1. Results and discussion

Glia development is a fundamental process in the nervous system throughout life. Thus, during the course of embryonic development, radial glial cells are found early and astrocytes and oligodendrocytes are found later. The PNS glia, Schwann and satellite cells, have a special origin; they are neural crest-derived: a group of cells that delaminate early in development from the dorsal neural tube, migrating extensively and giving rise to the PNS. The study examines the development of glial cells in the bamboo shark, *Chiloscyllium punctatum*, by identifying the expression pattern of several classic glial and myelin proteins. We show for the first time that glial development in the bamboo shark (*C. punctatum*) embryo follows closely the one observed in other vertebrates and that neural development seems to proceed at a faster rate in the PNS than in the CNS. In addition, we observed more myelinated tracts in the PNS than in the CNS, and as early as stage 32, suggesting that the ontogeny of myelin in sharks is closer to osteichthyans than agnathans. Published by Elsevier B.V.
In the late 1800s, elasmobranchs and especially sharks, held a pre-eminent position in the field of comparative embryology, but in the second half of the 20th century they were replaced by the use of more culture-friendly organisms such as chickens, frogs, mice and zebrafish (Balfour, 1874, 1880; Cole and Currie, 2007). However, in recent years a more detailed analysis of neurodevelopment in shark embryos is gaining value as new techniques such as orthologous gene identification and in situ hybridization are re-invigorating their use as models for evolution of development. Hence, the few molecular studies on the early development of shark nervous system are all quite recent. A good number of these have looked at the expression of some key ortholog transcription factors like Otx (Sauka-Spengler et al., 2001), Pax, NeuroD and Phox2B (Derobert et al., 2002; O’Neill et al., 2007) and FoxD (Wotton et al., 2008). These studies illustrate that the formation of the shark nervous system follows a pattern that is highly conserved among agnathans and gnathostomes, and the roles of the described transcription factors in brain regionalization have been highly conserved during vertebrate evolution (Derobert et al., 2002). These studies generally focused on neuronal and placodal development at stages 17–29, between the end of gastrulation and advanced organogenesis (Kuratani and Horigome, 2000). Gould and co-workers examined neural markers like O1, O4, GFAP and neurofilament in Squalus acanthias (dogfish) embryos on a 9 cm pre-hatching embryo (Gould et al., 1995), and demonstrated that the same relationships between oligodendrocytes and axons exist during early stages of myelination (Schweigreiter et al., 2006). In this study, we provide further insight into the appearance of glial cells in shark embryos before pre-hatching stages of development (stages 25–29 than in existing literature).

For glial development examination we chose two of the most widely used glial markers, glial fibrillary acidic protein (GFAP) and S100. GFAP, a member of the intermediate filament family, is known for its role in providing strength and support to cells (Kaneko and Sueoka, 1993). Specifically, GFAP forms the intermediate filaments that are characteristic of astrocytes and radial glia to regulate their shape and motility (Kaneko and Sueoka, 1993). This cytoskeletal component has a long phylogenetic history as it has immunoreactivity in the nervous systems of hagfish, lungfish, annelids and mollusks (Cardone and Roots, 1990; Dahl et al., 1985; Lazzari and Franceschini, 2004; Onteniente et al., 1983). The other glial marker we used was S100, a member of multi-member low-weight protein family with a variety of extracellular and intracellular functions, such as regulation of protein phosphorylation, calcium homeostasis, cell growth and differentiation, inflammatory responses, and transcription factor regulation (Donato, 2003; Riuzzi et al., 2006). Past research has demonstrated that GFAP (Ari and Kalman, 2008a,b; Kalman and Gould, 2001) and S100 (Ari and Kalman, 2008a; Chiba, 2000) clearly label glial cells in elasmobranchs making them ideal candidates to use for studies of glial cell development. However, these studies were performed on adult tissues and not embryonic tissues. Our study will delineate the embryonic development and distribution of glial cells in elasmobranchs.

1. The expression of gliogenic and neurogenic markers during central and peripheral nervous system development in shark

1.1. Stage 25 shark embryos

In order to begin assessing the presence of glial cells in shark embryos, we looked as early as stage 25 (1.3 cm).

1.1.1. CNS. The first glial cells that appear during development in gnathostomes are radial glia, the best known and most abundant type of glia found in developing vertebrates. This glial type is usually distinguished by expressing both nestin and GFAP (Malatesta et al., 2003). In stage 25 shark embryos we observed only weak staining using a polyclonal GFAP antibody (data not shown). In addition, we tested the glial precursor antibody, 7B3, against the transitin protein, also known to label neural crest cells that will later give rise to Schwann cells (Hennion et al., 2000) and found that as GFAP, it weakly stained cells in the neural tube (Fig. 1A). The GFAP and 7B3 staining was observed in both the ventricular and marginal layers of the developing neural tube. Interestingly, on the ectoderm/skin of these shark embryos there were scattered cells clearly positive for GFAP (arrows in Fig. 1B). Although we did not observe strong staining for GFAP or 7B3 in the neural tubes at this stage, we observed neuroblasts labeled with Dil extending along the width of the neural tube (Fig. 1D). We also observed that cells in the marginal zone were labeled with anti-beta III neuronal specific tubulin, TuJ1 (arrowheads in Fig. 1C and D) indicating that neurogenesis is actively taking place at this stage.

1.1.2. PNS. At stage 25, shark embryos have extensive CNS growing axons into the pharyngeal clefts (or branchial arches, Fig. 1C) and in the forming spinal nerves along the trunk (data not shown). More importantly, cells migrating on the branchial arches (presumptive neural crest cells) were positive for GFAP (inset in Fig. 1B) and the pre-migratory neural crest also weakly expressed GFAP (data not shown). We found positive staining for 7B3 along what is likely to be migrating lateral line primordium (arrow in Fig. 1A).

1.1.2.1. CNS. Fig. 2A shows stage 27 shark embryos with robust expression of GFAP in the neural tube and a pattern reminiscent of radial glia, especially at the most rostral levels. Myoblasts stained for GFAP as well (data not shown). Robust expression of 7B3 was seen in rostral neural tube cells extending throughout the neural tube (Fig. 2B) as was also shown for GFAP (Fig. 2A). Staining of thin sections with GFAP antibody in the forebrain showed a clear radial pattern of fibers extending throughout the width of the neural tube (arrows in Fig. 2C). GFAP and 7B3 staining at more caudal, less developed neural tubes were weak, as was seen in stage 25 embryos (data not shown).

1.1.2.2. PNS. GFAP surprisingly stained what could be cranial migrating neural crest cells (arrowhead in Fig. 2C) and 7B3 labeled mesenchymal cells in the shark embryos along the migratory pathways of neural crest cells (Fig. 2D). To identify the 7B3-positive mesenchymal cells as putative neural crest/glial cells we used FoxD3, a transcription factor that labels pre-migratory and early migrating neural crest in zebrafish (Lister et al., 2006). FoxD3 antibody strongly labeled Dil migrated neural crest along lateral line region (arrows in Fig. 2E), thus supporting the hypothesis that these cells are putative peripheral crest-derived glia. FoxD3 also labeled the ectoderm in shark embryos in a similar cellular pattern to that of ectoderm GFAP-expressing cells at stage 25 (data not shown) and what appears to be a developing cranial ganglia (arrow in Fig. 2F).

Wholemount staining with the neuronal marker TuJ1 at stage 27 showed a robust peripheral neuronal development. At the most rostral levels, there are already clearly defined spinal nerves and axons growing anterior to posterior in the embryo (yellow arrowhead in Fig. 2G). At the most caudal levels, where the nervous system of the embryo is still in early phases of development, thin section showed that the cells in the laminar layer as well as developing dorsal root ganglion express TuJ1 (Fig. 2H). However, observed weak GFAP-positive staining in these peripheral nerves at this stage (data not shown). In both wholemount and thin section, the TuJ1 immunostaining showed that peripheral ganglia have more TuJ1 positive cells than neural tubes (arrowhead in Fig. 2H).
1.1.3. Stage 29 shark embryos

In stage 29 embryos, the nervous system growth (PNS and CNS) is more advanced and the embryo is starting to change towards a more shark-like shape.

1.1.3.1. CNS. Embryo staining with GFAP showed that there are enough differentiated glial cells in the CNS at stage 29 to highlight the whole spinal cord in wholemount staining in a diffuse manner (Fig. 3A). At more rostral levels, the laminar part of the neural tube was positive for Tuj1 (Fig. 3C).

1.1.3.2. PNS. At the most rostral level, where the nervous system is more mature, GFAP highlighted a set of peripheral nerves (arrows in Fig. 3B). These nerves and lateral line (Fig. 3D) are well into differentiation as indicated by Tuj1 wholemount immunofluorescence (arrowhead in Fig. 3E). Peripheral nerves are beginning to enter the rostral fin (arrow in Fig. 3E). The developing myoblasts in the caudal fin also showed strong GFAP expression (arrow in Fig. 3D).

1.1.4. Stage 32 shark embryos

The CNS of shark embryo at stage 32 is well developed, as there are clearly visible neuronal layers in the hindbrain and the PNS seems to have reached its maximal sensory ganglia formation (we did not see DRG at the most caudal levels, only projecting nerves, data not shown). There are many nerves extending throughout the embryo, especially into the muscle tissues.

1.1.4.1. CNS. Immunohistochemistry on sections at the tail-most level in stage 32 embryos demonstrated that GFAP is strongly expressed in a radial pattern in the developing spinal cord (Fig. 4A) as well as in more rostral sections (inset in Fig. 4F). Similarly, 7B3/transitin is expressed in an identical radial pattern in the neural tube (Fig. 4B). In addition, we observed that both markers, GFAP and to a lesser degree, 7B3, continued to be present in the developing myoblasts, (arrows in Fig. 4A and B). The S100 glial marker was present at this stage in a similar radial pattern as GFAP and 7B3 (Fig. 4C). Interestingly, we were not able to determine with certainty the presence of S100-positive cells in the previous stages.

1.1.4.2. PNS. Peripheral nerves in stage 32 embryos were stained with S100 at the trunk level (arrow in Fig. 4C). At similar antero-posterior level Tuj1 labeled the neuronal component (arrowhead in Fig. 4D). The neuronal/stem cell marker Sox2 faintly stained peripheral nerve (arrowhead in Fig. 4D), and gave strong positive signal in younger myoblasts (Fig. 4D) at more caudal levels, along antero-posterior axis, whereas developmentally older myoblasts, at rostral levels, did not express Sox2 (data not shown).

HNK-1 labeled a group of cells under the skin (arrowhead in Fig. 4C) similar to FoxD3. FoxD3, a transcription factor responsible for neural crest and glia development (Kelsh et al., 2000; Kos et al., 2001), was expressed in cells underlying the skin ectoderm (arrowhead in Fig. 4E) (Dottori et al., 2001) and most importantly in peripheral nerve (red arrowhead in Fig. 4E).

GFAP wholemount staining in the trunk showed the same pattern as in stage 29 embryos; positive myoblasts (not shown) and a banded signal in the fin which partly overlapped with the Tuj1- and HNK-1 expression domains (Fig. 4F and data not shown).

We tested several commercially available antibodies against the Sox8 and SoxE transcription factors (Sox8 and Sox10) to see if we could label peripheral glia in shark embryos. But none of the antibodies against proteins expressed in glia (Sox8 and Sox10) yielded specific staining probably because they do not recognize the most...
conserved HMG domain in SoxE gene family members (Dutton et al., 2008).

1.2. Pre-hatching shark embryos

Once *Chiloscyllium punctatum* embryo has reached 6 cm of length, their body morphology resembles that of a small adult bamboo shark. Since development progresses in a rostral to caudal manner, we examined the expression of glial markers at the tail, mid-trunk and cephalic regions. Because at this stage, the rostral and most of the trunk regions of the nervous system are well advanced in development, we expected that the use of the glial markers would provide more structural rather than developmental information.

![Fig. 2. Neural markers expressed in the stage 27 shark embryos. Immunofluorescence of stage 27 shark embryo for GFAP (A and C), 7B3 (B and D), FoxD3 (E and F) and Tuj1 (G and H). By this stage neural tube shows robust staining for glia markers (GFAP in (A) and (C), 7B3 in (B)). GFAP was expressed in radial glia (arrows in (C)) and neural crest (arrowhead in (C)). 7B3 and FoxD3 labeled migrating neural crest (D), lateral line (E) and cranial ganglia (F). The neuronal marker Tuj1 was widely expressed by peripheral nerves (G and H) and by ventricular cells in the neural tube (H). (I) A cartoon of a stage 27 indicating the regions of the sections in each figure. (D–F) Neural tubes were vitally labeled with DiI, shown in red channel. Cell nuclei were stained blue with DAPI.](image-url)
1.2.1. CNS

At the most caudal end of these embryos, we detected extensive staining in radial glial fibers using GFAP and S100 antibodies (Fig. 5A and D). The expression of these GFAP- and S100-positive cells was prominent around the central canal and in fibers projecting to the periphery of the spinal cord, although none were “star-shaped”, as expected (Ari and Kalman, 2008a). S100 also showed some punctuated staining likely corresponding to glial cells in the ventricular zone of the spinal cord (Wicht et al., 1994) (inset in Fig. 5D), but we could not definitively identify them as either oligodendrocytes or astrocytes. To determine the level of development of neurons/neuroblasts at this anatomical level, we used the well known neural marker 3A10 that labels neurofilament in other species of sharks (Freitas and Cohn, 2004; Freitas et al., 2006; O’Neill et al., 2007). We observed that, at the trunk level, positive fibers in the spinal cord labeled the same discreet areas that myelin markers identified, which strongly suggests that they correspond to axons in the process of myelination (Potzner et al., 2007) (see Fig. 5G and also Fig. 7).

The expression pattern of GFAP and S100 at the rostral end of these embryos was quite different. GFAP- and S100-positive fibers were present in abundance in both head and trunk (Fig. 5B, C and E). GFAP staining was observed in a classic radial fashion (Dahl et al., 1985) and in great abundance in the ventricular zone along the length of the neural tube, much more pronounced than in stage 32 embryos (Fig. 5B and C). The definite presence of star-shaped cells are indicative of astrocytes (arrowheads in Fig. 5C) (Ari and Kalman, 2008a). S100 staining was very similar to GFAP especially in the head (Fig. 5E), and to lesser extend in the spinal cord (data not shown).

1.2.2. PNS

It is clear that by this stage neuronal development is well advanced, since Tuj1 staining was present along the entire neural tube including all the levels in the head and trunk. Fig. 5F shows a section at the trunk level stained with Tuj1, where the presence of mature neurons exist not only in the developing spinal cord, but also as motor axons, sympathetic ganglia, and the gut enteric nervous system. At this stage, GFAP levels in nerves was reduced in comparison to other stages and to the CNS (data not shown).

1.3. Study of myelin markers in the shark embryos

One of the main markers of myelination is myelin basic protein (MBP). This protein is the second most abundant myelin component in vertebrates, and is made by both oligodendrocytes and Schwann cells (Allinquant et al., 1991; Morell, 1984). Immunostaining of rostral sections of stage 32 embryos showed that MBP as well as Myelin Protein Zero (MPZ, Po) is starting to appear in the midbrain of these embryos (Fig. 6A and C). In a late stage
32 embryo, but not yet pre-hatching, we observed more specific staining of myelinated nerves using MBP antibody in the peripheral ganglia. Specifically, trigeminal ganglion at cranial level and dorsal root and spinal nerves in the trunk stained positive for MBP (Fig. 6B and D), while very little staining was found in CNS. However by pre-hatching embryo stage, myelin tracts positive for MPZ and MBP were distinctly present in the CNS (Fig. 6E and F), while few myelinated fibers were present in the brain (data not shown). MPZ was expressed not only in myelinated fibers, but also in the bodies of some cells in the CNS (squared area in Fig. 6E).

In addition to looking in the head regions, we also looked in the trunk for presence of myelinated tracts in the spinal cord and PNS. Interestingly, in the pre-hatching embryo stage, myelin tracts positive for MPZ and MBP were distinctly present in the CNS (Fig. 6E and F), while few myelinated fibers were present in the brain (data not shown). MPZ was expressed not only in myelinated fibers, but also in the bodies of some cells in the CNS (squared area in Fig. 6E).

1.4. Glial formation

The vast majority of studies on chondrichthyan glia have been on adult or pre-hatching embryo spiny dogfish (S. acanthias), a species phylogenetically related to bamboo shark, or skate species (Ari and Kalman, 2008a; Gould et al., 1992). These studies focused on the presence and distribution of astrocytes and radial glia in mature brains. Our findings showed that the first detectable glia appear between stage 25 and stage 27, using GFAP and 7B3 antibodies for identification. In stage 25 embryos, we observed 7B3 and GFAP in cells whose morphology suggests neuroepithelial cells instead of the radial cell as was observed in zebrafish embryos, further confirmed by live Dil labeling (Kim et al., 2008). By stage 27, GFAP and 7B3-positive cells spanned the rostral neural tube, and their morphology suggested that their phenotype corresponds to true radial glia instead of neuroepithelial (Malatesta et al., 2003). These cells have been already described in adult agnathans like lamprey and in adult sarcopterygians like lungfish (the sister group to the tetrapods), as well as in osteichthyans and actinopterygians embryos (Campbell and Gotz, 2002; Park et al., 2004; Rakic, 1971) based on being positive for glial markers (see Table 2). While radial glia appearance in mouse is known to hap-

Fig. 4. Glial and neural crest markers expressed in the stage 32 shark embryos. Sections through a stage 32 embryo at the tail-most end for GFAP (A), 7B3 (B), S100 (C), Tuj1 (D) and FoxD3 (E) labeled a specific group of cells. Radial glia and myoblasts were strongly stained with GFAP (inset for neural tube magnification in (A) and (F)). 7B3 labeled radial cells in the neural tube (inset in (B)) and to a lesser extent in the medial myoblasts (arrows). (C) S100 (green) also labeled radial cells; inset shows a magnification of the neural tube, while HNK-1 labeled skin (arrowhead). (D) Tuj1 (red) and Sox2 (green) weakly overlap and double labeled peripheral nerve (arrowhead in (D)). (E) FoxD3 was present in peripheral nerve (red arrow) as well as in dermal cells (arrowhead in (E)). GFAP labeled a group of cells in the fin (red arrows in (F)) while Tuj1 presented a banded staining in the fin (green). (G) A cartoon of a stage 32 embryo with line indicating the area for each figure. Cell nuclei were stained blue with DAPI.
pen around embryonic day 12 (Hartfuss et al., 2001), shortly fol-
lowed by neurogenesis, these cells soon after will disappear (Cai
et al., 2005; Malatesta et al., 2003). In agnathans and chondrich-
thians, these cells will remain through adulthood (Rovainen, 1979).
The early appearance of radial glia during neurogenesis indicates a
similarity in neural development between chondrichthians and
amniotes that does not seem to be shared with actinopterygians.
The expression of GFAP in pre-hatching embryos in the bamboo
shark (C. punctatum) was very strong along the ventricular layer
and in the numerous radial fibers present throughout the CNS as
has been observed in other vertebrates (Ari and Kalman, 2008a;
Kalman, 2002; Kalman and Gould, 2001). This pattern of GFAP
expression (and S100) in the pre-hatching embryo was more like
the one present in osteichthians and in hagfish (Wicht et al., 1994)
but it did not look like the one we found in earlier embryos
when using Dil. This difference in shape and timing of cell appear-
ance suggests there may be two different types of radial glia in
shark embryos. An early radial glia along which neurons climb like
those in osteichthians, and a later radial glia that remains in the
embryo through adulthood like those thought to be stem cells in
zebrafish (Kim et al., 2008).

There are few markers that indicate the presence of immature/
precursors of glial cells: for example A2B5, FoxD3, Krox20, Nkx2.2
and Sox10 (Kettenmann and Ransom, 2005; Nubaku and de Bel-
lard, 2008). We successfully achieved positive signal from only
two antibodies we tested: FoxD3 and 7B3/transitin. Suggesting
that both these transcription factors, or their close paralogs, are
present in the bamboo shark (Wotton et al., 2008). Recently, Appel
and co-workers demonstrated the existence of ventrally migrating
glia in zebrafish that give rise to nerve perineurium (Kucenas et al.,
2008). Unfortunately, even though the presence of these ventrally
migrating glia have been shown in lamprey, chicken and zebrafish
(Lunn et al., 1987; Nakao and Ishizawa, 1987), our use of Nkx2.2
gave ambiguous results for the presence of these cells in bamboo
shark.

The appearance of astrocytes must be a later phenomenon in
bamboo shark since no star-like shaped cells were observed in
our stage 27–32 embryos, though they were clearly present on
the pre-hatching embryos. Astrocytes appear late in development
in mammals, reptiles and birds (Kalman, 2002; Sanosaka et al.,
2008; Woodruff et al., 2001; Wu et al., 2006). The bamboo shark
may follow the osteichthyan program for astrocyte development
than the one found in agnathans (hagfish and lamprey). Past re-
search from Kalman's lab and others showed the presence of astro-
cytes across several species of adult elasmobranchs and that the
differences among them were mainly in their abundance through-
out brain regions (Ari and Kalman, 2008a,b; Kalman and Gould,
2001). Our study did not look into specific conserved brain regions
but focused on early shark embryos. Future studies using gluta-
mine synthetase (GS) and vimentin antibodies in stage 32 onwards
may provide more detailed information about shark astrocytes
development and origins (Ari and Kalman, 2008a,b).

It has been documented that glial cells and myelinated tracts of
the lizard Gallotia galloti, have S100 immunoreactivity (Romero-
Alemán Mdel et al., 2003) and that mature astrocytes and tany-
cytes in juvenile dogfish, Scyliorhinus canicula express S100 (Chiba,
Fig. 5. Neural markers expressed in the pre-hatching shark embryos. Shark embryo sections were immunostained for GFAP (A–C), S100 (D and E), 3A10 (G) and Tuj1 (F). GFAP
and S100 both labeled radial glia processes, while S100 also cell bodies in the ventricular zone (magnified inset in (D)). DRG, sympathetic ganglia (SG), motor axons (Ax) and
teric nervous (ENS) system were all clearly marked with Tuj1 in (F).
However, no studies have concentrated on the earliest stages of shark embryogenesis using this marker. Our study shows for the first time the presence of S100 in the developing nervous system of the shark, especially its presence in the radial glia of the developing neural tube and in peripheral nerves. The S100-positive cell bodies found in the spinal cord, most likely correspond to oligodendrocyte precursors as suggested by the overlap of S100 antibody staining and in situ hybridization with a probe specific for shark Sox8 (manuscript in preparation).

1.5. Myelin development

A very important aspect in the evolution of nervous system development of gnathostomes is the appearance of myelinated ax-
The ontogenesis of myelin has been a controversial issue. Some studies show that ontogenically myelin markers appear first in the PNS before CNS of fish, mice and chicken (Bromdale and Halpern, 2002; Fu et al., 2002; Martenson, 1992; Wang...)

Fig. 7. Myelin markers expressed in the trunk of pre-hatching shark embryo CNS. Spinal cord sections were immunostained for TuJ1 and MPZ (A–D), CNPase (E), MBP (F and H) and PLP (G). Spinal cord and nerves had abundant neuronal processes as attested by TuJ1 and myelinated fiber are beginning to delineate the butterfly shape of mammalian spinal cords observed in osteichthyans (B, F and to lesser extent E). At the tail-most end, MBP labeled fewer axons along the same locations as in more rostral regions (H). PLP did not give positive staining on these sections (G). Arrows point to myelinated tracts.
et al., 2006) although it is not settled if this pattern (first PNS then CNS) also happens phylogenetically. Other studies suggest that myelin first appears in the CNS, or simultaneously between the CNS and the PNS (Zalc, 2006). In the meantime, no definitive ontogeny has arisen on the development of myelin in sharks. Previous studies indicated that ventral spinal cord of pre-hatching dogfish (S. acanthias) have myelinated tracts (Gould et al., 1992) and that myelin MPZ and MBP proteins can be found in other shark species (Rotenstein et al., 2008). Our present study shows for the first time that bamboo shark embryos have myelinated fibers from stage 32 onwards, well before the pre-hatching stage, and that its appearance takes place in the PNS before the CNS. Therefore placing the ontogeny of myelination in sharks along that of fish, birds and mammals, which show myelin markers first in the CNS before the PNS.

Vertebrate myelin sheaths express either MPZ or PLP, usually accompanied by DM20 (Martenson, 1992; Snipes et al., 1993). While MPZ is found in the CNS myelin in fish and shark, PLP/DM20 is generally accepted as the myelin CNS marker in tetrapods, suggesting that myelin MPZ and MBP proteins can be found in other shark species (Rotenstein et al., 2008). Our present study found as expected from these past studies that shark embryos have much less PLP expression in myelin tracts compared with other myelin proteins (MPZ, MBP and CNPase).

We found that in addition to MBP in early spinal cord, SMP is present in CNS glia as well as in PNS ganglia. SMP is a protein used to differentiate myelin-producing glial cells from glial satellite cells (Cameron-Curry et al., 1991; Dulac et al., 1988) and is detectable in late neural crest-derived cells days before myelination begins (Dupin et al., 1990). Hence, it seems that in chondrichthyans not only MPZ protein, but also SMP is expressed both in PNS and CNS (Rotenstein et al., 2008). We observed that glial markers were present in shark nervous system starting by stage 25, with radial glia present by stage 27. Myelination was found as early as stage 32 embryos and was more abundant in the PNS than CNS in these shark embryos suggesting that myelin ontogeny in sharks follows the same pattern as in osteichthians rather than agnathans. Altogether, these data reinforce the relevance of using sharks as model organisms (Coolen et al., 2009).

Table 1

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+, ++, +++ or ++++ refers to the abundance/intensity of the staining with the antibodies.

Table 1: Summary table of antibody results.

CNS, central nervous system; NS, nervous system; PNS, peripheral nervous system; GFAP, glial fibrillary acidic protein; S100, calcium binding protein; MBP, myelin basic protein; MFZ, myelin P zero protein; SMP, Schwann cell myelin protein; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; PLP, proteolipid protein; 7B3, transitin; FoxD3, forhead family of transcription factors; TuJ1, anti-beta III tubulin; 3A10, neurofilament; NF-M, neurofilament medium; Sox2, SRY 2 transcription factor; p75, NGF receptor; HNK-1, antibody recognizes carbohydrate epitope; Cyto, cytoskeletal protein; TF, transcription factor; carb, carbohydrate.

2. Experimental procedures

2.1. Collection and staging of embryos

Bamboo shark cases, C. punctatum (Müller and Henle, 1838) or mermaid’s purses were harvested from the Long Beach Aquarium and Cabrillo Aquarium (kindly provided by Chris Plante and Kiersten Darrow, respectively), reared at 25 °C in sea water, and collected at different developmental stages. Embryos were removed from egg cases and fixed in 4% paraformaldehyde or Carnoy’s fixative overnight at 4 °C. The embryos were staged according to Ballard’s developmental table when feasible or by their length in cm (Ballard et al., 1993). The youngest embryos collected were 3-cm long and had accomplished the development of most of their forebrain. The oldest embryos were 10-cm long, a stage at which they show much physical activity and look like their adult counterparts. Embryos were either fixed in Carnoy’s (70% ethanol, 20% formaldehyde and 10% glacial acetic acid) or in 4% paraformaldehyde overnight at 4 °C and kept in 70% ethanol at –20 °C until histological preparation. For sufficient paraffin penetration, embryos needed very extensive dehydration steps (about 1 day per alcohol grade) and 2 full days in histosol for clearing. The tissues were then immersed in hot paraffin (McCormick Scientific ParaPlast Plus) in a vacuum oven for 2 days before preparing the blocks and sectioning. Embryos were sectioned (7–12 μm) with a microtome, collected on Super-Frost slides and dried overnight at 37 °C on a slide warmer. Dil injected embryos were cryoprotected in 15% and then 30% sucrose overnight, embedded in gelatin for 3 h at 38 °C, slowly frozen in liquid nitrogen, and sectioned at 12 μm.

For live labeling, stage 25 and stage 27 shark embryos were immobilized with tricaine and injected with DiI (cell tracker CM-Dil, C-7001, Invitrogen/Molecular Probes) (diluted in ethanol (1/10) in 10% sucrose) inside the neural tube along its length and hindbrain regions. The embryos were placed in a Petri dish after...
Ast, astrocyte; Ols, oligodendrocyte; SC, Schwann cell; RG, radial glia.

2.2. Immunohistochemistry on tissue sections

Shark tissue sections were de-waxed in histosol and re-hydrated in a graded series of ethanol washes (histosol, 100%, 90%, 70%, 50% and 25% ethanol washes in water) and then equilibrated in PBS (Dulbecco's) before blocking in PBS containing 10% fetal bo-

Table 2
Summary table of glia development in some vertebrates.

<table>
<thead>
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<th>Organism</th>
<th>Earliest glia found</th>
<th>Markers used</th>
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<td>Agnathan Lamprey (Lampeta japonica)</td>
<td>Adult lamprey: GFAP immunoreactivity observed in long straight processes that radiated through neuropil from ventricular wall, following nerve fiber bundles in white matter</td>
<td>GFAP</td>
<td>Onteniente et al. (1983)</td>
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<td>Chondrichthyan Shark (Squalus acanthias and Chiloscyllium punctatum)</td>
<td>Pre-hatching embryo: 5.5 cm (myelin)</td>
<td>Po, MBP, O1, O4</td>
<td>Gould et al. (1995), Gould et al. (1992) and Rotenstein et al. (2008)</td>
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<td>Osteichthyan Actinopterygian Fish (Danio Rerio)</td>
<td>Embryo: Radial glia show at 24 hpf</td>
<td>GFAP–GFAP promoter</td>
<td>Kim et al. (2008)</td>
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<td>Sarcopterygian Coelacanth (Latimeria chalumnae)</td>
<td>Adult: Glia observation was from myelin studies</td>
<td>Myelin proteins (PLP/DM20)</td>
<td>Linnington and Waehneldt (1990), Tohyama et al. (1999) and Waehneldt and Malotka (1989)</td>
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<tr>
<td>Lungfish (Protopterus annectens)</td>
<td>Adult lungfish: GFAP: Immunopositive structures represented by thin long fibers of cells were found in both grey and white matter of CNS, as well as thin fibers that converged on blood vessel walls. Vimentin immunoreactivity was observed in peripheral zone of brain, but weakly observed in the white and grey matter fibers of the brain and the white matter of the ventral region of spinal cord</td>
<td>GFAP and vimentin</td>
<td>Lazzari and Franceschini (2004)</td>
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<tr>
<td>Amphibian (Xenopus laevis)</td>
<td>Stage 21: Brain: NF expression in developing neurons occasionally observed at stage 21 and reliably in the neural tube and caudal regions of the brain at stage 23. At stage 23, GFAP expression was observed in the cytoplasm of spinal cord radial glial but only in the peripheral region of the brain</td>
<td>GFAP and NF</td>
<td>Messenger and Warner (1989)</td>
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<td>Reptiles (Eumeces fasciatus)</td>
<td>E32: Juvenile (Ols)</td>
<td>S100, electron microscopy</td>
<td>Monzon-Mayor et al. (1990) and Romero-Aleman et al. (2004)</td>
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<td>(Gallotia galloti)</td>
<td>Stage 29: CNS myelination</td>
<td>Vimentin and GFAP</td>
<td>Nalon et al. (1995)</td>
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<td>Stage 32: Telencephalon: Vimentin observed in the proliferative zone of the lateral ventricle and subpial end-feet in the marginal zone at stage 32. At stages 34 and 35, vimentin staining is at its peak of intensity in all radial glial. GFAP appears at stage 35 in the end-feet in the marginal zone and increases until adulthood</td>
<td>GFAP and vimentin</td>
<td>Romero-Aleman et al. (2004)</td>
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<tr>
<td>Turtle (T. sinensis)</td>
<td>5–6-cm juvenile (ependymal glial cell pattern throughout CNS; extraependymal star-shaped astrocytes in the spinal cord)</td>
<td>GFAP immunoreactivity observed in long straight processes that radiated through neuropil from ventricular wall, following nerve fiber bundles in white matter</td>
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<tr>
<td>Birds</td>
<td>Chicken (Gallus gallus)</td>
<td>Radial glia: Observed as early as E5 using recombinant virus</td>
<td>Recombinant virus</td>
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<td>Astrocyes: Observed as early as E10</td>
<td>CD44</td>
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<td>Oligodendrocytes: Precursors are early, but they have been observed by E6 using Nkx2.2-positive cells</td>
<td>Nkx2.2</td>
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<td>E12 using transplant and SMP antibody</td>
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<td>Schwann cells: Seen in neural crest population by HH19</td>
<td>Seraf</td>
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<td>cK14: Schwann cells precursors are detected with MPZ</td>
<td>1E8, S100, O4</td>
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<td>Radial glia: GFAP promoter directed expression in RG by E12. RC1 expression seen at E13.5–14.5. RC1+ cells were nestin immunoreactive at E18.5. RC1 expression partially overlapped with GFAP at this stage</td>
<td>RC1, GFAP, vimentin, nestin, CD44 (possible astrocyte marker)</td>
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<td>Astrocytes: GFAP expression partially detected first at E16. It was weakly expressed in dev axonal tracts. CD44 was detected as early as E11.5 in radial glial cord</td>
<td>CD44</td>
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<td>Oligodendrocytes: In rat spinal cord by E12</td>
<td>PLP–GFAP promoter</td>
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<td></td>
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<td>Schwann cells: E11</td>
<td>Rat Po mRNA in situ</td>
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<td>Primates (Macacus Rhesus monkey)</td>
<td>Radial glial appear in first third of gestation. They appear in brain and spinal cord by E41, in the diencephalon by E45; and in the telencephalon and cerebellum by E47. Bergmann glial cell of the cerebellar cortex appear by E54</td>
</tr>
</tbody>
</table>

Rinsing in sterile sea water and incubated with 5 ml of DMEM, 10% FBS, pencillin and streptomycin at 37 °C for 12 h. These embryos survived for this period of time. At the end of incubation embryos were fixed for 1 h without transplanting them in 4% PFA, then placed in a vial for overnight fixing at 4 °C and remained there until cryo-sectioning.
vive serum (FBS) and 1% Triton X-100 for 1 h at room temperature. Primary antibodies were added in a 1:500 dilution in PBS and slides were incubated overnight at 4°C. A battery of primary antibodies (see Table 1) was used at concentrations ranging from 1:100 to 1:500. After washing the sections in PBS 3 times for at least 5 min each, secondary antibodies (Alexa fluoroprobes conjugated to anti-rabbit or anti-mouse IgG, Invitrogen) were added for 30 min with DAPI to label the nuclei, washed in PBS for immunofluorescence visualization and coverslipped with Permount. In some instances we did DAB staining of embryos or sections (following the protocol from Innovex Technologies, Santa Clarita, CA). Pictures of sections were taken using Axiovision LE software (Zeiss™) with an AxioCam black and white camera attached to a Zeiss A-1 AxioImager upright fluorescent microscope and assembled into figures using Adobe Photoshop 7.

Antibodies used were polyclonal GFAP and S100 (DAKO), monoclonal TuJ1 (Sigma), polyclonal FoxD3 (courtesy of D. Raible (Lister et al., 2006)), polyclonal MBP and MPZ raised against shark myelin peptides (courtesy of B. Gould (Rosten et al., 2008)), monoclonal PLP (proteolipid protein, courtesy of D. Colman (Sinoway et al., 1994), SMP (Schwann cell myelin protein), monoclonal 7/38 (transitin, HK-1 and 3A10 (DSHB, Developmental Studies Hybridoma Bank, UI), polyclonal Sox2 (Abcam), monoclonal NF-M and CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase) (Chemicon), polyclonal p75 (courtesy of L. Reichardt).

2.3. Wholemount immunofluorescence

Embryos were blocked overnight in blocking buffer (phosphate-buffered serum (PBS) with 1% Triton X-100, 10% fetal bovine serum (FBS)), and then incubated with primary antibodies in PBS overnight at 4°C. The next day, embryos were extensively washed with PBS and incubated with secondary antibodies (anti-mouse or anti-rabbit-Alexa 594, Invitrogen, Molecular Probes). Because the nervous system of young embryos showed high levels of autofluorescence in the 488 wavelength we used 594 wavelength secondary antibodies whenever possible. The following day the embryos were washed extensively and photographed with either an A-1 AxioImager upright fluorescent microscope and assembled into figures using Adobe Photoshop 7.

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Appendix A. Supplementary data


References


