Journal of Chemical Neuroanatomy 39 (2010) 1–14

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas

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ARTICLE INFO

Article history:
Received 16 April 2009
Received in revised form 5 October 2009
Accepted 5 October 2009
Available online 12 October 2009

Keywords:
PCNA
Phospho-histone-H3
Rod opsin
Retinal pigment epithelium
Marginal retina
Development
Elasmobranchs

ABSTRACT

We studied the pattern of cell proliferation and its relation with photoreceptor differentiation in the embryonic and postembryonic retina of two elasmobranchs, the lesser spotted dogfish (*Scyliorhinus canicula*) and the brown shyshark (*Haploblepharus fuscus*). Cell proliferation was studied with antibodies raised against proliferating cell nuclear antigen (PCNA) and phospho-histone-H3, and early photoreceptor differentiation with an antibody raised against rod opsin. As regards the spatiotemporal distribution of PCNA-immunoreactive cells, our results reveal a gradual loss of PCNA that coincides in a spatiotemporal sequence with the gradient of layer maturation. The presence of a peripheral growth zone containing pure-proliferating retinal progenitors (the ciliary marginal zone) in the adult retina matches with the general pattern observed in other groups of gnathostomous fishes. However, in the shark retina the generation of new cells is not restricted to the ciliary marginal zone but also occurs in retinal areas that contain differentiated cells: (1) in a *transition zone* that lies between the pure-proliferating ciliary marginal zone and the central (layered) retina; (2) in the differentiating central area up to prehatching embryos where large amounts of PCNA-positive cells were observed even in the inner and outer nuclear layers; (3) and in the retinal pigment epithelium of prehatching embryos. Rod opsin immunoreactivity was observed in both species when the outer plexiform layer begins to be recognized in the central retina and, as we previously observed in trout, coincided temporally with the weakening in PCNA labelling.

1. Introduction

Eye morphogenesis involves a complex hierarchy of inductive processes and intercellular interactions that lead to the formation of the retina, optic stalk and lens. In vertebrates, the retina develops from the highly proliferating, pseudostratified neuroepithelium of the eye cup to a highly organized, laminated structure. Retinal lamination occurs after cell cycle withdrawal of neuronal precursors, and their subsequent migration to appropriate levels, where they become differentiated expressing the specific molecular markers and morphological characteristics of the cell type.

The presence of multipotent precursor cells is not restricted to the embryonic period of retinal development. A potential retinal stem cell population has been recently reported in the retina of mammals (including human) and birds (Kubota et al., 2002; Ahmad et al., 2004; Amato et al., 2004; Reh and Fischer, 2006), but adult neurogenesis appears rather limited in the retina of amniotes (reptiles, birds and mammals), in contrast to that occurring in anamniotes (fishes and amphibians), in which extensive retinal neurogenesis has been observed in adults that leads to continuous retinal growth throughout life. Retinal stem cells are contained within the ciliary marginal zone (CMZ), a peripheral ring of undifferentiated neuroepithelial cells located between the laminated neural retina and the ciliary region. Different subzones have been recognized in the amphibian and teleost CMZ, which reflect the temporal sequence of retinal development (Harris and Perron, 1998; Raymond et al., 2006), but the specific factors that regulate the timing of retinal differentiation are not entirely known. Additional adult proliferation is found along the differentiated neural retina, leading to formation of new rods (Johns, 1982; Mack and Fernald, 1997; Stenkamp et al., 1997; Julian et al., 1998). The time course of cell proliferation in the developing and adult retina has received much attention in teleosts (Schmitt and Kunz, 1989; Mansour-Robaey and Pinganaud, 1990; Negishi et al., 1990; Hagedorn and Fernald, 1992; Negishi and Wagner, 1995; Kwan et al., 1996; Mack and Fernald, 1997; Julian et al., 1998; Hu and Easter, 1999; Marcus et al., 1999; Li et al., 2000; Cid et al., 2002; Candal et al., 2005a; Raymond et al., 2006; Alunni et al., 2007), which are modern representatives of the large radiation of

Abbreviations: AC, amacrine cells; BP, bipolar cells; ce, ciliary epithelium; CMZ, ciliary marginal zone; GCL, ganglion cell layer; HC, horizontal cells; INL, inner nuclear layer; INLi, inner part of the INL; INLo, outer part of the INL; IPL, inner plexiform layer; L, lens; l, layered part of the TZ; nl, non-layered part of the TZ; OD, optic disc; ON, optic nerve; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptors; RGC, retinal ganglion cells; RPE, retinal pigmented epithelium; TZ, transition zone.

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doi:10.1016/j.jchemneu.2009.10.001

ray-finned bony fishes. However, it is not known whether these observations are extensive to other groups of fishes.

Cell cycle exit is closely linked to cell differentiation in the developing retina. A temporal correspondence between the loss of proliferation and the detection of differentiated cells has been reported in teleosts (Negishi et al., 1990). Further understanding of the process of retinal cell diversification is provided by the order in which the different types of neurons and glia appear during development. Among retinal cell types, photoreceptors have been often used in studies of cell differentiation, because they can be readily identified morphologically and by molecular markers. During development of the vertebrate retina, postmitotic cells in the outer neuroblastic layer differentiate in two types of photoreceptors: cones and rods. The sequence of expression of cone and rod opsins during development varies among the species examined (goldfish: Stenkamp et al., 1996, 1997; zebrafish: Raymond et al., 1995; Schmitt et al., 1999; salmonids: Cheng et al., 2007; chick: Bruhn and Cepko, 1996; Adler et al., 2001; Raymond and Barthel, 2004; Takechi and Kawamura, 2005; mammals: Szél et al., 1993, 1994; Seiler and Aramant, 1994; Dorn et al., 1995; Bumsted et al., 1997; Xiao and Hendrickson, 2000; Bumsted O'Brien et al., 2004; Chen et al., 2005), and the sequence in which the different photoreceptors are born does not necessarily correlate with the sequence in which opsins are expressed (Bumsted et al., 1997).

Cartilaginous fishes (Chondrichthyans) represent an ancient gnathostome lineage that includes sharks, skates and rays (Elasmobranchii) and chimaeras (Holocephala). Because of their key position as an out-group to all other living gnathostomes, knowledge of their retinal organization and development is essential to assess the ancestral condition in gnathostomes. Cartilaginous fishes (including sharks) are anamniotes and would be expected to have similar retinal development to other fish species. However, the scant developmental studies of the morphogenesis of their retina have revealed differences with teleosts in the order of cell differentiation (Fishelson and Baranes, 1999; Harahush et al., 2009) and in the development of the GABAergic system (Ferreiro-Galve et al., 2008).

In this study we focused on the pattern of cell proliferation and its relation with photoreceptor differentiation in the developing retina of two small sharks that inhabit shallow waters (*Scyliorhinus canicula* and *Haploblepharus fuscus*). The lesser spotted dogfish, *S. canicula*, a representative of the largest order of extant elasmobranchs (Order Galeomorpha, family Scyliorhinidae), has essential characteristics to become a model organism (Coolen et al., 2009). Moreover, the patterns of expression of some early developmental genes have been reported in the eyes of *S. canicula* in very early developmental stages (Sauka-Spengler et al., 2001; Plouhinec et al., 2005). The development of GABAergic structures in the retina of the two sharks subject of this work has been already published (Ferreiro-Galve et al., 2008).

While patterns of retinal cell proliferation have not been studied to date in elasmobranch fishes, over 40 species of elasmobranch have been investigated for their photoreceptor system. Ultrastructural studies performed in the developing retina of the shark *Chiloscyllium punctatum* have revealed that photoreceptors are the last retinal cells to differentiate (Harahush et al., 2009), although the time of expression of visual pigments (opsins) is not known. Many elasmobranchs possess at least one class of cone in the retina (Gruber et al., 1963, 1991; Gruber and Cohen, 1978, 1985; Cohen, 1990; Sillman et al., 1996; Hart et al., 2004, 2006; Theiss et al., 2007; Harahush et al., 2009) and some species of rays might have trichromatic colour vision (Govardovskii and Lychakov, 1977; Hart et al., 2004; Theiss et al., 2007; Bowmaker, 2008). However, other species may have pure-rod retinas (Govardovskii and Lychakov, 1977; Dowling and Ripps, 1970, 1991; Ripps and Dowling, 1991; Bozzano et al., 2001; Bozzano, 2004), and in two species of *Raja*, the rods function at both scotopic and photopic levels (Dowling and Ripps, 1991; Ripps and Dowling, 1991), which indicates that rods are the only photoreceptor common to all elasmobranchs. To our knowledge, the photoreceptor system has not been investigated in *H. fuscus*, and the presence of cones in the genus *Scyliorhinus* is controversial, as *Scyliorhinus* spp. has been classically reported to posses cones (Neumayer, 1897; see also Gruber and Cohen, 1985; Theiss et al., 2007; Davies et al., 2009), while *S. canicula* has been defined as a pure-rod species (Bozzano et al., 2001; Gačić et al., 2007).

For proliferation we examined the expression of the proliferating cell nuclear antigen (PCNA, a subunit of DNA polymerase) and of the phospho-histone-H3. PCNA has been widely used as a proliferation marker in the retina (for a review, see Candal et al., 2005a), as it can be detected in proliferating cells throughout the cell cycle except in the mitotic phase (M-phase). Its expression begins in late G1 phase, is maximal during the S phase and decreases from the S/G2 transition, the levels of PCNA being also low in resting cells (G0-phase). The use of PCNA as a proliferation marker has some advantages with respect to other assays to detect cell proliferation, including the use of DNA precursors such as tritiated thymidine (^{3}H-T) or nucleotide analogues such as bromodeoxyuridine to detect their incorporation into DNA (for a review, see Ortego et al., 1994). These DNA precursors are incorporated only during the S phase and, for slow-growing animals, require a long exposition to these substances for evaluating the results, which can affect their viability, while PCNA detects cycling cells without previous treatments of living specimens. Although PCNA immunohistochemistry (unlike the above mentioned methods) does not allow to study the dynamic evolution of cell populations, it labels basically the same cells and regions evidenced by the use of ^{3}H-T or BrdU as markers of proliferation (for a review, see Candal et al., 2005b). In addition, we examined the expression of the phospho-histone-H3 (PH3) currently used as a mitotic marker, since H3 phosphorylation is associated with chromosome condensation and dynamics during mitosis (Hendzel et al., 1997; McManus and Hendzel, 2006; Carney et al., 2007). For characterizing photoreceptor differentiation we used an anti-rod opsin antibody (CERN 922), which has been found useful to label early developing photoreceptors in the retina of agnathans (Meléndez-Ferro et al., 2002; Villar-Cheda et al., 2008) and teleost fishes (Candal et al., 2005a). In addition, we performed two-colour immunolabelling with PCNA and rod opsin antibodies in order to examine the spatiotemporal sequence of photoreceptor differentiation in relation to cell cycle exit (PCNA-immunonegativity).

Knowledge of the pattern of cell proliferation and photoreceptor differentiation in the retina of elasmobranchs may improve comparative knowledge about the development of the visual system in vertebrates.

2. Materials and methods

2.1. Experimental animals

Embryos, juveniles and adults of the lesser spotted dogfish (*S. canicula*) and the brown shyshark (*H. fuscus*) were kindly provided by the Aquário Vasco da Gama and the Oceanário in Lisbon (Portugal), and the Aquarium Finisterrae in A Coruña (Spain). Additional embryos of *S. canicula* were supplied by the Station Biologique de Roscoff (France). Eggs from different broods and juveniles were raised in fresh sea water tanks in standard conditions of temperature (16–18 °C) and 12:12 h day/night cycle.

The embryonic stages of *S. canicula* were identified by external features, following Ballard et al. (1993), and similar features were used for staging the *H. fuscus* embryos (see Table 1). The following embryonic stages were analysed: stage 26 (1 *S. canicula*), stage 28 (1 *H. fuscus*), stage 29 (1 *S. canicula*, and 1 *H. fuscus*), stage 30 (1 *H. fuscus*), stage 31 (3 *S. canicula* and 6 *H. fuscus*), stage 32 (7 *S. canicula* and 5 *H. fuscus*), and stages 33 and 34 (prehatching; 4 *S. canicula* and 6 *H. fuscus*). Moreover, 5 juveniles (3 *S. canicula* and 2 *H. fuscus*, from 10 to 14 cm in total length) and 1 adult *S. canicula* (about 50 cm total length; provided by a local fisherman) were also processed. Adults were sacrificed immediately after incoming to the lab.