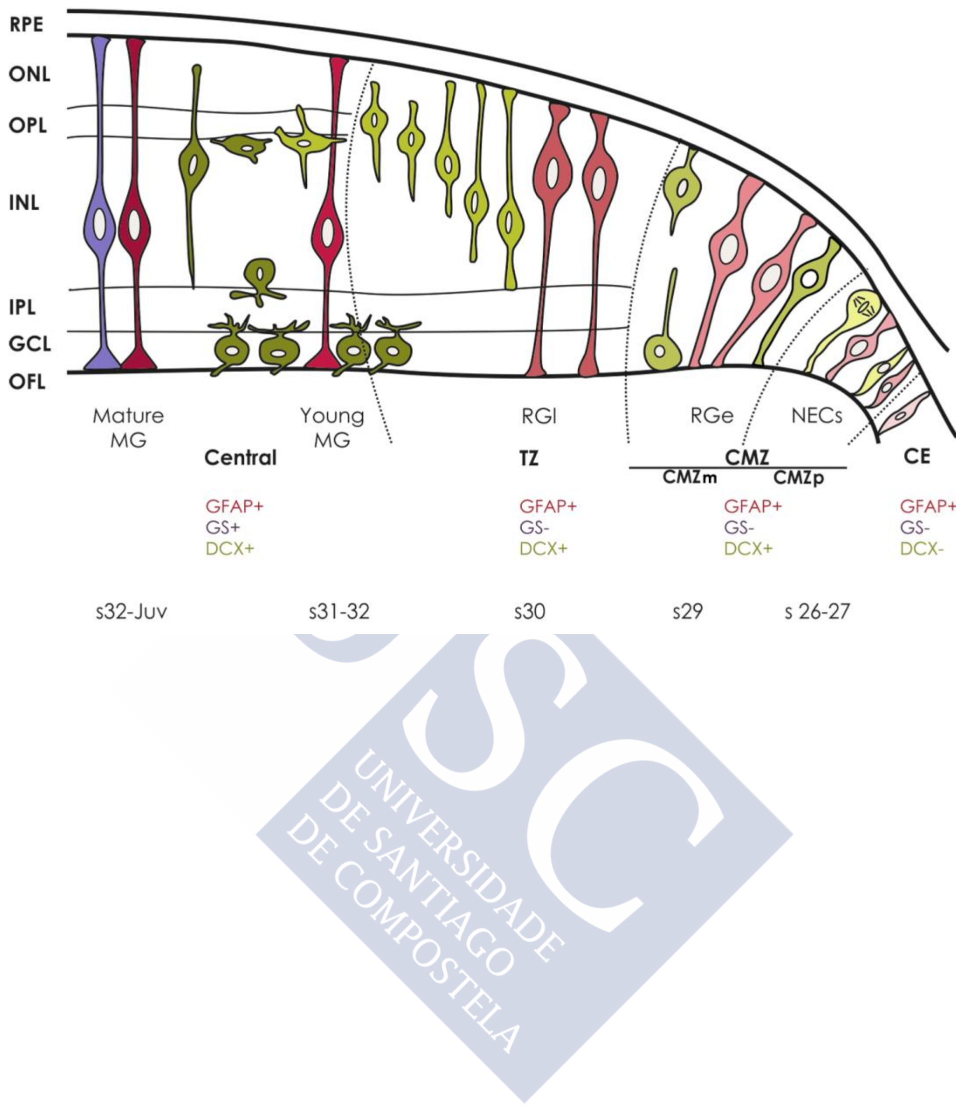

Figure 5

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GENERAL DISCUSSION

GENERAL DISCUSSION

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General discussion

During the development of the central nervous system (CNS), billions of cells proliferate, migrate and differentiate in a precisely coordinated sequence of events known as neurogenesis. During neurogenesis, primary progenitor cells (PCs) give rise to new neurons and glia. Identifying PCs and the lineages derived from them, is essential for understanding the heterogeneity of the different cell types produced, and how to use them for CNS repair. Neurons and glial cells have typically been considered to be derived from separated pools of PCs during development. However, recent works have demonstrated that radial glia (RG) and a subpopulation of astrocytes, both being part of the glial lineage, give rise to neurons and glial cells during development and also in the adult brain (De Filippis and Binda, 2012; De Juan Romero and Borrell, 2015; Götz et al., 2015; Kriegstein and Alvarez-Buylla, 2009; Taverna et al., 2014). Among all neurogenic systems in the CNS, the neural retina constitutes an excellent system to study key events regarding

neurogenesis. Particularly, the retina of zebrafish has been one of the most used models in this field (Bernardos et al., 2007; Goldman, 2014; Lenkowski and Raymond, 2014; Otteson and Hitchcock, 2003; Raymond et al., 2006; Than-Trong and Bally-Cuif, 2015). However, the fast development of the CNS of this teleost fish, including the retina, and the small size of the embryos (Kimmel et al., 1995; Schmitt and Dowling, 1994), might make some processes remain unnoticed. Instead, the retina of the lesser spotted dogfish *Scyliorhinus canicula* offers an exceptional model to approach the study of embryonic and adult neurogenesis. The big size and the slow-development of the brain and retina in this elasmobranch fish represent a great advantage in contrast to teleost fishes for identifying processes in further detail. Despite some studies have been focused on proliferation and differentiation patterns during the development of the retina in *S. canicula* (Bejarano-Escobar et al., 2012; Ferreiro-Galve et al., 2008, 2010a, 2010b, 2012), this thesis goes an step further, covering other neurogenic events in the retina including cell positioning in the mature retina, the analysis of the transition from neuroepithelial cells (NECs) to radial glial cells (RGGs) and the heterogeneity of PCs and/or neural stem cells (NSCs) present in the main neurogenic niche of the retina of fishes, the ciliary marginal zone (CMZ).

In this thesis, we have extended the knowledge about neurogenesis using the retina of *S. canicula* as a model, analyzing the distribution pattern of the microtubule associated protein (MAP) doublecortin (DCX), involved in regulating cytoskeleton components during migration processes (Friocourt et al., 2007, 2003; Geoghegan and Carter, 2008; Moores et al., 2004, 2006; Reiner et al., 2004; Weimer and Anton, 2006). This thesis has reported for the first time the distribution of DCX throughout the lifespan of a fish by using immunohistochemistry (**Chapter 1**). This allowed us to differentiate three regions within the peripheral retina: a peripheral CMZ (CMZp), which showed no signs of layering, containing weak DCX immunoreactivity; a middle non-layered

CMZ (CMZm) adjacent to it that contained intense DCX immunoreactivity; and a transition zone (TZ) bordering it, which contained intense DCX-ir cells in the inner layered part and at different locations within the neuroblastic part. Then, we extended this work analyzing the neurogenic state of DCX-labeled cells during retinal development and also in the adulthood, to gain a better understanding of the possible roles of this MAP during neurogenesis (**Chapter 2**). For that, we explored DCX immunoreactivity and its co-localization with proliferation and neuronal differentiation markers. We also studied its relation with glial cells and characterized DCX-expressing cells in mature areas where it co-localized with mature neuron markers.

We detected DCX immunoreactivity very early in the developing retina, specifically in proliferating NECs, a fact that has not been described before. The nuclei of NECs were located at different positions as a result of the interkinetic nuclear migration (IKNM; Sauer, 1936; Spear and Erickson, 2012a, 2012b; Taverna and Huttner, 2010). The fact that DCX is functionally related with Lis1 and dynactin, two regulators of the dynein motor protein in the mouse cortex and cerebellum (Caspi et al., 2000; Hu et al., 2013; Spear and Erickson, 2012b; Tanaka et al., 2004), lead us to consider the possibility that DCX may also be involved in IKNM in the retina of fishes. Although the role of the DCX in IKNM has not been studied so far, an oriented microtubule cage has been also described surrounding the nuclei of NECs in the retina of zebrafish (Norden et al., 2009). Apart from that, DCX-immunoreactive (-ir) cell populations have been isolated by flow cytometry from developing and adult brains of mouse, containing a sizeable population of low DCX-expressing precursors that retain their multipotentiality (Walker et al., 2007). Indeed, we observed the same pattern in the CMZp, which is maintained as a proliferating pseudostratified epithelium throughout life. Our findings point to a significant increase in DCX immunoreactivity during cell cycle withdrawal, coinciding with early stages of differentiation of a subpopulation of

neurons, which indicates that DCX in the neuroblastic retina is a marker for differentiating neuronal precursors. Our results are compatible with previous findings in the developing rat (Lee et al., 2003)and chick (Kim and Sun, 2012) retina, as well as in other regions in the developing mammalian brain (Des Portes et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Kim et al., 2006). Intense DCX immunoreactivity has been also described in a row of peripheral cells bordering the CMZ in the mature retina of *S. canicula* (present results) and in adults of the teleost *Nothobranchius furzeri*, a short-lived annual fish (Tozzini et al., 2012). As the layering of the retina progressed, we observed DCX immunoreactivity in different cell types and processes, preceding the developmental period when cells become settled in their definitive cell layers. Our results support the idea that DCX is decisive for the organization of the inner retina, since it is present in cells while they are setting new connections (Horesh et al., 1999; Lee et al., 2003; Nacher et al., 2001). This is in agreement with that described in the developing inner retina of chick (Kim and Sun, 2012) and mammals (Gleeson et al., 1999; Lee et al., 2003). In addition, our results show that DCX immunoreactivity is maintained in the inner retina even in the adult. However, this is different from that reported in rat (Lee et al., 2003), wherein after P1, DCX immunoreactivity decreased in the inner retina, not only in cell somas, as in sharks, but also in the inner plexiform layer (IPL). Concerning the outer retina, we also showed DCX immunoreactivity in mature cells (horizontal and bipolar cells). Despite the presence of DCX in adult tissues has been mostly related to adult neurogenesis, mature horizontal and bipolar cells in the retina of *S. canicula* were localized in areas far away from the neurogenic niches (see Ferreiro-Galve et al., 2010a). DCX expression in differentiated neurons could be related to microtubule reorganization linked to dynamic neuronal plasticity, axonal outgrowth or synaptogenesis (Geoghegan and Carter, 2008; Nacher et al., 2001; Vellema et al., 2013; Wakabayashi et al., 2008). Indeed, in the retina of mouse, DCX appears to be independent

from migration and has been related to cell differentiation and microtubule remodeling during neurite formation and synaptogenesis in the IPL (Lee et al., 2003). As the retina of fishes expand actively throughout the life by addition of new cells, the connectional pattern among cells must change accordingly to accommodate new neurons. Consequently, the possibility should be considered that DCX-mediated processes of synaptogenesis or synapse remodeling are required through life. Regarding DCX expression in horizontal cells, DCX has also been reported in horizontal cells in the developing retina of chick (Kim and Sun, 2012) and rat (Lee et al., 2003). DCX-immunoreactivity was also found in horizontal cells in the adult retina of rat (Wakabayashi et al., 2008), but not in the teleost *Nothobranchius furzeri* (Tozzini et al., 2012). Dendrite arborization and remodeling is a usual feature of mature horizontal cells in mammals (see Wakabayashi et al., 2008 and references therein) and phosphorylation of DCX facilitates neurite outgrowth (reviewed in Reiner et al., 2004), which could explain why DCX is present also in the retina of mammals. On the other side, we reported DCX immunoreactivity in mature bipolar cells in *S. canicula.* Although most bipolar cells retract their apical process during maturation, some of them maintain a thick process known as Landolt's club. In the retina of chick, retraction of apical processes seems to occur only after the dendritic arbors form, which had led to suggest that apical processes are important for the process of synaptogenesis (see Morgan et al., 2006). Alternatively, DCX could be involved in nuclei stabilization, as the cellular location of DCX-immunoreactivity in these cells is strongly reminiscent of the perinuclear microtubule cage described during nuclear migration and stabilization processes (e.g. Koizumi et al., 2006; Tanaka et al., 2004). Of note, DCX-ir bipolar cells have not been described in the mature retina of teleosts or rat (Tozzini et al., 2012; Wakabayashi et al., 2008), which suggest that mechanisms regulating synaptogenesis or nuclei stabilization may vary between species. Additionally, our results showed DCX immunoreactivity in some cells in the retinal pigment epithelium (RPE). Although the

embryonic RPE is capable of proliferation and transdifferentiation into a neural retina in many vertebrates, this capability is lost in the majority of cases. Only adult urodeles and post-hatched birds retain this capability (Araki, 2007; Barbosa-Sabanero et al., 2012; Del Rio-Tsonis and Tsonis, 2003; Moshiri et al., 2004; Reh and Fischer, 2006; Wohl et al., 2012). It has been largely believed that regenerative potential in the adult retina of fishes was restricted to the CMZ and Müller cells, and that the RPE of fishes was unable to transdifferentiate into neurons (e.g. Wohl et al., 2012). Nevertheless, proliferating cells have been reported in the RPE of juveniles of *S. canicula (*Ferreiro-Galve et al., 2010a), so we suggesting that RPE cells in elasmobranch fishes have certain neuronal differentiation potential. Future studies are necessary to explore the ability of RPE cells as neuronal progenitors in the retina of elasmobranch fishes. This work shows that the retina of *S. canicula* constitutes a valuable model to further future investigations about the possible roles of DCX in relation with neurogenic events, since different levels of DCX immunoreactivity have been detected in NECs and neuroblasts or young migrating cells. DCX can be also detected in mature cells, all in the same neurogenic system. The retina of this elasmobranch specie compiles all the neurogenic events that can take place in other regions of the CNS (e.g. cortex), wherein DCX may be involved.

In this thesis, we also aimed at analyzing changes in RG during neurogenesis since radial glial cells (RGCs) are now accepted as the major source of neurons in several regions of the CNS (**Chapter 3**). We used the retina of *S. canicula* to analyze the processes underlying the transition from NEC to RGC, and RG differentiation from development to the adulthood. For that, we analyzed the distribution of the glial fibrillary acidic protein (GFAP) during the lifespan of *S. canicula* and explored its localization with respect to other cell markers, including glutamine synthetase (GS), specifically expressed by mature Müller cells. Our work showed GFAP immunoreactivity very early in development in the vitreal

endfeet of NECs at the time that all cells are proliferating, with nuclei located at different positions (which again matches the definition of IKNM; Baye and Link, 2007; Del Bene, 2011; Kosodo, 2012; Reiner et al., 2012; Spear and Erickson, 2012a, 2012b), and before the onset of the neurogenic process (since DCX expression is still not present at this point). Our observations in the neuroepithelial retina of *S. canicula* are very similar to that observed in other neuroepithelia along the CNS before the neurogenesis process begins (Götz and Huttner, 2005; Paridaen and Huttner, 2014). Shortly after, when neurogenesis start, we reported GFAP immunoreactivity in RGCs that could correspond to ***early RGCs***, according to the nomenclature used in the cortex (Alvarez-Buylla et al., 2001; Kriegstein and Alvarez-Buylla, 2009; Kriegstein and Götz, 2003; Lenkowski and Raymond, 2014; Sild and Ruthazer, 2011). Our results represent the first evidence of GFAP in early RG in the developing retina, also indicating that GFAP could be a marker for PCs in the early neuroepithelial retina of sharks. In the present work we also reported the transition from early RG to mature Müller glia. GFAP immunoreactivity increased in the inner third of cell processes that acquired an organized aspect. We defined these cells as ***late RGCs***. Our results based on GFAP and GS immunoreactivity, showed several changes in the appearance of these cells that acquiring the typical morphology of ***young Müller cells,*** coinciding with the maturation of the central retina and finally, these cells were identified as ***mature Müller cells*** in the basis of observations in the retina of other species. This expression pattern of GFAP and GS is maintained even in juveniles and adults. Our results about the presence of GFAP in radial processes in the mature Müller glia in *S. canicula* are in line with that reported in the mature retina of a few teleosts (Arenzana et al., 2011; Bignami, 1984; Linser et al., 1985; Nona et al., 1989; Yazulla and Studholme, 2001). However, in the adult retina of mammals, GFAP has been reported in astrocytes, while Müller cells do not contain or contain only low levels of GFAP (Björklund and Dahl, 1985; Gariano et al., 1996; Lemmon and Rieser, 1983; Sarthy et al., 1991). The presence of GFAP in

these cells could be related to their role as progenitors as has been proposed for the postnatal RG found in neurogenic niches in the adult brain of mammals. Additionally, our results showed an increase in GFAP immunoreactivity with age, probably because Müller cells play functions typical of astrocytes or oligodendrocytes in other regions (Bringmann et al., 2006; Newman and Reichenbach, 1996; Reichenbach and Bringmann, 2013; Tsacopoulos and Magistretti, 1996). This increase is in agreement with that shown in other brain areas in mammals (Masliah et al., 1993; Morgan et al., 1999; Nichols et al., 1993; O'Callaghan and Miller, 1991; Rozovsky et al., 2005). Regarding the CMZ, we found GFAP immunoreactivity in proliferating cells with radial morphology, which might represent transient progenitor RG. Our results suggest that the contribution of the CMZ to adult neurogenesis in *S. canicula* implies a transition through the RG state. So far, the fact that in teleosts neuroepithelial progenitors in the CMZ express both NSC and PC markers, but not mature glial markers as GFAP, has led to think that the chief contribution of the CMZ (non-glial) cells to generate all types of adult-born neurons does not implies an obligatory transition through the RG state. Of note, our results also shown GFAP immunoreactivity in the ciliary epithelium (CE), which pointed the possibility that cells of this region could serve as quiescent source of stem cells.

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Abbreviations

CE	ciliary epithelium
CMZ	ciliary marginal zone
CMZm	middle CMZ
CMZp	peripheral CMZ
CNS	central nervous system
DCX	doublecortin
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
IKNM	interkinetic nuclear migration
IPL	inner plexiform layer
-ir	immunoreactive
MAP	microtubule associated protein
NEC	neuroepithelial cell
NSC	neural stem cell
PC	progenitor cell
RG	radial glia
RGC	radial glia cell
RPE	retinal pigment epithelium
TZ	transition zone

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RESUMEN

RESUMEN

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Introducción

Es bien sabido que las células madre o células tronco neurales y las células progenitoras generan neuronas mediante el proceso denominado neurogénesis. Durante el desarrollo del sistema nervioso central (SNC) en los vertebrados, la neurogénesis ocurre en todas las regiones del tubo neural siguiendo un patrón espacio-temporal específico. La neurogénesis incluye además los procesos de migración y diferenciación neuronal, crecimiento axónico y arborización dendrítica, sinaptogénesis, y el establecimiento de las conexiones neuronales; todo ello interconectado y relacionado con la gliogénesis (generación de astrocitos y oligodendrocitos), la mielinización, la angiogénesis, y la formación de la barrera hematoencefálica.

Uno de los avances más recientes y emocionantes en el campo de la neurogénesis, ha sido el reconocimiento de la glía radial (o células gliales radiales) como progenitora neurogénica. A medida que avanza el desarrollo, las células madre neurales y/o las células progenitoras, que

tienen un potencial de proliferación menor que las primeras, van a generar diferentes tipos de neuronas y células gliales, siempre bajo el control de factores intrínsecos y extrínsecos. Las células madre neurales expresan marcadores como Sox2, GFAP (*glial fibrillary acidic protein*) y Nestina, pero hasta el momento no se conoce una combinación específica de marcadores que nos permita distinguir entre las células madre y las células progenitoras neurales. Dos tipos de células progenitoras neurales se han descrito durante el desarrollo del SNC en mamíferos: las células neuroepiteliales y las células gliales radiales, derivadas de las primeras y a partir de las cuales se generarán neuronas y células gliales. Tanto las células madre neurales, como las células gliales radiales, presentan morfología radial y polaridad; además, ambos tipos de células progenitoras comparten muchos marcadores. Al final de la neurogénesis, en la mayor parte de las regiones del cerebro no se conserva esta glía radial progenitora, sino que ésta dará lugar a células gliales maduras (astrocitos o células ependimarias).

A medida que el potencial neurogénico disminuye, las células progenitoras quedan recluidas a regiones concretas del cerebro conocidas como *nichos germinales*. Es en estos nichos donde se generarán nuevas neuronas en la edad adulta. Los progenitores postnatales, denominados células madre adultas, tienen capacidad para producir neuronas o células gliales, y comparten características y marcadores con la glía radial y con los astrocitos, lo que parece indicar que son, en realidad, progenitores embrionarios quiescentes que persisten en la etapa adulta reactivándose para producir diferentes tipos de neuronas y células gliales. Además, cuando se produce un daño, la neurogénesis también puede reactivarse en regiones no neurogénicas, como ocurre en el ojo o en la médula espinal. Sin embargo, los progenitores del SNC adulto de mamíferos, no son capaces por sí mismos de reemplazar las neuronas perdidas en las enfermedades

neurodegenerativas (reparación endógena). La glía radial en mamíferos se considera, por tanto, transitoria.

Contrastando con la situación en mamíferos, la glía radial se mantiene ampliamente en el SNC adulto de vertebrados anamniotas, que poseen además mecanismos de regeneración endógena. Algunos de estos mecanismos son comunes a los procesos que tienen lugar durante el desarrollo, mientras que otros difieren. Es en los peces teleósteos donde se han identificado el mayor número de regiones neurogénicas en la etapa adulta, lo cual se ha atribuido al crecimiento indeterminado mediante la adición de nuevas neuronas que presentan la mayor parte de las estructuras encefálicas y sensoriales. Sin embargo, la mayor parte de estos procesos neurogénicos se limitan a algunos nichos germinales, contradiciendo la hipótesis de que la neurogénesis adulta es sólo una cuestión de crecimiento continuado. Entre los teleósteos, el pez cebra es la especie en la que más se han estudiado los procesos de neurogénesis y regeneración adulta. A diferencia de los mamíferos, su rápido desarrollo y la temprana expresión de marcadores típicos de la glía radial dificultan la identificación de procesos como la transición de célula neuroepitelial a glía radial. A medida que avanza el desarrollo, la glía radial expresa otros marcadores, similares a los identificados en el SNC de los mamíferos durante el desarrollo. Sin embargo, a diferencia de éstos, el cerebro adulto del pez cebra presenta tanto células neuroepiteliales como glía radial, que podría actuar como progenitora, tanto durante la neurogénesis *constitutiva*, como en la neurogénesis *regenerativa*. Los mecanismos celulares que gobiernan estos procesos están aún por resolver.

De entre todos los sistemas neurogénicos del SNC del pez cebra, uno de los más estudiados ha sido, y es, la retina, por la alta tasa de neurogénesis adulta que presenta y por la implicación de la glía radial en los procesos de reparación.

En la formación de la retina neural están implicados numerosos genes. Inicialmente, la retina neural está formada por células progenitoras multipotenciales a partir de las cuales derivarán en un orden particular y relativamente conservado en los vertebrados, los seis tipos de neuronas retinianas y un tipo especializado de célula glial, la glía radial de Müller. La región vítrea (basal) se diferencia primero, siguiendo la retinogénesis un gradiente de maduración centro-periférico.

Además de la proliferación, la migración de las neuronas a sus posiciones finales es esencial para que éstas sean maduras y funcionales. Defectos en la migración causan diversas malformaciones del desarrollo en regiones como la corteza. La migración neuronal es, por tanto, un proceso crítico en el desarrollo del SNC, incluida la retina. La migración nuclear intercinética es uno de los modos de migración que presentan las células neuroepiteliales y también la glía radial. Los mecanismos moleculares no se conocen del todo, pero se sabe que los microtúbulos y las proteínas asociadas a microtúbulos están implicados en el movimiento del núcleo acorde con las fases del ciclo celular.

Los procesos de neurogénesis adulta en la retina del pez cebra tienen lugar en dos regiones diferentes: la glía de Müller, que puede reactivarse tras ciertos estímulos y que es, además, la fuente primaria de las células fotorreceptoras bastones; y la zona marginal ciliar (*CMZ*), que consiste en un anillo de células progenitoras en el margen de la retina y junto al epitelio ciliar, capaz de generar todos los tipos celulares de la retina, incluida la glía de Müller. Esta región es muy interesante por recapitular los eventos que tienen lugar durante la retinogenesis. En ella se distinguen tres zonas: una *CMZ*-periférica (junto al epitelio ciliar), que contiene los progenitores multipotenciales, una *CMZ*-media, donde se disponen los progenitores en proliferación con potencialidad más restringida, y una *CMZ*-central que contiene células postmitóticas pero aún indiferenciadas. Curiosamente, las células de la *CMZ* en el pez cebra

adulto expresan BLBP, un marcador de glía radial y astrocitos que se encuentra en las células neuroepiteliales y en la glía radial de los mamíferos durante el desarrollo y en progenitores en la etapa adulta. El hecho de que las células de Müller generadas a partir de la *CMZ* entren rápidamente en quiescencia, ha llevado a pensar que no existe una transición obligatoria a través de un estado de glía radial progenitora. Si las células de la *CMZ* en la retina adulta son, o no, verdaderas células madre, sigue siendo hoy en día un tema controvertido con muchas cuestiones por resolver. Así, dentro de la *CMZ*, ¿qué células son las que realmente contribuyen a la neurogénesis continua? Se sabe que estas células neuroepiteliales mantienen una alta tasa de proliferación, característica que no es típica de las células madre adultas. La identificación de marcadores que permitan la distinción entre las células neuroepiteliales quiescentes y los progenitores que están proliferando, proporcionaría una información relevante en torno a las jerarquías celulares dentro del nicho germinal. Además, en mamíferos se ha definido la multipotencialidad de las células madre adultas como una característica poblacional y no a nivel de células individuales. Por tanto, ¿cuál sería la situación de estas células en la *CMZ*?

Hoy en día, la degeneración de la retina es la principal causa de pérdida de visión en los seres humanos durante el envejecimiento. Desafortunadamente, no poseemos mecanismos de reparación endógena que nos permitan la restauración de las células fotorreceptoras. Los mecanismos de regeneración de la retina han sido objeto de extensas investigaciones en los últimos años. El bajo potencial regenerativo de los mamíferos se relaciona con la restricción de algunas vías de señalización, pudiendo estimularse en algunos casos, aunque la respuesta proliferativa sigue siendo muy limitada. La identificación de los procesos que subyacen a la neurogénesis en la retina, sobre todo en retinas con capacidad de regeneración endógena (principalmente en peces y algunos anfibios), es esencial para llegar a comprender el

potencial y las limitaciones de la regeneración retiniana en mamíferos, incluidos los seres humanos. Estos mecanismos no sólo incluyen los procesos de proliferación y diferenciación, sino también los procesos de migración y posicionamiento celular. Así, el estudio comparado de los mecanismos conservados y específicos de los nichos germinales en los diferentes grupos de vertebrados es crucial para diseñar estrategias que estimulen la neurogénesis adulta en mamíferos. Estudios recientes han evidenciado que la neurogénesis constitutiva en etapas adultas es específica de las regiones y de las especies, por lo tanto, la cuestión tendría que ver más con la regulación específica de estos mecanismos en cada región, así como los mecanismos de desarrollo que determinarán que estas células continúen generando neuronas a lo largo de la vida del organismo, o bien que se detenga en determinado momento. Lo que sí se sabe es que la capacidad neurogénica en etapas adultas ha disminuido a lo largo de la evolución.

Los peces elasmobranquios presentan neurogénesis adulta, lo cual señala, debido a su posición filogenética, que la neurogénesis adulta es un rasgo evolutivamente conservado. De entre los peces cartilaginosos, la pintarroja (*Scyliorhinus canicula*) ha sido muy utilizada en estudios de desarrollo, principalmente por el fácil mantenimiento de los embriones y su lento desarrollo. La organización de la retina de los peces elasmobranquios es prácticamente la misma que presentan otros vertebrados, y su patrón espacio-temporal de proliferación y diferenciación ha sido estudiado previamente. Particularmente, la retina de *S. canicula* es un excelente modelo para estudiar los procesos de neurogénesis embrionaria y adulta por los siguientes motivos: (1) es una estructura laminada y muy organizada, como en todos los vertebrados; (2) al igual que en otros peces, la *CMZ* presenta proliferación durante toda la vida del animal; (3) el tipo de célula glial predominante es la glía de Müller, como en otros peces; (4) a diferencia del rápido desarrollo que presenta la retina de peces teleósteos como el pez cebra, la retina de la

pintarroja presenta un período prolongado de proliferación en la retina central y una zona de transición que bordea la *CMZ* y que contiene células tempranas diferenciadas y células neuroepiteliales. En particular, la presencia y el tamaño de la *CMZ*, y la zona de transición (*TZ*), hacen que la retina de la pintarroja se presente como un buen modelo para resolver algunas de las cuestiones planteadas en el contexto de la neurogénesis: podría facilitar la identificación de los cambios moleculares en la transición de célula madre a célula progenitora y a célula diferenciada. También podría servir para definir mejor la transición de célula neuroepitelial a célula glial radial progenitora durante el desarrollo, algo que, hasta el momento, no parece obvio en peces teleósteos. Asimismo, sería interesante la caracterización de las células madre y/o las células progenitoras presentes en la *CMZ*. Finalmente, podría ayudar a resolver si la contribución a la neurogénesis adulta en la *CMZ* implica, o no, una transición a través de un estado de glía radial.

Nuestro objetivo, en sentido amplio, ha sido contribuir a llenar el vacío de conocimiento que existe en torno a la neurogénesis constitutiva, utilizando la retina como sistema por las características tan extraordinarias que posee y, en particular, la retina de la pintarroja como organismo modelo, aportando además información sobre aspectos de la neurogénesis más conservados evolutivamente. Debido a la importancia de la migración y el posicionamiento celular en la neurogénesis, hemos analizado el patrón de expresión espacio-temporal de la doblecortina (DCX) en la retina de la pintarroja, ampliamente considerado como un marcador de neuroblastos en migración en el desarrollo del SNC y también en etapas adultas (Capítulos 1 y 2). Además, hemos tratado de diferenciar las células neuroepiteliales de la glía radial progenitora y de la glía radial madura, durante el desarrollo y también en la retina madura, con especial atención a la *CMZ* y a la *TZ* (Capítulo 3).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CAPÍTULO 1: Organización espacio-temporal de la inmunorreactividad a DCX en la retina de *Scyliorhinus canicula.*

Durante los procesos de migración celular, las células van a sufrir una serie de cambios morfológicos que dependen de la reorganización de los componentes del citoesqueleto y en el que se ven implicadas numerosas proteínas. Deleciones y mutaciones en genes relacionados con la organización del citoesqueleto, causarán defectos en la migración cortical. Uno de estos genes, es el gen que codifica para la proteína de unión a microtúbulos DCX. Estudios inmunohistoquímicos han mostrado la expresión de DCX mayormente en células que no expresan Nestina (marcador de células madre), y además, su expresión disminuye conforme avanza la maduración celular. Por eso, durante el desarrollo, ha sido muy utilizado como marcador de neuroblastos en migración, pero también en nichos germinales en el cerebro adulto. Frente a este patrón general de expresión, se han descrito algunas excepciones, encontrándose DCX en células inmunorreactivas para Nestina, además de en algunas regiones adultas no asociadas a neurogénesis adulta, por lo que se le han atribuído funciones relacionadas con la plasticidad neuronal. A pesar de la importancia de la DCX en el desarrollo y posicionamiento neuronal, se le ha prestado muy poca atención a su expresión en la retina, una estructura laminada. Tan sólo se ha estudiado su distribución en la retina en desarrollo de pollo y rata, y en la retina adulta de una especie de pez teleósteo y en rata.

Como hemos expuesto en la introducción (ver arriba), la retina de la pintarroja se ha utilizado para el estudio de diversos aspectos relacionados con la neurogénesis, por eso nos ha parecido interesante estudiar la distribución de la DCX. Estudios previos de nuestro grupo han dividido el desarrollo de la retina de *S. canicula* en tres períodos: un *primer período* que comprende los estadíos 26-29, caracterizado por la apariencia neuroepitelial de la retina y la ausencia de laminación; un

segundo período que incluye los estadios 30-32 que se define por la formación progresiva de las capas en la retina central y permite la distinción de la *CMZ* (adyacente al el epitelio ciliar) y la *TZ* (entre la *CMZ* y la retina central laminada; presenta una región interna laminada y una región externa con apariencia neuroepitelial); y un *tercer período* que comprende desde el estadio 33 en adelante, en el que la laminación progresa siguiendo un gradiente centro-periférico y en el que la retina adquiere la organización característica de estadios postembionarios.

Este trabajo ha mostrado por primera vez la distribución de DCX a lo largo del desarrollo en la retina de un pez, aportando información acerca de las posibles funciones de esta proteína durante la neurogénesis del SNC. Curiosamente, hemos detectado una expresión débil de DCX en las células de la *CMZ* más periférica (células neuroepiteliales), tanto durante el desarrollo como en la retina madura, indicando su presencia en regiones donde se generan nuevas células (nichos germinales). Su presencia en la retina neuroepitelial, un hecho no descrito previamente, nos ha llevado a considerar su posible implicación en la migración nuclear intercinética en la retina de los peces. Además de estar presente en células y prolongaciones de la retina interna e interna en el momento en el que las células se diferencian y se establecen nuevas conexiones sinápticas, hemos visto que este patrón se mantiene en el tiempo (juveniles), incluso después de que los procesos de migración celular hayan terminado. Este es el caso en las células horizontales y en las células bipolares en la retina adulta, donde la DCX podría desempeñar funciones relacionadas con la plasticidad neuronal y la sinaptogénesis en células ya diferenciadas y maduras. Sin embargo, serían necesarios estudios de co-localización con marcadores de proliferación y diferenciación celular para elucidar mejor su participación en los procesos de neurogénesis.

CAPÍTULO 2: Doblecortina y neurogénesis durante el desarrollo y en la retina madura de tiburones: un estudio inmunohistoquímico comparado con marcadores de proliferación y diferenciación.

En el capítulo anterior describimos por primera vez el patrón de distribución de esta proteína en la retina de peces. En este trabajo hemos estudiado su relación con marcadores de proliferación (PCNA) y diferenciación (HuC/D) celular, además del marcador glial GFAP. Debido a su presencia en células maduras y diferenciadas (Capítulo 1), hemos querido además caracterizar estas células utilizando CB (marcador de células horizontales y bipolares) y Pax6 (regulador de la proliferación, diferenciación y diversificación neuronal en la retina de *S. canicula*). Asimismo, hemos querido confirmar si la expresión de DCX se mantiene incluso en la retina de ejemplares adultos. Además, teniendo en cuenta los resultados de un trabajo previo que muestra la expresión de DCX en células del epitelio retiniano pigmentario *in vitro* de mamíferos, hemos prestado especial atención a esta estructura.

El estudio conjunto de la DCX con el marcador de proliferación PCNA nos ha permitido localizar la expresión de DCX en los pies de las células neuroepiteliales de la retina neuroblástica de la pintarroja, rodeando el núcleo de las mismas (PCNA+). Aunque DCX suele detectarse justo al final de la mitosis, se han aislado diferentes poblaciones de células de cerebro adulto de ratón con diferentes niveles de DCX, una de ellas con capacidad multipotencial, lo que indica que es posible encontrar bajos niveles de DCX en células que están proliferando. En estas células, la DCX podría estar implicada en procesos de migración nuclear intercinética, ya que se ha visto que el núcleo de las células que experimentan este movimiento lo hacen rodeadas de una "cesta de microtúbulos". En este trabajo hemos mostrado que el aumento de la expresión de DCX coincide con el momento en que las células empiezan a diferenciarse (PCNA-/HuC/D+) en la retina interna, confirmando así su

papel como marcador de precursores neurales. Asimismo, su aumento en la capa plexiforme interna y en la capa de fibras del nervio óptico, coincide con el momento en el que comienza la laminación de la retina, manteniéndose en etapas maduras. En la retina madura, hemos mostrado que la expresión de DCX se mantiene en células ganglionares, amacrinas, bipolares, y horizontales. A pesar de que DCX en etapas adultas se ha relacionado mayormente con la neurogénesis adulta, en pintarroja se encuentra en células alejadas de los nichos germinales, donde co-localiza con los marcadores postmitóticos HuC/D, Pax6 y CB. Nuestros resultados descartan que DCX sea un marcador específico de neurogénesis en la etapa adulta, y es compatible con las funciones sugeridas en estudios previos para esta proteína, incluyendo la estabilización nuclear, la remodelación sináptica y el crecimiento de neuritas, procesos requeridos durante el crecimiento continuo propio de la retina de los peces. En relación al epitelio retiniano, en este capítulo mostramos como unas pocas células del mismo son positivas para DCX. Aunque hacen falta más estudios en torno a esto, estas células podrían tener cierta capacidad de diferenciación neuronal. Este trabajo ha revelado que la retina de la pintarroja es un valioso modelo para investigar las posibles funciones de la DCX en la neurogénesis, migración y sinaptogénesis. Además, la retina periférica recapitula el patrón de distribución de DCX observado a lo largo del desarrollo. El ritmo con el que crece la retina y el tamaño de la CMZ y TZ suponen una gran ventaja de la retina de este tiburón con respecto a los peces teleósteos.

CAPÍTULO 3: Identificación de la glía radial progenitora en la retina de *Scyliorhinus canicula*.

Tal y como describimos brevemente en la introducción (ver arriba), la identificación de la glía radial como progenitora neurogénica ha constituido un reciente y apasionante avance en el campo de la neurogénesis. La contribución de la glía radial a la neurogénesis adulta ha sido y es motivo de intensas investigaciones. La retina es uno de los pocos sistemas donde se mantiene la glía radial (glía de Müller) en el adulto, una célula glial que resulta en sí misma determinante, particularmente en la retina de peces, no sólo por sus relevantes funciones en la función retiniana, sino por las propiedades neurogénicas que posee. La glía de Müller participa en la proliferación en el adulto, generando células precursoras de fotorreceptores bastones durante toda la vida del pez, pero además, tras daño, es capaz de desdiferenciarse y generar todos los tipos celulares de la retina, actuando como auténticas células madre multipotenciales.

Estudios previos en la retina del pez cebra no han evidenciado una transición obligatoria desde las células progenitoras neuroepiteliales a través del estado de glía radial en la neurogénesis continua que tiene lugar en la retina periférica. La retina de la pintarroja ofrecía un marco excepcional para analizar en profundidad la transición de célula neuroepitelial a glía radial progenitora, así como su evolución a glía madura de Müller. Además, la presencia de células neuroepiteliales en la *CMZ*, la presencia de una *TZ* que contiene tanto células neuroblastos como células en diferenciación temprana, y el desarrollo lento que presenta la retina de esta especie, hizo que la retina de este tiburón se nos presentara como un excelente modelo para este estudio. Para identificar la glía radial utilizamos dos marcadores, GFAP y GS (*glutamine sinthetase*), y estudiamos su relación con la neurogénesis utilizando

marcadores de proliferación (PCNA; *proliferating cell nuclear antigen*), y neuroblastos en migración (DCX).

En este capítulo mostramos como la expresión de GFAP aparece tempranamente en los pies de las células neuroepiteliales, cuando toda la retina está proliferando y los núcleos se encuentran a diferentes alturas confiriéndole el aspecto pseudoestratificado característico de los neuroepitelios. En este momento aún no detectamos expresión de DCX, por lo tanto no podemos conocer si esas células pertenecerán o no al linaje neuronal. Nuestras imágenes muestran que con el comienzo de la neurogénesis (PCNA+, DCX+), las células neuroepiteliales se alargan transformándose en células gliales radiales, que en estos estadíos tempranos hemos denominado ***células gliales radiales tempranas***, acorde con el nombre que reciben en la corteza, un hecho no descrito antes en la retina. Sabiendo que las células gliales radiales poseen características de células progenitoras en el cerebro en desarrollo, hemos visto que la GFAP podría ser un marcador de células progenitoras en la retina neuroepitelial de los tiburones. Hemos mostrado que, a medida que comienza la diferenciación en la retina interna (PCNA-, DCX+), se produce un aumento en los niveles de GFAP en dicha región, aunque todavía se observa un aspecto desorganizado de la inmunorreactividad a GFAP en las prolongaciones celulares. Consideramos a estas células como ***células gliales radiales tardías*** (GFAP+) las cuales adquieren poco después, y coincidiendo con la maduración de la retina central, el aspecto de ***células de Müller jóvenes*** (GFAP+, GS+). Nuestros resultados muestran que estas células adquieren la morfología típica de ***células de Müller maduras*** (GFAP+, GS+) cuando toda la retina adquiere la madurez típica de estadíos postembrionarios. Además, en este capítulo hemos mostrado la expresión de GFAP en células neuroepiteliales de la CMZ (PCNA+), por lo que hemos propuesto que estas células GFAP+ con morfología radial podrían representar células radiales progenitoras que se originan a partir de las células neuroepiteliales. En definitiva, este trabajo

representa la primera evidencia de que la contribución de la *CMZ* a la neurogénesis adulta, efectivamente implica una transición vía estado de glía radial. Además, hemos mostrado como el epitelio ciliar muestra un fuerte marcaje para GFAP (PCNA- o con muy baja expresión de PCNA), lo que nos ha llevado a pensar que las células del epitelio ciliar podrían ser una fuente de células madre quiescentes implicadas en el desarrollo y crecimiento continuo de la retina de peces.

CONCLUSIONS

CONCLUSIONS

1. Our results have shown the distribution pattern of DCX protein for the first time in the retina throughout the lifespan of a fish.

2. The distribution pattern of DCX protein in the developing and mature retina of sharks contributes to gain knowledge about the involvement of DCX in the neurogenic process, not only in the retina, but also in other structures of the CNS. The retina of *S. canicula* appears as a valuable model to further investigate the significance of DCX in promoting cell differentiation, migration or synaptogenesis.

3. We reported for the first time the presence of DCX in neuroepithelial cells in the retina of *S. canicula*, a fact that has never been described before in any other species. Thus suggesting that DCX protein could be involved in interkinetic nuclear migration in the retina of sharks.

4. We reported DCX in cells and their processes in the inner plexiform layer from the time that cell differentiation and retinal lamination begins in the embryonic retina up to the adulthood. The increase in labeling coincides with early stages of differentiation of a subpopulation of ganglion, amacrine, horizontal and bipolar cells at the time that connections are established during development, suggesting that DCX is an early marker of differentiation of specific cells.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

5. DCX labeling is found in ganglion, amacrine, horizontal and bipolar cells in the mature retina, long after cell differentiation and positioning has finished. The fact that these cell types are located away from germinal niches pointed out that this protein might be involved in roles other than adult neurogenesis in the mature retina of sharks, such as nuclear stabilization and synaptic remodeling, both required for the continued growth of the retina in fishes.

6. DCX pattern distribution pattern in the ciliary marginal zone (CMZ) has and allowed us to distinguish two regions within CMZ: the peripheral CMZ containing low levels of DCX, and the middle CMZ where DCX immunoreactivity increases.

7. DCX is found in some cells of the retinal pigment epithelium, thus suggesting that some retinal pigment epithelial cells in sharks may display neural progenitor identity.

8. Differences in the intensity and appearance of GFAP immunoreactivity, together with the combined distribution of GFAP with that of GS, DCX and PCNA within the spatio-temporal frame provided by the peripheral retina allows to differentiate different types of progenitors in the retina of sharks.

9. Neuroepithelial cells are found in the peripheral CMZ and share phenotypic properties with neuroepithelial cells elsewhere in the CNS. They present GFAP immunoreactivity in their endfeet and co-distribute with weakly DCX-immunoreactive cells.

10. Early and late radial glia are located in the middle CMZ and TZ, respectively, co-distributing with strongly DCX-immunoreactive cells. They are characterized by the presence of disheveled GFAP-

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immunoreactivity in endfeets and in the inner third of cell processes extending towards the outer retina.

11. Early and late Müller cells are found in progressively more central regions of the mature (layered) retina. These cells are characterized by the presence of neatly arranged GFAP-immunoreactivity in cell processes and GS-immunoreactivity in somas and cell processes.

12. The same types of progenitors are found in juvenile specimens, which suggest that the contribution of the CMZ cells to generate all types of adult-born neurons implies a transition through the radial glial state even in adults.

13. The presence of GFAP in cells of the ciliary epithelium suggests that these cells could contribute to the continuous renewal of the pool of stem cells present in the CMZ.

14. The detailed neurochemical characterization of different progenitor cells within each region of the peripheral retina of sharks could serve as a useful starting point for characterizing progenitor cells in other regions of the CNS, where the absence of a spatio-temporal frame makes difficult to differentiate the transition from one type of progenitor to another.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CONCLUSIONES

1. Nuestros resultados han mostrado el patrón de distribución de la proteína DCX por primera vez en la retina a lo largo de la vida de un pez.

2. El patrón de distribución de la proteína DCX durante el desarrollo y en la retina madura de tiburones contribuye a mejorar el conocimiento sobre la implicación de la DCX en los procesos neurogénicos, no sólo en la retina, sino también en otras estructuras del SNC. La retina de *S. canicula* aparece como un valioso modelo para futuras investigaciones en torno al significado de la DCX en promover la diferenciación celular, migración y sinaptogénesis.

3. Hemos mostrado por primera vez la presencia de DCX en células neuroepiteliales en la retina de *S.* canicula, un hecho que no había sido descrito antes en otras especies. Ésto sugiere que la proteína DCX podría estar implicada en la migración nuclear intercinética en la retina de los tiburones.

4. Hemos mostrado DCX en células y en sus prolongaciones en la capa plexiforme interna a partir del momento en que comienza la diferenciación y la laminación de la retina hasta la etapa adulta. El aumento del marcaje coincide con las etapas tempranas de diferenciación de subpoblaciones de células ganglionares, amacrinas, bipolares y horizontales, en el momento en el que se establecen las conexiones durante el desarrollo, sugiriendo que la DCX es un marcador de diferenciación tempana de poblaciones celulares específicas.

5. En la retina madura el marcaje de DCX se encuentra en células ganglionares, amacrinas, bipolares y horizontales en la retina madura, mucho tiempo después de que los procesos de diferenciación y posicionamiento de las células hayan terminado. El hecho de que estas células se localicen lejos de los nichos germinales indica que esta proteína podría estar implicada en funciones distintas de la neurogénesis adulta en la retina madura de tiburones, tales como la estabilización nuclear y la remodelación sináptica, ambos procesos necesarios para el crecimiento continuo de la retina en los peces.

6. El patrón de distribución de DCX en la zona marginal ciliar (CMZ) nos ha permitido distinguir dos regiones dentro de la misma: la CMZ periférica, que presenta bajos niveles de DCX, y la CMZ central, donde la inmunorreactividad a DCX aumenta.

7. DCX se encuentra en algunas células del epitelio retiniano pigmentario, lo que sugiere que algunas células del epitelio retiniano en tiburones podrían mostrar identidad de progenitor neural.

8. Las diferencias en la intensidad y en el aspecto de la inmunorreactividad a GFAP, junto con la distribución combinada de GFAP con GS, DCX y PCNA en marco espacio-temporal que proporciona la reina periférica, permite diferenciar diferentes tipos de progenitores en la retina de los tiburones.

9. Las células neuroepiteliales se localizan en la CMZ periférica y comparten propiedades fenotípicas con las células neuroepiteliales de otras regiones del SNC. Estas células presentan

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inmunorreactividad a GFAP en sus pies y co-distribuye con células débilmente inmunorreactivas para DCX.

10. La glía radial temprana y tardía se localizan en la CMZ central y en la zona de transición (TZ), respectivamente, co-distribuyendo con células fuertemente inmunorreactivas para DCX. Se caracterizan por presentar inmunorreactividad a GFAP en los pies y en el tercio inferior de las prolongaciones que se extienden hasta la retina externa.

11. Las células tempranas y tardía de Müller se localizan en regiones cada vez más centrales de las retina madura (laminada). Estas células se caracterizan por la presencia de prolongaciones bien ordenadas inmunorreactivas para GFAP y para GS, también presente en los somas.

12. Los mismos tipos de progenitores se encuentran en ejemplares juveniles, lo que sugiere que la contribución de las células de la CMZ para generar todos los tipos de neuronas nuevas implica una transición a través del estado de glía radia incluso en adultos.

13. La presencia de GFAP en células del epitelio ciliar sugiere que estas células podrían contribuir a la renovación continua de la reserva de células madre presentes en la CMZ.

14. La caracterización neuroquímica detallada de diferentes células progenitoras dentro de cada región de la retina periférica de tiburones podría servir como un punto de partida útil para la caracterización de las células progenitoras en otras regiones del SNC, donde la ausencia de un marco espacio-temporal hace difícil diferenciar la transición de un tipo de progenitor a otro.

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APPENDIX

Retinal regeneration in sharks

In addition to collecting the main issues surrounding the different sources of regeneration in vertebrates, this appendix includes some of the experimental approaches focusing on retinal damage and pupil dilation in *Scyliorhinus canicula,* and a brief summary of my training stay in the MOKALAB (CRTD, Dresden, Germany).

APPENDIX: RETINAL REGENERATION IN SHARKS

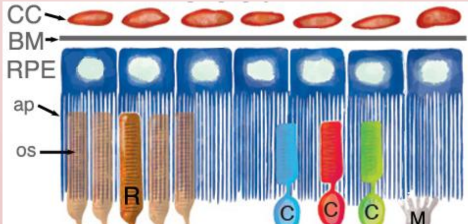
1. Retinal regeneration and diseases

The neural retina is a part of the central nervous system (CNS) located at the back of the vertebrate eye, containing neurons which capture the light and send electrical impulses to the visual cortex in the brain. The neural retina contains six major classes of neurons including photoreceptor cells (cones and rods), which transmit the visual sensory information to the inner retina through interneurons (**see BOX 3 in General introduction** and Masland, 2012). In vertebrates, the light detecting unit comprises two elements: the photoreceptor cell and the pigmented cell (Lamb et al., 2007). The monolayer of pigmented cells constitutes the retinal pigment epithelium (RPE; **BOX 1**), whose interaction with photoreceptor outer segments is essential for visual function (Chiba, 2014; Martínez-Morales et al., 2004; Strauss, 2005).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

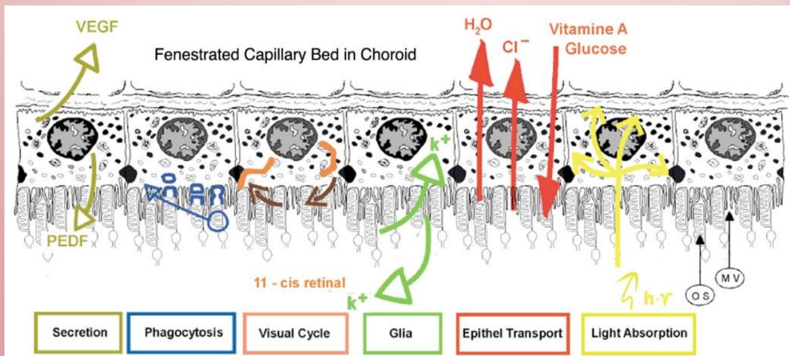
BOX 1: RPE

The RPE is a monolayer of pigmented cells (characterized by the presence of the melanosome organelle, a store of melanin pigment (Marks and Seabra, 2001) located between the outer segments of the photoreceptor cells and the choroid (**see picture below**). In *S. canicula* the RPE is not-pigmented.



CC, choriocapillaris; BM, Bruch's membrane; ap, apical processes of RPE enwrapping photoreceptor outer segments; os, outer segments, R, rods; C, cones. Taken from: webvision.med.utah.edu.

During development of the vertebrate eye, the RPE differentiates from the neuroectoderm of the optic vesicle as a partner of the neural retina (Martínez-Morales et al., 2004). Although the RPE is not a defined part of the neural retina, after maturation, it constitutes an indispensable element for the function and survival of this tissue (see RPE roles summarized in the schematic drawing above).



VEGF, vascular endothelial growth factor; PEDF, pigment epithelium-derived factor. Taken from Strauss (2005).

Analyses of different types of inherited retinal degenerations have shown a strong interdependence between the RPE and the photoreceptor cells (Garweg et al., 2013; Ramsden et al., 2013).
Differentiated cells of the RPE are typically mitotically quiescent. However, after damage in the neural retina, cells of the RPE in various groups of vertebrates begin to proliferate and differentiate into retinal progenitor cells (**see Section 2.3**).

As happens in other regions of the CNS, many acquired or inherited neuronal degenerative diseases that affect the neural retina result in visual detriment and compromised vision, often causing complete blindness (Farrar et al., 2015). Some of the most common retinal degenerations are diabetic retinopathy, retinitis pigmentosa, glaucoma and age-related macular degeneration, the latter being one of the major causes of blindness in the elderly (Dang et al., 2015; Enzmann et al., 2009). Besides these, there are many other inherited and acquired conditions that cause vision loss, all of them mediated by an irreversible loss of photoreceptors. Nowadays, retinal degenerative diseases are an important health problem and the main reason of vision loss in the aging human (www.vision2020.org). Several studies have reported that visual recovery depends on restoring photoreceptor cells. Unfortunately, mammals (including humans) do not possess self-endogenous regeneration and therefore, when retinal ganglion cells (GCs) are lost in glaucoma or photoreceptors in age-related macular degeneration, these neurons cannot be restored. Diverse strategies for different types of retinal diseases have been developed in humans in the last few years (Alexander et al., 2015; Baker and Brown, 2009; Dang et al., 2015; Garcia et al., 2015; Humayun et al., 2003; Limb and Daniels, 2008; MacLaren et al., 2006; Radtke et al., 2008), and important progress has been made by successful transplantation of RPE and photoreceptor cells in the adult retina (reviewed in Osakada and Takahashi, 2015). However, no therapies are available up to date to stimulate endogenous retinal regeneration and to prevent epi-/sub-retinal gliotic scar formation (Karl and Reh, 2010; Lamba et al., 2008, 2009), and it remains largely unknown how new photoreceptors integrate within retinal circuits and contribute to vision restoration (Osakada and Takahashi, 2015).

However, it has been reported that non-mammalian vertebrates (e.g. birds) shown some capacity for endogenous retinal regeneration

(Fischer and Bongini, 2010; Fischer et al., 2014; Moshiri et al., 2004; Reh and Fischer, 2001). In this context, the comparison of the mechanisms underlying retinal development and regeneration among different vertebrate groups with different regeneration capacities appears as a valuable strategy to identify the limitations of retinal regeneration in mammals. Throughout the life of fishes, amphibians, reptiles and birds, different cell sources generate new neurons in the adult retina, not only in response to retinal damage, but also under physiological conditions (**see Section 4.2** and **Fig. 6 in General introduction**).

In response to injury, the retina of the different classes of vertebrates possesses different strategies to restore the visual function. In some vertebrates, retinal regeneration mechanisms are spontaneously triggered to replace neurons. After damage, the retina can respond with regeneration mechanisms or with the formation of a glial scar. Regeneration involves the addition on new cells which replace damaged cells restoring the functional retina. The formation of a glial scar (**see below in BOX 2**) does not restore the retinal functions, but involve the repayment of the damaged retina with reactive astrocytes and proteoglycans (Fischer and Bongini, 2010).

2. Potential sources of neurogenesis in the adult retina of different vertebrates

Retinal stem cells present in the ciliary marginal zone (CMZ), the ciliary epithelium (CE), the RPE and the Müller glia (MG), are the main cellular sources of retinal regeneration in different vertebrate classes (Amato et al., 2004; Barbosa-Sabanero et al., 2012; Fischer and Bongini, 2010; **summarized in Table 1**). Many reviews published in the last years describe retinal regeneration in non-mammalian vertebrates (Fischer et

al., 2014; Gallina et al., 2014; Karl and Reh, 2010; Moshiri et al., 2004; Otteson and Hitchcock, 2003; Stenkamp, 2007; **Fig. 1**).

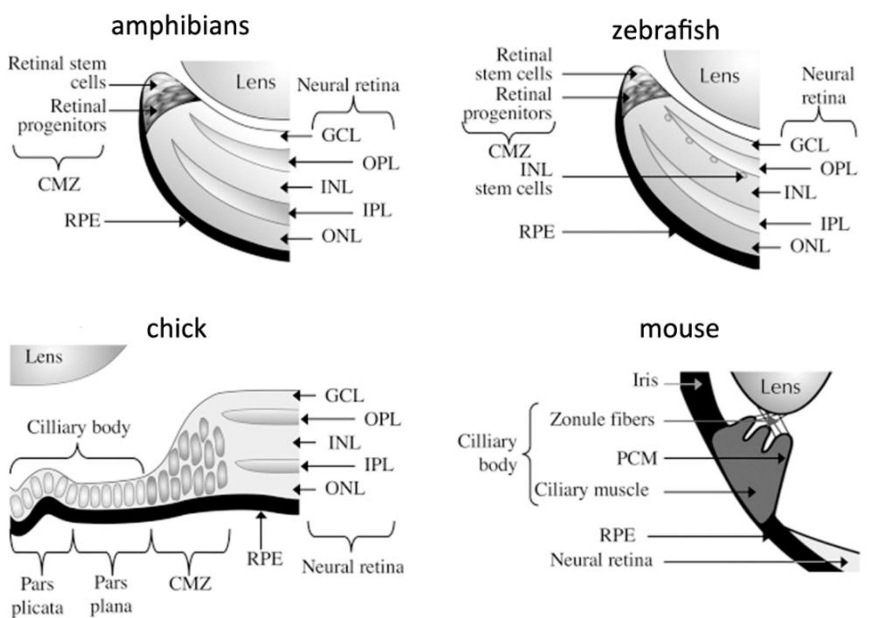


Figure 1. This scheme represents the different locations of stem cells in the retina of different vertebrates. Taken from Amato et al. (2004).

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.1. Regeneration from the CMZ

Amphibians and teleost fishes are important models to study adult retinal neurogenesis since in both cases the retina continues to grow in parallel to overall eye growth throughout life (Amato et al., 2004; Fischer and Bongini, 2010). The retina grows by the addition of new cells produced by neural stem cells clustered within the most peripheral zone of the CMZ (Otteson and Hitchcock, 2003; Perron et al., 1998; Reh and Levine, 1998). The CMZ of teleost fishes and frogs contains a population of true retinal stem cells that, after damage, contribute to retinal regeneration and are capable of generating most of the mature retina

(Fischer et al., 2014; Perron et al., 1998; Perron and Harris, 2000). However, an unexpected trait of cells in the CMZ is the high proliferating rate that does not match with the long-lasting maintenance of stem cells, which raised the hypothesis of a continuous renewal of the pool from a different quiescent source (**see General introduction**). In chick, most of the retina is formed during earlier embryogenesis (Prada et al., 1991) and at the time of hatching the retina is fully functional. Similar to that found in the retina of amphibians and teleost fishes, a proliferative marginal zone has been described in postnatal birds, although the potential to generate new cells is more limited, as they can generate bipolar and amacrine cells but not ganglion or photoreceptor cells (Fischer and Reh, 2000; Kubota et al., 2002; Tood et al., 2015). However in birds, there is evidence that cells of the postnatal proliferative marginal zone does not take part in regeneration (Fisher, 2005; Fischer and Reh, 2000). Regarding reptiles, turtles also have progenitors in the marginal zone that persists into the adulthood (Todd et al., 2015). There are no evidences of proliferation at the peripheral zone in the normal mammalian retina, although in young marsupials, newborns neurons have been shown to be generated from a rudimentary proliferative marginal zone similar to that of birds and turtles (Kubota et al., 2002).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.2. Regeneration from the CE

The ciliary body is another structure derived from the optic vesicle during embryonic development, adjacent to the CMZ, between the neural retina and the iris. Depending on the species two regions can be distinguished in this epithelium (**Fig. 2**): the *pars plana* and the *pars plicata*. The epithelium of the ciliary body, the CE, comprises two layers: a pigmented epithelium and a cuboidal non-pigmented epithelium. In birds (chick), the non-pigmented epithelium of the CE contains quiescent stem cells with neurogenic potential which can proliferate and generate GCs

when they are stimulated with growth factors (Fischer and Reh, 2003). It has been found that the pigmented CE of mammals contains cells which can be induced to acquire properties of stem cells in culture (Tropepe, 2000). In rats and mice, some cells of the pigmented CE proliferate *in vivo* postnatally for one week (Kubota et al., 2002), however, it is under debate if these cells are real retinal stem cells or they are differentiated pigmented cells that proliferate under given conditions in culture (Cicero et al., 2009).

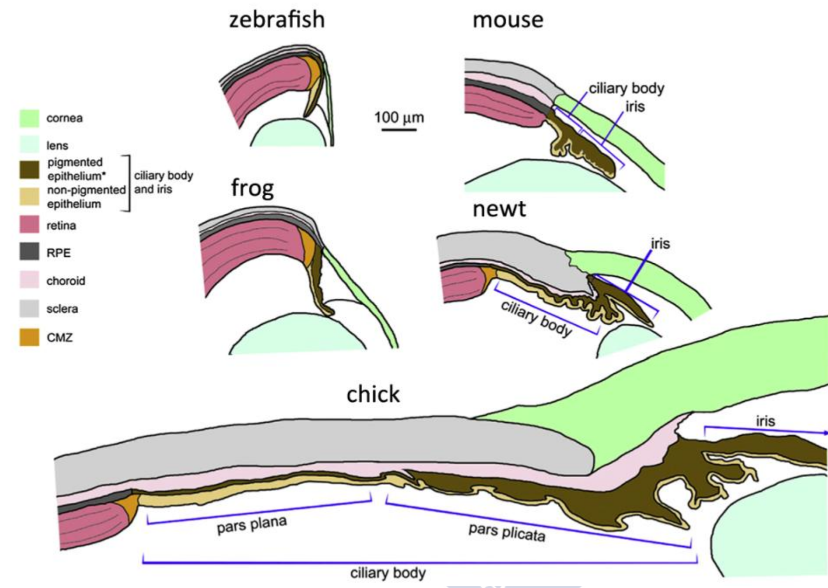


Figure 2. The ciliary body constitutes a large structure in newts and birds, but is relatively small in mice. In the eyes of zebrafish and frogs, the ciliary body is extremely reduced and no clear anatomical distinction exists between the pars plana, pars plicata or even the iris. Modified from Fischer et al. (2014).

2.3. Regeneration from the RPE

The RPE is a source of retinal stem cells in amphibians; in fact, in these animals the RPE constitutes the most important source of regeneration. Following retinal injury, RPE cells dedifferentiate, loss their

pigmentation and generate neurons in a process named transdifferentiation (Araki, 2007; Barbosa-Sabanero et al., 2012; Moshiri et al., 2004; Reh and Fischer, 2006), which is capable to form a complete retina (Chiba, 2014). RPE transdifferentiation has been also reported during embryonic stages in birds (chick) and mammals (rodents) though they lose the proliferative capacity of RPE cells during embryogenesis (Zhao et al., 1995). However, the mechanism in mammals is quite different from that reported in amphibians, since it depends on exogenous factors and the RPE does not replenish itself because it loses its plasticity (reviewed in Araki, 2007; Barbosa-Sabanero et al., 2012). In teleost fishes, it has been largely assumed that RPE cells do not have any regeneration potential (Raymond and Hitchcock, 2000; reviewed in Wohl et al., 2012). However, previously results from our group have been reported that some cells of the RPE shown proliferation capacity in the small shark *S. canicula* (Ferreiro-Galve et al., 2010a; **see also Chapter 2**).

2.4. Regeneration from the MG

In addition to the CMZ, the other important source of proliferating cells in the neural retina in birds (chicks), amphibians (frogs) and fishes are the MG (Centanin and Wittbrodt, 2014; Fischer and Reh, 2001). Under normal conditions, MG are the predominant type of support cell in the retina (reviewed in Bringmann et al., 2009; Reichenbach and Bringmann, 2013). In birds, MG serve as a source of new neurons after retinal injury and also in the presence of exogenous growth factors (insulin or FGF) either without damage or after the injection of a cytotoxic substance such as NMDA (N-methyl-D-aspartate). Of note, the neurogenic potential of MG in absence of exogenous growth factors is very limited (Barbosa-Sabanero et al., 2012; Fischer and Bongini, 2010). In fishes, rod progenitors are continually derived from MG (Bernardos et al., 2007). In

addition, upon damage, MG dedifferentiate and proliferate producing progenitor cells which can give rise to all retinal cell types (Goldman, 2014; Gorsuch and Hyde, 2014; Lenkowski and Raymond, 2014; **see Fig. 7 in General Introduction**). The regenerative potential of the MG in both birds and fishes make the MG a really interesting target for the treatment of retinal degenerative diseases in mammals. Despite the regeneration potential is much more restricted in mammals than in other animals, it has been reported that MG in mammals, even if it do not react spontaneously after damage, can be induced to proliferate using growth factors, transcription factors or amino acids (Karl et al., 2008; Karl and Reh, 2010; Lamba et al., 2008, 2009; Ooto et al., 2004). Recently, some evidence of progenitor properties has been shown in the MG of the primate retina (Fischer and Bongini, 2010; Lawrence et al., 2007).

Table 1. Sources of adult neurogenesis in the vertebrate retina. NPECB, non-pigmented epithelium of the ciliary body; PECB, pigmented epithelium of the ciliary body.

SOURCE	MODEL	PROLIFERATION CAPACITY
CMZ	**fishes** Centanin and Wittbrodt, 2014; Perron and Harris, 2000; Stenkamp, 2011; Than-Trong and Bally-Cuif, 2015	• **teleosts** **constitutive**: YES **induced:** YES (following retinal injury) • **elasmobranchs** **constitutive**: YES **induced**: NO data available
	amphibians Del Rio-Tsonis and Tsonis, 2003; Fischer and Bongini, 2010; Kubo et al., 2003; Perron and Harris, 2000; Todd et al., 2015	**constitutive**: YES **induced**: YES (following retinal injury)
	reptiles Todd et al., 2015	**constitutive**: YES (only turtles) **induced**: uncertain (following retinal injury)
	birds Fischer and Bongini, 2010; Fischer and Reh, 2000; Fischer et al., 2005; Kubota et al., 2002	**constitutive**: YES (only amacrine and bipolar cells) **induced**: NO (following retinal injury)
	mammals Ahmad et al., 2004; Del Rio-Tsonis and Tsonis, 2003; Fischer and Bongini, 2010; Kubota et al., 2002; Perron and Harris, 2000; Todd et al., 2015; Tropete et al., 2000	**constitutive**: YES (only young marsupials) **induced**: uncertain (following retinal injury)

CE	**birds** Fischer, 2011; Fischer and Bongini, 2010; Fischer and Reh, 2003	**-PECB** **constitutive**: NO **induced**: NO **- NPECB** **constitutive**: NO **induced:** YES (when stimulated with growth and exogenous factors; only ganglion cells)
	mammals Ahmad et al., 2000; Cicero et al., 2009; Fisher et al., 2001; Kubota et al., 2002; MacNeil et al., 2007; Tropete et al., 2000; Wohl et al., 2012	**- PECB** **constitutive**: YES (rats and mice, but only 1 week postnatally) **induced**: YES (monkeys following retinal injury) **-NPECB** **constitutive**: NO data available **induced**: YES (monkeys following retinal injury; but lesser than PECB
RPE	**fishes** Del Rio-Tsonis and Tsonis, 2003; Ferreiro-Galve et al., 2010a; Raymond and Hitchcock, 2000	• **teleosts** **constitutive**: NO **induced**: NO • **elasmobranchs** **constitutive**: YES (PCNA-ir cells) **induced**: NO data available
	amphibians Araki, 2007; Barbosa-Sabanero et al., 2012; Del Rio-Tsonis and Tsonis, 2003; Moshiri et al., 2004; Reh and Fischer, 2006	**constitutive**: YES **induced**: YES (following retinal injury)
	birds Barbosa-Sabanero et al., 2012; Zhao et al., 1995	**constitutive**: NO **induced**: YES (following retinal injury and when stimulated with exogenous factors)
	mammals Tropete et al., 2000; Zhao et al., 1995	**constitutive**: NO **induced**: YES (*in vitro*, when stimulated with exogenous factors)
MG	**fishes** Barbosa-Sabanero et al., 2012; Bernardos et al., 2007; Centanin and Wittbrodt, 2014; Goldman, 2014; Lenkowski and Raymond, 2014; Otteson and Hitchcock, 2003; Raymond et al., 2006; Than-Trong and Bally-Cuif, 2015	• **teleosts** **constitutive**: YES (slow dividing INL cells → rods) **induced**: YES (following retinal injury: all retinal cell types) • **elasmobranchs** **constitutive**: YES (slow dividing INL cells → rods) **induced:** NO data available
	amphibians Todd et al., 2015	**constitutive**: NO **induced**: YES (newts and some species of salamander when stimulated with exogenous factors)
	birds Barbosa-Sabanero et al., 2012; Fischer and Bongini, 2010; Fischer and Reh, 2001, 2005; Gallina et al., 2014	**constitutive**: very limited **induced**: YES (following retinal injury, when stimulated with exogenous factors)
	mammals Bringmann et al., 2009; Gallina et al., 2014; Jadhav et al., 2009; Karl et al., 2008; Karl and Reh, 2010; Lamba et al., 2008, 2009; Ooto et al., 2004	**constitutive**: NO **induced**: YES (when stimulated with exogenous factors)

3. Fishes *vs.* Mammals

Summarizing, in the retina of mammals none of the regeneration processes described above occur spontaneously. The knowledge of mechanisms underlying neurogenesis in the retina, especially in retinas with endogenous regeneration capacity, is essential to understand the potential and limitations of retinal regeneration in mammals, including humans.

The retina of fishes offers an exceptional model for studying retinal regeneration because of several reasons: first, it constitutes an excellent system to understand retinogenesis; second, it exhibits persistent neurogenesis in the adult by the generation of rod precursors and also by the addition of new cells from the CMZ throughout life; and third, it regenerates after retinal injury, since it contains MG that dedifferentiate upon damage to generate all retinal cell types.

One of the main questions to solve in the field of retinal regeneration is why MG in fishes show a strong ability to generate new retinal neurons while MG in mammals, including humans, do not. Understanding the mechanisms involved in the response of MG to retinal injury and comparing injury responses in fishes and mammals are essential to answer this question. While in fishes, MG dedifferentiate after retinal injury and proliferate generating multipotent progenitor cells which can differentiate into all retinal cell types, in mammals, upon injury, the MG respond by undergoing several morphological, biochemical and physiological changes (**see reactive gliosis in BOX 2**) aimed to protect neurons after retinal damage (Bringmann et al., 2006, 2009).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

BOX 2: Reactive gliosis

Gliosis is a biological process that occurs during repair after injury in the CNS. The overexpression of the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin is a hallmark of reactive gliosis in the CNS (Bargagna-Mohan et al., 2010; Brenner, 2014; Eddelston and Mucke, 1993; Pekny et al., 1995). Glial cells (astrocytes, macro- and microglia) become reactive following injury as a result of disease, trauma, genetic disorder, or chemical damage. This process implies an increase in the number and size of cells that express GFAP (Brenner, 2014; Bringmann et al., 2009; Eddelston and Mucke, 1993).
Reactive gliosis includes hypertrophy, increase in intermediate filaments and, if the damage persists, proliferation and migration leading to the formation of a glial scar, a potential barrier for regeneration mechanisms (Karl and Reh, 2010; Lenkowski and Raymond, 2014).
Reactive gliosis depend on the species, tissue, glial cell type and the injury. In the retina o mammals, the typical response upon damage is the reactive gliosis of the MG, that provide a rapid repair of the retina (Karl and Reh, 2010). However, the formation of a glial scar in mammals may block tissue regeneration after retinal injury (Bringmann et al., 2009).
Summarizing, all the features of the MG under the reactive gliosis in both the retina of fishes and mammals appear to be the same with the exception of the formation of a glial scar, which only occurs in mammals (Lenkowski and Raymond, 2014).

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.1. *Scyliorhinus canicula* as a possible model for studying retinal regeneration

The retina of fishes, especially the retina of the lesser spotted dogfish *S. canicula,* may be a good model to investigate retinal regeneration mechanisms since it has been an excellent system to understand proliferation and differentiation patterns (Bejarano-Escobar et al., 2012a; Ferreiro-Galve et al., 2008, 2010a, 2012) for several reasons: first, as in all fishes, the retina of *S. canicula* has a CMZ that contain retinal stem cells which continually added new cells throughout life; second, the embryos are large in size and present an extremely slow developmental period, in contrast to what happen in teleosts; four, its phylogenetic

position make them very suitable for identifying ancestral features of gnathostomes and for obtaining information about the evolution of retinogenesis in vertebrates; and five, the evidence of a protracted period of scattered cell proliferation in mature regions of the retina of *S. canicula*, and some proliferating cells in the RPE, make it an interesting model for identifying regeneration mechanisms.

In this context, we performed retinal damage assays in the retina of *S. canicula* in order to compare the pattern of retinogenesis with that of retinal repair in sharks. This analysis could shed light on the conservation (or not) of molecular mechanisms that guide both processes. Comparison between teleosts, elasmobranchs and mammals become crucial to identify the limitations in regeneration in mammals, and also to develop strategies to enhance endogenous regeneration in the retina of mammals.

4. Accessing the retina through the pupil in *S. canicula*

The iris is the structure that regulates the entrance of light towards the retina by modifying the diameter of the pupil. In most vertebrates, from elasmobranchs to humans, the size and shape are regulated by iris muscles, the *sphincter* (circular muscle that contracts when the acetylcholine acts over it) and *dilator papillae* muscles (radial muscles; its contraction cause pupil dilation), which are innervated by the autonomic nervous system with some notable differences between vertebrate groups and species. In *S. canicula*, cholinergic innervation from the ciliary ganglion contracts the dilator muscle, while the sphincter contracts in response to light exposure (Neuhuber and Schrödl, 2011; Young, 1933). Most teleost fishes possess fixed pupils; however elasmobranchs possess

mobile pupils (Bunt, 1991; Hart et al., 2006; McComb et al., 2009; Neuhber and Schrödl, 2011). It is known that pupillary movements in elasmobranchs are associated with environmental light levels which are highly related with depth. In low-light levels (deep habitats) pupils are predominantly fixed, compared with mobile pupils characteristic in high-light levels (shallow lit habitats). In addition, species with diurnal habits typically have faster rates of pupillary constriction and dilation compared with nocturnal species (Kaijura, 2010; Lisney et al., 2012; Litherland et al., 2009). Sharks usually have slit-shaped pupils with the ability to close. In *S. canicula* under day light conditions, pupils close almost completely. It has been reported that diurnal species such *S. canicula* dilate their pupils in 1-30 min and constrict them maximally in 2-5 min as the result of the effect of light on the sphincter muscle. Re-opening of the pupils under dark conditions also proceeds slowly (Hart et al., 2006; Kajiura, 2010; Kuchnow, 1970; Young, 1933).

4.1. Pupil dilatation assay

In order to achieve an approximation to degeneration assays in teleost fishes, *we* tried to induce pupil dilatation aimed to solve the limitation of the mobile pupils in *S. canicula* (**Table 2**). Prior to treatments, juveniles (size between 108 and 125 mm) of *S. canicula* were isolated in individual small tanks within a flow through system. Then, animals were anesthetized in 0.02 % tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in a small tank in seawater.

Juveniles 1, 2, and 3 were treated with atropine. Atropine was directed applied to each right eye under scope visualization without light to minimize the pupil and the eyelids closing motion. Animals were kept over an ice bed and gills were covered with wet paper in order to allow respiration. Juvenile 4 was treated with vecuronium bromide (**see details**

in Table 2). Thereafter, fishes were transferred to a fresh water tank with GeneralTonic (TetraMedica®, 150µL/2Lwater), a broad-spectrum antibiotic.

Specimen	CD	Treatment	Pupil response	Animal response
Juv. 1	5 h	R eye: 10 % AS in ERS, 20 µL L eye: 20 % AS in ERS, 15 µL	R eye: fully closed L eye: fully closed	Returned to the fresh water tank †
Juv. 2	24 h	R eye: 50 % AS in ERS, 15 µL + Returned to the fresh water tank + 50 % AS in ERS, 15 µL L eye: control	R eye: not overlapped, but closed	Returned to the fresh water tank †
Juv. 3	24 h	R eye: 80 % AS in ERS, 3 applications (15 min intervals). Each application: 15 µL	R eye: closed	† during procedure
Juv. 4	24 h	R eye: 0,4 % Vecuronium bromide administered 4 times (15 min intervals), each application: 10µL L eye: control, treated with saline (0.9 % NaCl). 4 applications (15min intervals), each application: 10µL Procedure as described in Dearworth et al., 2007.	Both pupils close in response to light	Returned to the fresh water tank Complete recovery

Table 2. Summary of pupil dilation assays in the eyes of *S. canicula* juveniles. AS, atropine sulfate (COLIRCUSI ATROPINA 10mg/mL); CD, under constant darkness; ERS, elasmobranch Ringer's solution (see **Material and methods** section in this thesis); L, left; PFA, paraformaldehyde; R, right.

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4.2. Preliminary data and discussion

We have investigated the effects of a muscarinic cholinergic antagonist (atropine) and a nicotinic cholinergic antagonist (vecuronium bromide), on the pupil of juveniles of *S. canicula*. Our data shown that neither atropine nor vecuronium bromide had effect on the pupil size. After treatments with different concentrations of both atropine and vecuronium bromide, we found that the pupil constrict them maximally in 2-5 min. In all cases, even in low-light conditions, the pupil became closed so that dorsal and ventral margins meet, except in two narrow apertures at opposite ends (**red arrowheads in Fig. 3**).

Figure 3. Pupil of a juvenile *S. canicula* token under the stereomicroscope after treatment with atropine sulfate. Note the small narrow apertures in both ends of the slit (red arrowheads).

A previous study on the physiology of the iris in elasmobranchs using different drugs (Young, 1933), shown that atropine sulfate seemed to have no effect on light or dark-adapted pupils and did not inhibited the closure caused by the light exposure. We increased dark adaptation times (5 h and 24 h) compared with the 30 min used in other previous studies of the pupillary response in elasmobranchs (Kajigura, 2010), in order to yield a slightly greater maximum pupil area. Drops of atropine in

different concentrations (**see Table 2**) were topically administered to the cornea. Our results shown that atropine had no effect on pupil size in *S. canicula*. This is in line with results obtained by Young (1933) in Salachians. In addition, atropine had no effect on pupil size in turtles (Dearworth et al., 2007) and birds (Loerzel et al., 2002; Mikaelian et al., 1994; Ramer et al., 1996). However, in other reptilian species such as alligator, it produced pupillary dilation (Iske, 1929). The sphincter muscle in this species is controlled similarly to that of pigeons (Vershschueren et al., 1991). We also used vecuronium bromide. Drops of the agent were topically administered to the cornea four times, at 15 min intervals, using a syringe with a needle. One eye was selected for treatment by the drug (0.4 %), while the other was used as a control and was treated with a saline solution (0.9 % NaCl). Number of applications, time intervals and doses (**see Table 2**) were chosen based on the protocol used in turtles by Dearworth et al. (2007). Our results shown that vecuronium bromide had no effect on pupil size in *S. canicula*. To our knowledge, previous assays with vecuronium bromide in elasmobranchs have not been reported so far. Vecuronium bromide is an effective cholinergic antagonist that blocks the parasympathetic pupillary light response in turtles, causing mydriasis, although the pupil dilation is less than in birds (Dearworth et al., 2007). In addition, it has been reported in birds that this drug has no side effects, which could make it a good candidate to try to minimize the risk of death associated with drugs treatments in *S. canicula*. More experimentation will be necessary to find possible drugs that cause changes in the pupil dilation of *S. canicula* without causing major damages to the animal.

MSS-222 was used to anesthetize the fishes. This drug could affect the accommodative musculature causing its relaxation in some species of teleost fishes (Sivak, 1982). However in sharks, MSS-222 does not seem to influence pupillary response (Kajigura, 2010; Kuchnow, 1971), so it is highly improbable that MSS-222 dramatically affected the pupillary response.

5. An experimental approach

To study the regeneration capacity of the retina of fishes, particularly the retina of teleost fishes, a great number of different types of lesion have been produced depending on the species: cryolesion (Jimeno et al., 1999; 2003; Lillo et al., 2001), light-induced (Allen et al., 1999; Bejarano-Escobar et al., 2012b, 2014; Thomas et al., 2012; Vera and Migaud, 2009; Vihtelic and Hyde, 2000) or laser-induced (Braisted et al., 1994; Wu et al., 2001) photoreceptor ablation, mechanical lesion [e.g. retinal puncture in (Fausett and Goldman, 2006; Senut et al., 2004)], surgical lesion (Cameron, 2000; Cameron and Easter, 1995), chemical or citotoxic lesion (Fimbel et al., 2007; Sherpa et al., 2008), and thermal damage (Raymond et al., 2006). In teleost fishes, after damage, MG re-enter the cell cycle regenerating all retinal cell types (**see above**).

The eye of elasmobranch fishes shows several differences with respect to that of teleost fishes. As in teleosts, the light enters the eye through the pupil, a small aperture in the iris, which is mobile in most elasmobranchs, in contrasts to fixed pupils typical of teleosts (Gruber, 1977; Hart et al., 2006; Lisney et al., 2012; Sivak, 1990). The eye of teleost fishes is similar to the eye of mammals but teleosts do not possess eyelids and the pupil aperture is fixed, which is why the retina of teleosts is more vulnerable to light-induced damage. For light intensity protection, teleosts have developed different adaptations such as photoreceptor motility and migration of melanin granules (Kusmic and Gualtieri, 2000). Additionally, the retina of teleosts and elasmobranchs differs in the composition and proportion of photoreceptor cells. Both possess rods and cones photoreceptors in the retina (duplex retina), although teleosts possesses double cones organized in a regular mosaic pattern (Kusmic and Gualtieri, 2000). However, in many elasmobranch species such in *S. canicula*, the proportion of rods in much higher (rod-cone ration >100 : 1), providing higher visual sensitivity, which can be enhanced in dim light by

the *tapetum lucidum* (**see General introduction**). In addition, when cones are present, these are single cones instead of the paired or double cones present in most teleosts (Hart et al., 2006; 2011; Lisney et al., 2012; Schieber et al., 2012).

To our knowledge, data available from studies on light-induced retinal damage and regeneration in fishes were only carried out in teleost species (Allison et al., 2006; Bejarano-Escobar et al., 2012b, 2014; Vera and Migaud, 2009; Vihtelic and Hyde, 2000), but not in elasmobranch species.

5.1. Experimental light treatment design

Before starting light treatments (**Table 3**), juveniles (size between 108 and 125 mm) of *S. canicula* were isolated in individual small tanks within a flow through system. Then, fishes were kept under CD to increase retinal sensitivity.

Light treatment consisted of exposing fishes to constant high intensity light for 24, 48 or 72 h using a Versatile Environmental Test Chamber in which the water temperature can be regulated to standard conditions (15-16 °C). For evaluating light intensity we used a hand-held light meter. Intensity recorded in the small tanks was 16290 lx at the edge of the tank.

Different times of CD adaptation and times of light exposure are shown in **Table 3**. Three specimens died during light treatment and only one (Juv. 8 in Table 3) survived and was returned to a fresh water tank under normal 12L:12D photoperiod.

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Specimen	CD	Light treatment	Animal response
Juv. 5	5 days	CL 72 h, 16290 lx	†
Juv. 6	4 days	CL 48 h, 16290 lx	†
Juv. 7	4 days	CL 24 h, 16290 lx	†
Juv. 8	4 days	CL 72 h, 16290 lx	Alive Returned to a fresh water tank under normal photoperiod 12L:12D

Table 3. Summary of retinal degeneration light treatments in *S. canicula* juveniles. CD, under constant darkness; CL, constant high intensity light; lx, luxes.

Sharks are predatory animals and visual communication must be important to survive. The visual system in these animals has received less attention comparing with the olfaction or electrosensory senses. The death of these specimens during light treatments may be produced by the stress caused by constant high intensity light. However, their pupils and eyelids were completely closed under bright conditions, which is the reason why we think that the retina of elasmobranch fishes is less vulnerable to light-induced damage comparing with the retina of teleost fishes.

After that, eyes were removed and fixed by immersion in 4 % PFA in elasmobranch phosphate buffer (EPB) (**for more information about tissue processing see Section 3 of this thesis: Material and methods**). Sections were obtained on a cryostat.

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5.2. Preliminary data and discussion

We aimed to induce light damage by long term light exposure. However, the lack of an optimized protocol for pupil dilatation and hence, for light- induced damage in this species, has not allowed us to obtain a significant number of treated specimens and accordingly, to deeply analyze the response of the retina to damage. Results described below therefore correspond to preliminary observations in the damaged retina of *S. canicula* maintained for 24 h or 72 h under constant light and fixed immediately or 4 days after treatment, respectively. Results were compared with those obtained in non-treated retinas from specimens at the same developmental stage (**Fig. 4**).

The control retina of juveniles shows some Pax6 immunoreactive (-ir) cells in the innermost part of the inner nuclear layer (INLi). Some of them were labeled for Pax6 and GAD (**Fig. 4A**; see also Ferreiro-Galve et al., 2012). In addition, DCX-ir cells were also positioned in the GCL, INLi and INLo (**Fig. 4B**; **see also Chapters 1-3**). The specific MG marker glutamine synthetase (GS) labeled the nuclei of Müller cells which were positioned just above the cell somas immunoreactive for Pax6, GAD or DCX (**open arrows in Fig. 4B**). Following light-induced damage, notable changes in the structure of the retina were noted. The thickness of the retina decreased and its aspect was disorganized. This aspect is very similar to that described in zebrafish when exposed to constant intense light for 51 h (see Figure 4 in Thummel et al., 2008). Pax6-ir cells were still observed in the INLi and GCL (**Fig. 4C**), though their shape was slightly different (swollen-like) from that observed in control specimens (**compare C and C' with A**). In the treated retina, some Pax6-ir cells were also labelled for Reelin (Reln) (**arrowheads in C'**), an extracellular matrix protein related with laminar organization in several brain areas (D'Arcangelo et al., 1995). Although Reln is expressed during normal retinogenesis in trout and mouse (Candal

et al., 2005; Rice et al., 2001, its expression is upregulated in mice retina following injury, including cells in the INLi (Pulido et al., 2007).

In the retina of control juveniles, GS and GFAP were observed in cell somas and/or processes of MG, respectively (**Fig. 4D and E; see also Chapter 3**). It is known that retinal regeneration response after damage is characterized by cell cycle re-entering of MG cells to generate neuronal progenitor cells (Bernardos et al., 2007; Fimbel et al., 2007; Thummel et al., 2008). Albeit we did not observe a highly proliferative response after 72 h treatment, we detected some PCNA-ir dividing cells in the same position occupied by the nuclei of MG cells (**arrowheads in Fig. 4F**). This phenomenon was previously reported in rod progenitors in the INLi of the normal retina of the teleost *T. tinca* (Bejarano-Escobar et al., 2012b) and also in dedifferentiated MG in the INL in zebrafish after light-induced (Thummel et al., 2008). In treated retinas, the appearance of GFAP-ir processes of the MG seem to be different, showing shorter or incomplete wider processes, morphologically hypertrophied, in comparison with the control retina (**Fig. 4G, H**). Of note, we have observed a few Pax6-ir cell nuclei and processes located in outer positions with respect to Pax6-ir cell nuclei in the INLi (**curved arrow in Fig. 4H, H'**). The position of the soma of these Pax6-ir cells appears to coincide with that of the soma of MG cells (**open arrows in Fig. 4E**). Interestingly, some of these Pax6-ir cells were observed undergoing cell division (**arrowheads in detail in Fig. 4H'**) a fact that has been previously reported in the retina of teleosts preceding damage-induced retinal regeneration (**see below**).

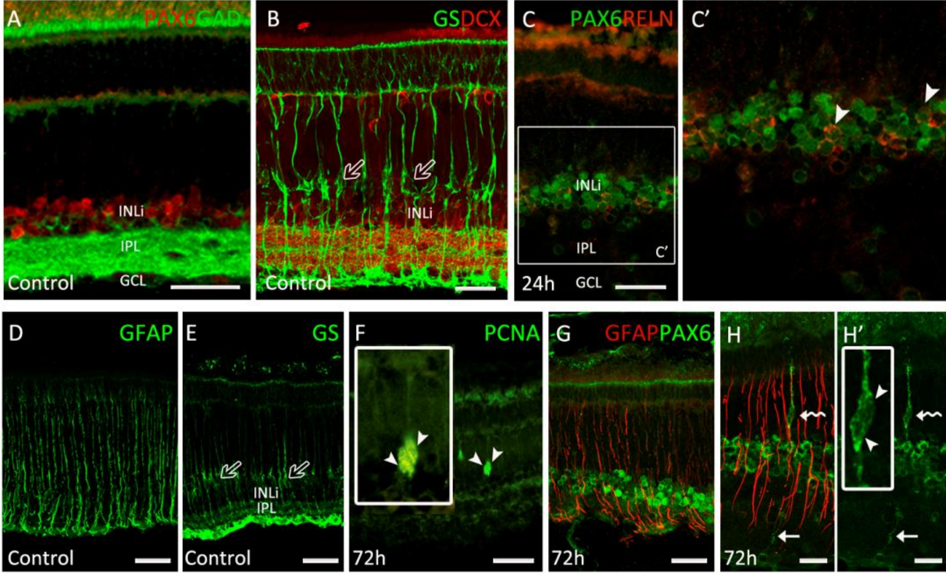

Figure 4. Patterns of Pax6, GFAP and GS in control (A, B, D, E) and dark-adapted juveniles of S. canicula exposed to constant intense light for 24 (C, C') and 72h (F, G, H, H'). (A) The control juvenile retina shows the typical distribution of Pax6-ir cells in the GCL (GCs) and in the INLi (amacrine cells). (**B**) GS in control juvenile retina labels the soma of the MG in the INLi (**open arrows**) and their processes. (**C, C'**) Pax6 immunoreactivity in treated retina exposed to constant intense light for 24 h. Pax6 is observed in amacrine (some of these are also Reln-ir, **arrowheads**) and GCs. (**D, E**) Distribution of GFAP in Müller cell processes and GS in processes and also in the nuclei of Müller cells (**open arrows**) in the control retina of juveniles. (**F**) PCNA expression at 72 h of constant light treatment in a few cells in the INL. Arrowheads point to a dividing cell. (**G**) Distribution of Pax6-ir cells and GFAP-ir MG at 72 h under constant light. Note that the number of Pax6-ir cells in the INLi appears to be increased and the appearance of these cells are quite different from that of the control retina (**compare with A**). The morphology of Müller cell processes is different from the control (**compare with D**). (**H, H'**) A few Pax6-ir dividing cells (**arrowheads**) in the INLi at 72 h under constant light extend their processes to the IPL (**arrows**) and OPL. GCL, ganglion cell layer; INLi, inner part of the inner nuclear layer; IPL, inner plexiform layer. Scale bars: 25 µm in C, H, H'; 50 µm in A, B, D, E, F, G.

Unlike in mammals, differentiated MG in teleosts (Bernardos et al., 2007; Braisted et al., 1994; Vihtelic and Hyde, 2000; Wu et al., 2001) and sharks (Sánchez-Farías and Candal, 2015; **see also Chapter 3**), express GFAP in the intact retina. Several studies have reported neuronal markers in MG that proliferate after retinal injury not only in adult rodents (Das et al., 2006; Ooto et al., 2004), but also in early postnatal chick (Fischer and Reh, 2003; Fischer, 2005) and teleosts (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007; Raymond et al., 2006; Yurco and Cameron, 2005). However, although MG proliferation (reactive gliosis) is the typical response in mammals, in teleosts, MG do not phagocytose *in vivo*, suggesting that MG may play a different role in fishes (Wagner and Raymond, 1991).

Pax6 has been reported in the brain in radial glia associated with the maintaining of the multipotent stage of retinal progenitors (Marquardt et al., 2001) and with the neurogenic capacity (Götz and Barde, 2005). In the retina, Pax6 has been shown in neurogenic MG that generate rod precursors in the growing and adult teleost retina (Bernardos et al., 2007; Hitchcock et al., 1996; Otteson et al., 2001) and also proliferating MG express several transcription factors, including Pax6, during retinal regeneration after damage (Raymond et al., 2006; Thummel et al., 2008). After retinal injury, columns of proliferating cells named “neurogenic clusters” that express markers such as Pax6 have been identified in the INL in teleosts (Fausett and Goldman, 2006; Thummel et al., 2008) and precedes retinal regeneration (Braisted et al., 1994; Vihtelic and Hyde, 2000; Wu et al., 2001; Yurco and Cameron, 2005). Although we have observed a reduced number of PCNA-ir cells in the damaged retina of *S. canicula*, the presence of dividing Pax6-ir cells with radial morphology could correspond to neurogenic clusters described in zebrafish. The presence of Pax6 in cell nuclei in the INL and radial processes directed to the IPL and OPL (present results, **arrow in Fig. 4H, H’**) has been also

reported in MG when they re-enter cell cycle during retinal regeneration in zebrafish (Thummel et al., 2008).

Our results represent the first data on the response of the retina of a shark to an intense light treatment. Changes observed in the retina of *S. canicula* following light treatment are similar to that reported in the damaged retina of teleosts (Thummel et al., 2008), though the magnitude of the response is notably lower than in this species. This reduced response might be related with the almost complete closure of the mobile pupil of the shark in response to light. An optimized protocol to induce localized damage in the retina of *S. canicula* deserves further investigation.

6. Training stay in the MOKALAB: Laboratory of the Professor Mike O. Karl. Center for Regenerative Therapies TU Dresden (CRTD)

mokalab

Our laboratory has previously analyzed the spatial and temporal patterns of cell proliferation and differentiation during retinal histogenesis in *S. canicula* (Ferreiro-Galve et al., 2008, 2010a, 2010b, 2012; Sueiro et al., 2007), showing that the retina of this elasmobranch fish offers an exceptional model to study the development of the retina and also adult neurogenesis (**see 4.2 in General introduction**). Moreover, this thesis gives a glimpse of cell positioning using DCX antibody because of its importance in neural development and layering (**Chapters 1 and 2**) and how neuroepithelial cells transform to radial glial progenitors during development and also in the adult retinal stem cell niche (**Chapter 3**). All this information will be useful to identify and develop methods to stimulate endogenous neurogenesis in the retina of mammals. Looking at how to increase or stabilize neurogenesis in pathological processes, we are currently interested in finding molecular determinants that can stabilize the neural stem stage, serve as fate determinants towards the neuronal lineage or can reverse a glial precursor into a neuronal precursor. We are also aim to test a selected number of potential candidates by inhibiting/activating its expression. However, genetic manipulation of *S. canicula* and retinal *ex-vivo* cultures in this species are far from being optimized in our laboratory.

6.1. Lab Project: Identify chemical strategies to stimulate MG neurogenic competence and prevent reactive gliosis in the mammalian retina

The overall aim of the MOKALAB is to understand the basic mechanism of cell regeneration in the retina, with the goal to develop strategies for its protection and repair. It is well known that fishes and frogs are capable to replace lost neurons by an endogenous self-healing

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regeneration process. Unfortunately, mammals do not possess this ability, although some regeneration capacity has been reported in higher vertebrates (**see above**).

The laboratory of the professor Mike O. Karl has two main objectives: (1) Identify molecular mechanisms that stimulate neurogenic capacity of the MG, and (2) prevent epi-/sub-retinal gliotic scar formation. In the last few years, this group has essentially advanced in the first one, developing new strategies for monitoring the process of regeneration from the MG using different mitogens. In the last few years several groups have reported that some types of retinal neurons can be regenerated in the mammalian retina both *in vivo* and *in vitro*, and that regeneration of these neurons can be stimulated using growth factors or transcription factors. However, the number of newly generated progenitor cells, and thus regenerated retinal neurons, is still very limited (Karl et al., 2008; Karl and Reh, 2010, 2012; Lamba et al., 2008; Nelson et al., 2011). In this way, two major cell types have been identified as sources for retinal regeneration: the MG and the RPE. More recently, they have found that in the retina of young mice, more than 50% of the MG can be stimulated and acquire stem cell characteristics, although this potential decreases with age (1% in adults). Along these lines, reasons and mechanisms that limit and regulate retinal regeneration in mammals need to be identified.

6.2. Brief summary of the rotation

The major intention of my rotation in the MOKALAB was focused on identifying the detailed outcome of the MG-derived neuronal progeny and mature new born neurons in mouse retina using an *ex-vivo* model. It is well known that MG is post-mitotic and quiescent at P10 retina. Previously, they observed on one side MG cell progeny-derived new born amacrine cells [calbindin (CB) + 5-bromo-2'-deoxyuridine (BrdU) +] and, on the other

side, MG cell progeny subpopulation that might be precursors for bipolar and photoreceptor cells [Otx2+ 5-ethynyl-2'-deoxyuridine (EdU)+]. However, which factors induced the transition from MG-derived neuronal progeny (immature) to mature neurons (amacrine, bipolar or photoreceptor cells) is still unknown. At this juncture, I was integrated in two aims. Because of my commitment of not data dissemination, the description below is a brief description of what I did.

Aim 1: Find out which mitogens differentially stimulate MG cell proliferation and de-differentiation in mouse retina *ex-vivo*

The strategy consisted in analyzing the differential MG response upon various mitogens treatment (DAMP molecules): VEGF (vascular endothelial growth factor), PEDF (pigment epithelium-derived factor), AGE-BSA (bovine serum-derived advanced glycation end product), PDGF-AA (platelet-derived growth factor-AA) and EGF (epidermal growth factor).

We identified MG proliferation using Sox2, BrdU and Ki67 immunohistochemistry (**see an example of the images taken from these experiments in Fig. 5**), and MG de-differentiation with Sox2, Sox9, Pax6 and PH3.

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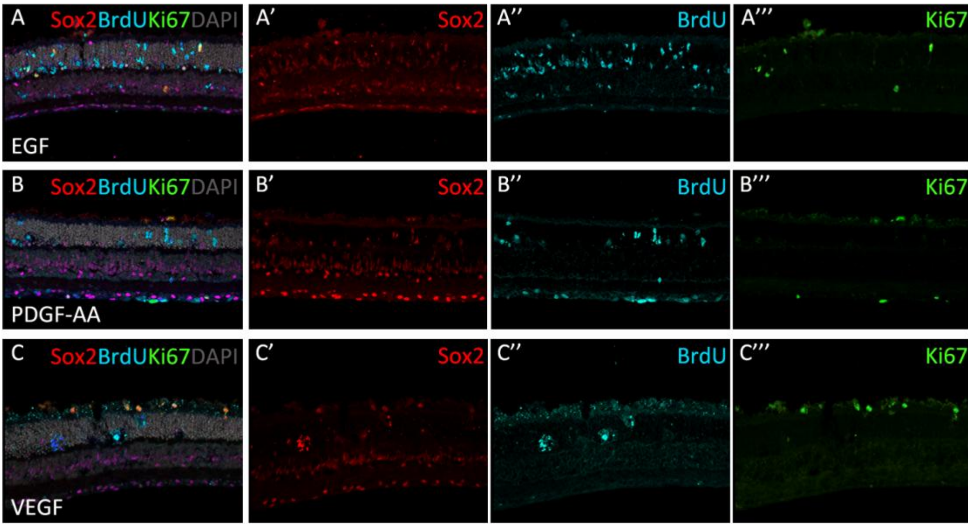

Figure 5. Examples of some images taken from *ex-vivo* adult (P21) mice retinas treated with different factors: (A-A''') EGF, (B-B''') PDGF-AA, (C-C''') VEGF (Pran-Babu et al., *unpublished*).

Aim 2: Stimulate the generation of MG-derived progeny

Since previous studies have found that the activation of Notch might stimulate MG-derived neurogenesis in mouse (Das et al., 2006) and zebrafish retina (Bernardos et al., 2005), the major question was to find out if MG generate neuronal progeny upon defined stimulation in *ex-vivo* approach.

The strategy consisted of analyzing the maturation of MG-derived Otx2 neuronal progeny to photoreceptors using differentiation approach comparing short term experiment (DEV 7) with long term experiment (DEV 12) in Nrl-GFP mice. The *Nrl* gene encodes a retinal transcription factor that plays an essential role in the differentiation and homeostasis of rods. In this transgenic strain, the *Nrl* promoter drives the expression of EGFP (enhanced green fluorescent protein) in rods. In the adult retina, GFP is detected in the outer nuclear layer (ONL) and in the outer segments. Staining with anti-rhodopsin antibody shows complete co-localization with

GFAP, whereas no overlap is observed between GFP and cone-specific markers. Fluorescence is detected shortly after terminal cell division, corresponding with rod genesis.

We carried out the analysis of Otx2+EdU and CB+EdU progeny in various differentiation assays. *Ex-vivo* cells were cultured in appropriate media and growth factors (HB-EGF+FGF2; HB-EGF+FGF2 with DAPT+EC23; HB-EGF+FGF2 with DKK+IGF-1) were removed on day 4. Data at day 7 and day 12 were compared. Data are now being processed.

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Abbreviations

AS	atropine sulfate
AGE-BSA	bovine serum-derived advanced glycation end
ap	apical processes of RPE enwrapping photoreceptor outer segments
BM	Bruch's membrane
BrDU	5-bromo-2'-deoxyuridine
C	cones
CB	calbindin
CC	choriocapillaris
CD	constant darkness
CE	ciliary epithelium
CL	constant high intensity light
CMZ	ciliary marginal zone
CNS	central nervous system
DAPT	Notch inhibitor
DEV	day *ex-vivo*
DKK	Dickkopf (Wnt/beta catenin signalling inhibitor)
EC23	alternative for retinoic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EPB	elasmobranch phosphate buffer
ERS	elasmobranch Ringer's solution
FGF-2	basic fibroblast growth factor
GCs	ganglion cells
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GS	glutamine synthetase
GT	GeneralTonic
H	Histoacryl®
HB-EGF	heparin binding epidermal growth factor
IGF-1	Insulin growth factor-1
INLi	inner part of the inner nuclear layer
IPL	inner plexiform layer
-ir	immunoreactive
lx	luxes
MG	Müller glia
NMDA	N-methyl-D-aspartate
MS-222	tricaine methane sulfonate

NPECB non-pigmented epithelium of the ciliary body
ONL outer nuclear layer
os outer segments
Otx2 orthodenticle homeobox 2
PDGF-AA platelet-derived growth factor-AA
PECB pigmented epithelium of the ciliary body
PEDF pigment epithelium-derived factor
PFA paraformaldehyde
PH3 phospho-histone H3
R rods
Reln Reelin
RP recovery period
RPE retinal pigment epithelium
T tobramicine
VEGF vascular endothelial growth factor

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