

Spatiotemporal Distribution of Chondroitin Sulfate Proteoglycans in the Developing Mouse Retina and Optic Nerve

Safwat Ali Mohamed ALI^{1,2,3)}, Yoshinao Z. HOSAKA^{1,2)} and Masato UEHARA^{1,2)*}

¹⁾Laboratory of Basic Veterinary Science, United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753–8515

²⁾Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan and ³⁾Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Menoufia University, Sadat City, Egypt

(Received 11 May 2010/Accepted 1 August 2010/Published online in J-STAGE 12 August 2010)

ABSTRACT. The aim of the present study was to determine the distribution of chondroitin sulfate proteoglycans in the mouse retina and optic nerve of the prenatal and postnatal mouse by immunohistochemistry. At embryonic day (E) 18, chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S) and biglycan were detected in the retina and optic nerve. However, aggrecan was seen in the retina but not in the optic nerve. At postnatal day (P) 7, aggrecan and biglycan were clearly observed in the optic nerve, inner nuclear layer and ganglion cell layer and diffuse in the outer retina. C4S diffusely distributed in the retina and optic nerve, but C6S was mainly confined to the photoreceptor layer and optic nerve sheath. At P42, biglycan showed diffuse distribution in the retina and optic nerve with intense staining in nerve-fiber rich layers. Aggrecan showed weak staining at the inner plexiform layer with higher density in the outer and inner nuclear layers, outer plexiform layer and ganglion cell layer. Both C4S and C6S were detected in the optic nerve and retina, but C6S showed strong immunostaining in the photoreceptor layer. The distributions of these proteoglycans with respect of time course during development of the retina and optic nerve suggest that they may have unique or overlapping roles in development and maintenance of the retina and optic nerve.

KEY WORDS: chondroitin sulfate proteoglycan, development, optic nerve, retina, spatiotemporal distribution.

J. Vet. Med. Sci. 73(1): 13–18, 2011

The extracellular matrix plays an integral role in the pivotal processes of development, tissue repair, and metastasis by regulating cell proliferation, differentiation, adhesion, and migration [14, 15, 20, 34]. The major components of the extracellular matrix are collagens, proteoglycans, glycosaminoglycans (GAGs), fibronectin, and numerous less abundant proteins and glycoproteins. Proteoglycans are complex macromolecules composed by a core protein with one or more covalently bound GAG chains. GAGs, including chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate, are a widely distributed structurally diverse family of sulfated, unbranched polysaccharides that are expressed abundantly on the surface of cells and the extracellular matrix. GAGs have emerged as important regulators of the signaling involved in cell growth, tumorigenesis and inflammation [16, 29, 43].

Previously, it had been believed that proteoglycans were like cement simply filling the extracellular spaces in connective tissue and they were never the focus of research on neural tissues. As many unique proteoglycans have now been identified in neural tissues and the roles they play in cell–cell and cell–substratum interactions, cell proliferation and cell differentiation have been clarified, their major involvement in neural network formation has been indicated [13]. Proteoglycans have recently been shown to not only provide shape and biomechanical strength of organs and tissues but also to have direct and indirect cell signaling prop-

erties [20].

Chondroitin sulfate is uniquely important in morphogenesis, cell division and cartilage development and is the carbohydrate component of chondroitin sulfate proteoglycans (CSPGs), molecules that are spatiotemporally regulated during brain development [11, 18, 23, 38] and upregulated after injury in the central nervous system [6, 37]. CSPGs have also been found to be expressed in the retina [10, 20]. Keratan sulfate was localized in growing axons from chick retinal explants [27], however there is no reports about the presence of keratan sulfate in the mouse retina and optic nerve.

However, to the best of our knowledge, there are no reports about localization and developmental changes of proteoglycans in the mouse retina and optic nerve. The present study represents an attempt to assess the diversity and timing of the expression and distribution of 4 members of chondroitin sulfate, namely, chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), aggrecan and biglycan, and keratan sulfate, and to elucidate their functional roles in the development and maintenance of the retina and optic nerve.

MATERIALS AND METHODS

Animal care and experimental procedures were approved by the Animal Research Committee, Tottori University, Japan. Approval number is 09-T-19.

Animals and tissue processing: Jcl: ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were maintained in a 12:12 hr light-dark cycle with free access to food and water throughout the whole experiment.

* CORRESPONDENCE TO: UEHARA, M., Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan.
e-mail: muehara@muses.tottori-u.ac.jp

One male mouse was placed to mate with many female mice overnight and pregnant females were identified by the presence of vaginal plug in the next morning. Thus, the embryonic day zero (E0) was setup on the first day the vaginal plug was observed. Mouse embryos having embryonic age of 12 days (E12, $n=8$) and 18 days (E18, $n=8$) obtained by cesarean section from these pregnant mice were enucleated. In addition, eyes of mice having postnatal age of 7 (P7, $n=6$), 14 (P14, $n=4$), 42 (P42, $n=8$), and 120 days (P120, $n=6$) were also used in this experiment.

The mice were euthanized by cervical dislocation, and both eyes of each mouse were collected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight at 4°C with gentle shaking. Serial sagittal sections of the central retina were cut at 4 μm .

Antibodies: Polyclonal anti-biglycan, polyclonal anti-aggrecan and monoclonal anti-keratan sulfate antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A), monoclonal anti-C4S antibody was purchased from Abnova Corporation (Taipei, Taiwan), and monoclonal anti-C6S antibody was purchased from Seikagaku Biobusiness Corporation (Tokyo, Japan).

Immunohistochemistry: Four- μm -thick paraffin sections of mouse retina were deparaffinized, rehydrated, and rinsed with PBS. Indirect immunostaining was performed using a Histofine SAB-PO (M) (for mouse primary antibodies) or SAB-PO (R) (for rabbit primary antibodies) immunohistochemical staining kit (Nichirei, Japan). Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in methanol for 5 min. After washing with PBS, the retinal sections were treated sequentially with blocking serum from the same animal in which the secondary antibody was raised. Blocking times were 25 min for polyclonal antibodies and 1 hr for monoclonal antibodies at room temperature. The sections were then incubated at 4°C overnight either with specific primary antibodies (1: 50 in PBS for polyclonal antibodies and 1:50 1% bovine serum albumin in PBS for monoclonal antibodies) or with a buffer solution for control sections after treating with the blocking serum. After washing with PBS, biotinylated anti-mouse and rabbit IgG was applied for 30 min at room temperature. After washing with PBS, peroxidase-conjugated streptavidin solution was applied for 30 min. Peroxidase labeling was visualized using 0.05% 3–3' diaminobenzidine (Sigma). Micrographs of the sections were taken with a digital camera for a microscope (DP-71, Olympus, Tokyo, Japan).

RESULTS

The present study was done to investigate the distributions of CSPGs in the retina and optic nerve of mice at embryonic, early postnatal and adult stages.

Retina (Fig. 1): At E12, the retina was composed of homogenous neuroblasts. No immunostaining for CSPGs was detected in the retina. At E18, the ganglion cell and innerplexiform layers (GCL and IPL, respectively) were distinguishable from other neuroblasts. C4S, aggrecan and

biglycan showed diffused distribution throughout the retina, but C6S was detected mainly in the GCL.

At P7, all layers in the retina were distinguishable, but the photoreceptor layer (PRL) and IPL was thin. The distribution of chondroitin sulfate changed with retinal differentiation. Aggrecan immunostaining was clearly observed in the GCL and inner nuclear layer (INL) and was diffuse in the outer retinal layer. C4S and biglycan showed moderate diffuse immunostaining throughout the retina. C6S showed strong immunostaining in the PRL.

At P14 and P42, the retinal layers were complete. Biglycan showed diffuse distribution in the retina with higher staining affinity in nerve fiber-rich layers, including the IPL, outer plexiform layer (OPL) and nerve fiber layer (NFL), but did not show immunostaining in the PRL. Aggrecan showed moderate immunostaining in the GCL, INL, outer nuclear layer (ONL) and OPL with weak immunostaining in the IPL and PRL. C4S was distributed in the GCL, INL, ONL, OPL and retinal pigment epithelium (RPE) and not detected in the PRL. C6S was moderately distributed in the PRL and RPE and faint in the ONL.

At P120, the distributional pattern of these CSPGs was similar to that in P42 but in lesser intensity. However, aggrecan showed higher immunostaining in the ONL with faint immunostaining in the PRL and C6S showed higher immunostaining in the PRL.

No immunostaining for keratan sulfate was detected in the retina from E12 to P120.

Optic nerve (Fig. 2): At E12, the optic nerve was not formed yet as the previous studies [6, 7]. At E18, the optic nerve had formed. The optic disc showed diffuse immunostaining for C4S and biglycan, however, C6S showed weak immunostaining at the optic disc and the optic nerve with moderate staining at the optic nerve sheath. Aggrecan showed moderate immunostaining at the optic nerve head (Fig. 2).

At P7, C4S and biglycan were diffusely distributed in the optic disc and optic nerve with high density at the lamina cribrosa and confined to the laminar beam of the optic nerve. C6S showed weak immunostaining at the optic nerve head and moderate-staining at the optic nerve sheath. Aggrecan showed strong immunostaining in the optic disc, lamina cribrosa and optic nerve proper (Fig. 3).

At P14 and P42, the optic nerve showed diffuse immunostaining for C4S, C6S and biglycan and stronger immunostaining for aggrecan, especially in P42. The distribution of these proteoglycans at P120 was similar to that at P42 (Fig. 2). No immunoreactivity for keratan sulfate was detected in the optic nerve at any observed stage.

DISCUSSION

There are few reports on distribution of CSPGs in the retina and optic nerve [18, 31, 45]. In this study, we estimated the presence and distribution of C4S, C6S, aggrecan and biglycan in addition to keratan sulfate. We tried to determine the possible importance of such distributions on the

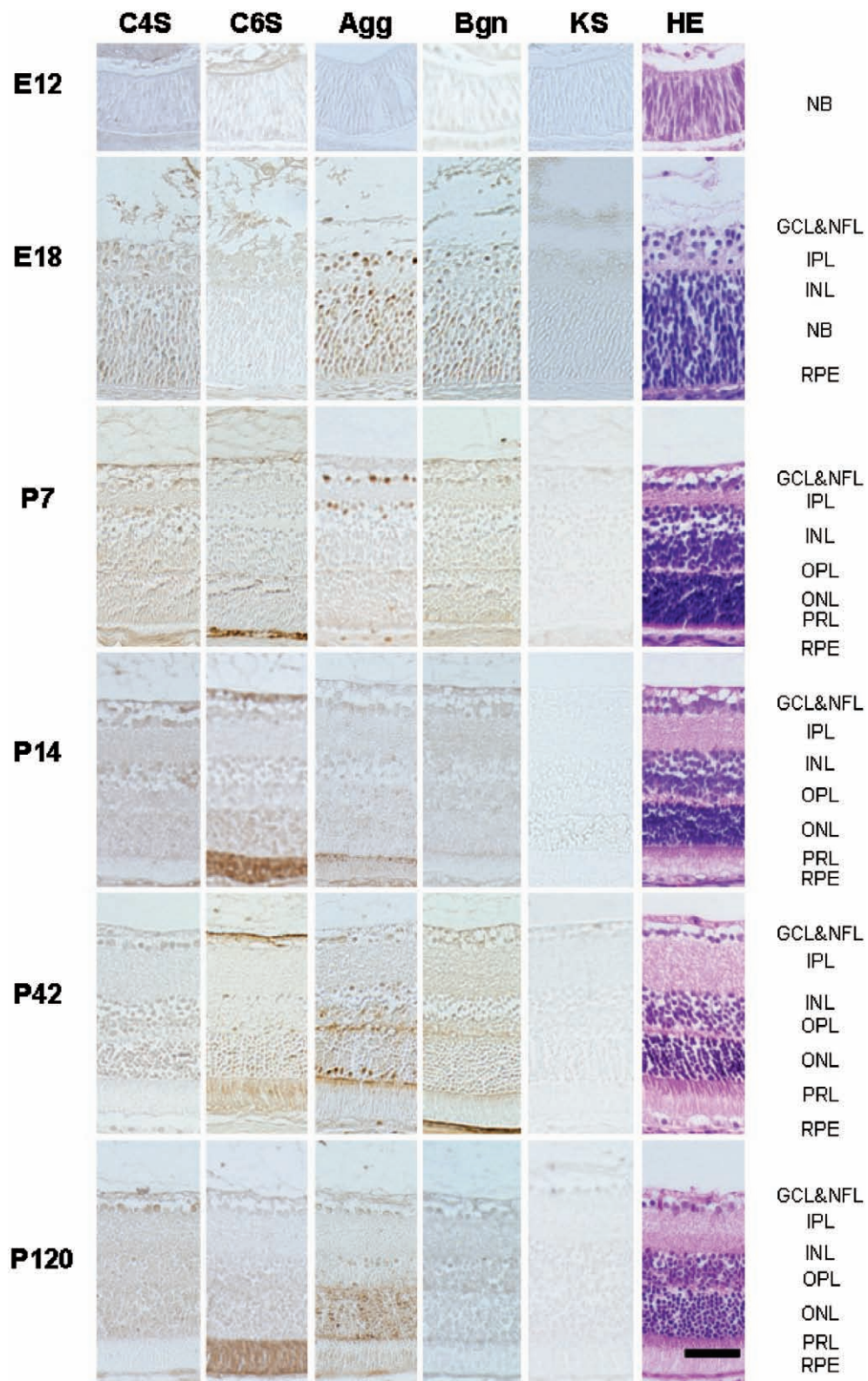


Fig. 1. HE staining and immunostaining of CSPGs in the retina of mice at E12, E18, P7, P14, P42 and P120. C4S, chondroitin-4-sulfate; C6S, chondroitin-6-sulfate; Agg, aggrecan; Bgn, biglycan; KS, keratan sulfate; HE, hematoxylin and eosin; NB, neuroblast cell mass; GCL&NFL, ganglion cell layer and nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium. Scale bar=50 μ m.

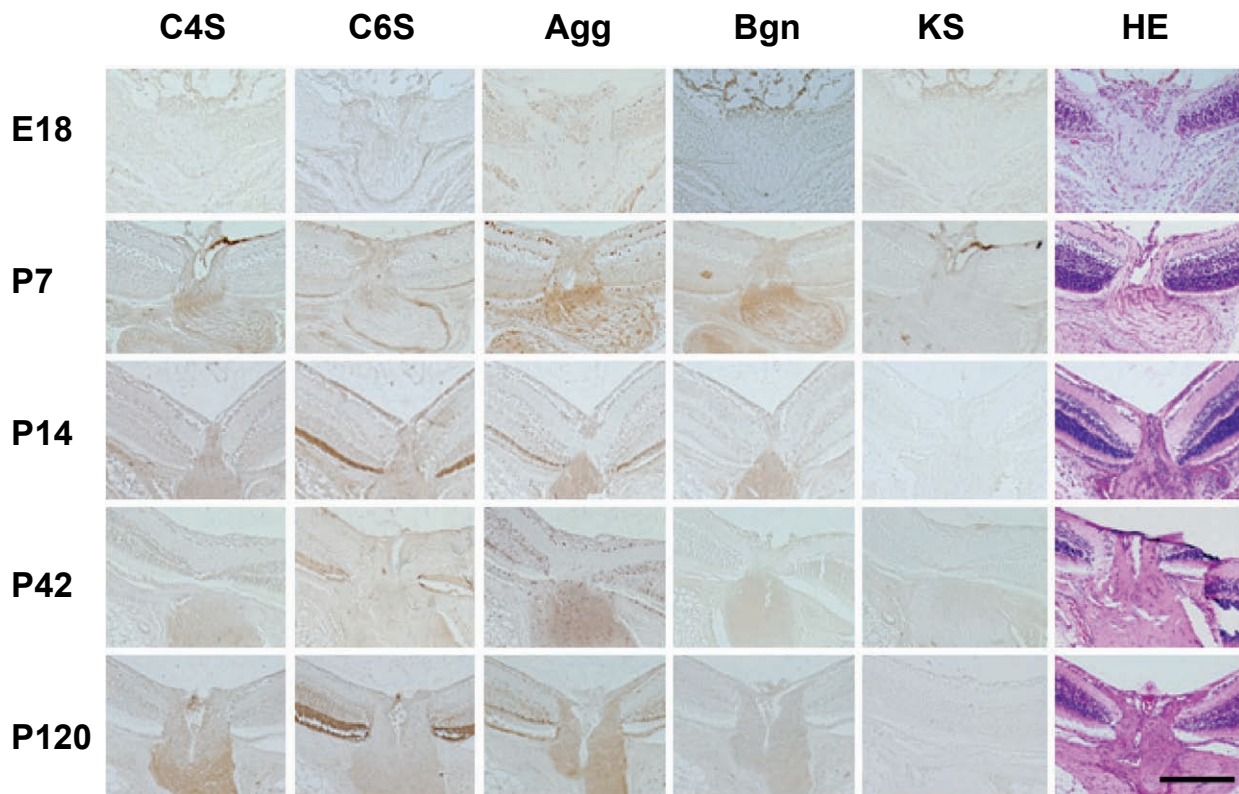


Fig. 2. HE staining and immunostaining of CSPGs in the optic nerve of mice at E18, P7, P14, P42 and P120. HE; hematoxylin and eosin. Scale bar=200 μ m.

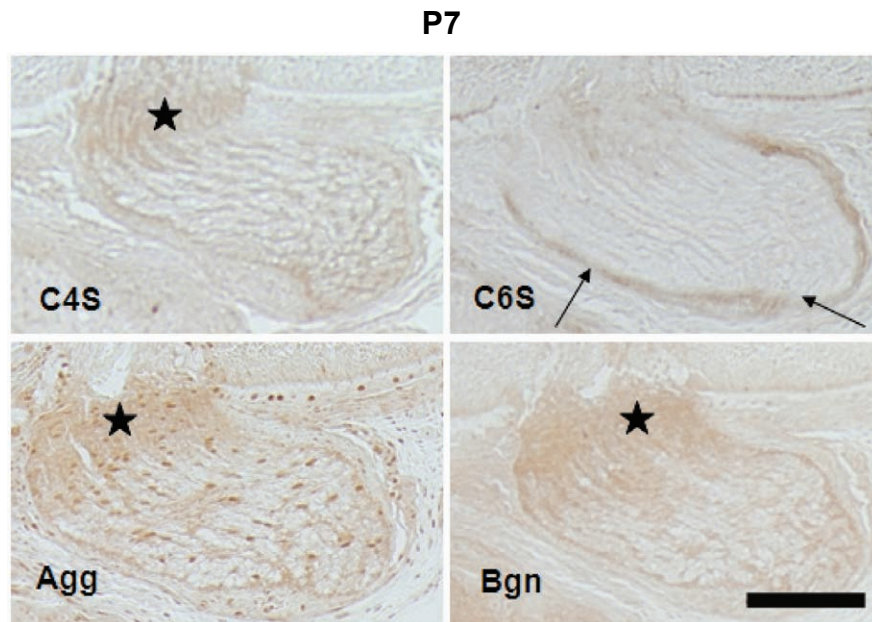


Fig. 3. Immunostaining of CSPGs in the optic nerve of mice at P7 showing a difference in the immunostaining pattern at the optic nerve. Lamina cribrosa (star), optic nerve sheath (arrows). Scale bar=100 μ m.

development of retina and optic disc. It has been suggested that CSPGs may regulate the pattern of retinal ganglion cell outgrowth in the developing retina because of CSPGs having an inhibitory effect on elongation of retinal ganglion cell axons [3, 39–41].

The retina was mainly composed of homologous neuroblasts at E12 as the previous study [36] and the NFL, GCL and IPL in the retina were distinguishable at E18 as the previous studies [22, 36]. CSPGs showed no specific staining in the eye at E12 but at E18 C4S and C6S appeared in the retina. At P14 and P42, C4S was not detected in the PRL. In contrast to C4S, C6S was distributed in the PRL at these ages and increased in the PRL at P120. Thus, CSPGs appeared in the retina with differentiation of retinal neuroblasts. C4S might be regulator of axonal outgrowth [4]. C6S distributes in the interphotoreceptor matrix of the rat retina [32] and modulates retinal ganglion cell body polarity and stimulates retinal axon elongation [2]. In addition, photoreceptor degeneration is found in mice with mucopolysaccharidosis type VII, which is a lysosomal storage disorder and caused by a deficiency of specific lysosomal enzyme required for the degradation of glycosaminoglycans including chondroitin sulfate [25]. Little expression of chondroitin sulfate was also found in the interphotoreceptor matrix of rds mice which photoreceptor cells progressively loses [42]. Thus, C6S may be involved in the maintenance of photoreceptor cells.

In this study, biglycan was present in the retina in a diffuse manner at E18–P7 and showed higher affinity to nerve fiber rich layers from P14–P42. The distribution of biglycan in the mouse retina in our study was similar to that reported for decorin in the rat retina [12]. Biglycan and decorin are closely similar in molecular structure [21]. Either biglycan or decorin is capable of interacting with collagen fibrils [17, 19]. It is supposed that biglycan and decorin are not only neurotrophic factors for retinal cells but also relate to their differentiation.

Aggrecan is one of the major CSPGs expressed in the central and peripheral nervous system of embryos and adults [28, 35]. In this study, aggrecan appeared at E18 in the retina. More over at P7, the strong immunostaining of aggrecan with cells was consistent with astrocyte in the inner retina and optic nerve. Aggrecan is produced by glial precursors [1, 9]. Astrocyte-precursor cells migrate into the mouse retina at E17 [44]. Thus, these previous studies confirm the time of appearance of aggrecan in the mouse retina. Aggrecan have multiple effects on neurite outgrowth [5, 8, 40] and is required for normal astrocyte differentiation [39]. It is suggested that aggrecan is a mediator in neural development and may be important in maintenance of these layers of the mature retina.

In this study C6S was detected in the GCL at E18 but showed strong to moderate immunostaining in the PRL at P7, 14 and 42. C6S is localized at the apical surface of the RPE and in adjacent photoreceptors [32]. In contrast, the apical surface of the RPE of RCS rats, which is a widely

studied animal model of a condition similar to retinitis pigmentosa, is barely immunopositive [33]. C6S plays an important role in adhesion between the neural retina and RPE [24]. It seems reasonable, therefore, that C6S was positive in the developed PRL at P7 or later.

We compared developmental changes in the localization and levels of immunostaining of C4S, C6S, biglycan and aggrecan in the mouse eye from E12 up to P120. The presence and distribution of aggrecan in the late embryonic rat retina and optic nerve have been reported [26], but there is no information on ocular aspects of the investigated CSPGs in mice. Although the neurobiological significance of C4S, C6S, aggrecan and biglycan in the retina and optic nerve remains to be more clearly delineated, our results indicate that the individual proteoglycans probably have unique, perhaps partially overlapping, functions in the retina and optic nerve as well as in the central nervous system [30].

REFERENCES

1. Afshari, F. T., Kwok, J. C., White, L. and Fawcett, J. W. 2010. Schwann cell migration is integrin-dependent and inhibited by astrocyte-produced aggrecan. *Glia*, **58**, 857–869.
2. Brittis, P. A. and Silver, J. 1994. Exogenous glycosaminoglycans induce complete inversion of retinal ganglion cell bodies and their axons within the retinal neuroepithelium. *Proc. Natl. Acad. Sci.* **91**: 7539–7542.
3. Brittis, P. A., Canning, D. R. and Silver, J. 1992. Chondroitin sulfate as a regulator of neuronal patterning in the retina. *Science* **255**: 733–736.
4. Challacombe, J. F. and Elam, J. S. 1997. Chondroitin 4-sulfate stimulates regeneration of goldfish retinal axons. *Exp. Neurol. Res.* **142**: 169–175.
5. Chan, C. C., Roberts, C. R., Steeves, J. D. and Tetzlaff, W. 2008. Aggrecan components differentially modulate nerve growth factor-responsive and neurotrophin-3-responsive dorsal root ganglion neurite growth. *J. Neurosci. Res.* **86**: 581–592.
6. Chung, K. Y., Taylor, J. S., Shum, D. K. and Chan, S. O. 2000. Axon routing at the optic chiasm after enzymatic removal of chondroitin sulfate in mouse embryos. *Development* **127**: 2673–2683.
7. Collello, R., J. and Guillery, R. W. 1990. The early development of retinal ganglion cells with uncrossed axons in the mouse: retinal position and axonal course. *Development* **108**: 515–523.
8. Condie, M. L., Snow, D. M. and Letourneau, P. C. 1999. Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression. *J. Neurosci.* **19**: 10036–10043.
9. Domowicz, M. S., Sanders, T. A., Ragsdale, C. W. and Schwartz, N. B. 2008. Aggrecan is expressed by embryonic brain glia and regulates astrocyte development. *Dev. Biol.* **315**: 114–124.
10. Hocking, A. M., Shinomura, T. and McQuillan, D. J. 1998. Leucine-rich repeat glycoproteins of the extracellular matrix. *Matrix Bio.* **17**: 1–19.
11. Hwang, H. Y., Olson, S. K., Esko, J. D. and Horvitz, H. R. 2003. *Caenorhabditis elegans* early embryogenesis and vulval morphogenesis require chondroitin biosynthesis. *Nature* **423**: 439–443.
12. Inatani, M., Tanihara, H., Honjo, M., Hangai, M., Kresse, H.

- and Honda, Y. 1999. Expression of proteoglycan decorin in neural retina. *Invest. Ophthalmol. Vis. Sci.* **40**: 1783–1791.
13. Inatani, M. and Tanihara, H. 2002. Proteoglycans in retina. *Prog. Retin. Eye Res.* **21**: 429–447.
 14. Iozzo, R. V. 1998. Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem.* **67**: 609–652.
 15. Iozzo, R. V. 1999. The biology of the small leucine-rich proteoglycans: functional network of interactive proteins. *J. Biol. Chem.* **274**: 18843–18846.
 16. Iozzo, R. V. 2005. Basement membrane proteoglycans: from cellar to ceiling. *Nat. Rev. Mol. Cell. Biol.* **6**: 646–656.
 17. Keene, D.R., San Antonio, J. D., Mayne, R., McQuillan, D. J., Sarris, G., Santoro, S. A. and Iozzo, R.V. 2000. Decorin binds near the C terminus of type I collagen. *J. Biol. Chem.* **275**: 21801–21804.
 18. Knudson, C. B. and Knudson, W. 2001. Cartilage proteoglycans. *Semin. Cell Dev. Biol.* **12**: 69–78.
 19. Kobe, B. and Deisenhofer, J. 1994. The leucine rich repeat: a versatile binding motif. *Trends Biochem. Sci.* **19**: 415–421.
 20. Kresse, H. and Schönherr, E. 2001. Proteoglycans of the extracellular matrix and growth control. *J. Cell Physiol.* **189**: 266–274.
 21. Krusius, T. and Ruoslahti, E. 1986. Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. *Proc. Nat. Acad. Sci.* **83**: 7683–7687.
 22. Kuwabara, T. and Weidman, T. A. 1974. Development of the prenatal rat retina. *Invest. Ophthalmol. Vis. Sci.* **13**: 725–739.
 23. Laabs, T., Carulli, D., Geller, H. M. and Fawcett, J. W. 2005. Chondroitin sulfate proteoglycans in neural development and regeneration. *Curr. Opin. Neurobiol.* **15**: 116–120.
 24. Lazarus, H. S. and Hageman, G. S. 1992. Xyloside-induced disruption of interphotoreceptor matrix proteoglycans results in retinal detachment. *Invest. Ophthalmol. Vis. Sci.* **33**: 364–376.
 25. Lazarus, H. S., Sly, W. S., Kyle, J. W. and Hageman, G. S. 1993. Photoreceptor degeneration and altered distribution of interphotoreceptor matrix proteoglycans in the mucopolysaccharidosis VII mouse. *Exp. Eye Res.* **56**: 531–541.
 26. Luo, Y. and Raper, J. A. 1994. Inhibitory factors controlling growth cone motility and guidance. *Curr. Opin. Neurobiol.* **4**: 648–654.
 27. McAdams, B.D. and McLoon, S.C. 1995. Expression of chondroitin sulfate and keratan sulfate proteoglycans in the path of growing retinal axons in the developing chick. *J. Comp. Neurol.* **352**: 594–606.
 28. Oakley, R. A. and Tosney, K. W. 1991. Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. *Dev. Biol.* **147**: 187–206.
 29. Parish, C. R. 2006. The role of heparan sulphate in inflammation. *Nat. Rev. Immunol.* **6**: 633–643.
 30. Popp, S., Andersen, J. S., Maurer, P. and Margolis, R. U. 2003. Localization of aggrecan and versican in the developing rat central nervous system. *Dev. Dyn.* **227**: 143–149.
 31. Popp, S., Maurer, P., Andersen, J. S. and Margolis, R. U. 2004. Developmental changes of aggrecan, versican and neurocan in the retina and optic nerve. *Exp. Eye Res.* **79**: 351–356.
 32. Porrello, K. and LaVail, M. M. 1986. Immunocytochemical localization of chondroitin sulfates in the interphotoreceptor matrix of the normal and dystrophic rat retina. *Curr. Eye Res.* **5**: 981–993.
 33. Porrello, K., Yasumura, D. and LaVail, M. M. 1989. Immunogold localization of chondroitin 6-sulfate in the interphotoreceptor matrix of normal and RCS rats. *Invest. Ophthalmol. Vis. Sci.* **30**: 638–651.
 34. Ruoslahti, E. 1988. Structure and biology of proteoglycans. *Annu. Rev. Cell Biol.* **4**: 229–255.
 35. Schwartz, N. B., Domowicz, M., Krueger, R. C. J., Li, H. and Mangoura, D. 1996. Brain aggrecan. *Perspect. Dev. Neurobiol.* **3**: 291–306.
 36. Sharma, R. K., O'Leary, T.E., Fields, C. M. and Johnson, D.A. 2003. Development of the outer retina in the mouse. *Brain Res. Dev. Brain Res.* **145**: 93–105.
 37. Silver, J. and Miller, J. H. 2004. Regeneration beyond the glial scar. *Nat. Rev. Neurosci.* **2**: 146–56.
 38. Sirko, S., von Holst, A., Wizenmann, A., Gotz, M. and Faissner, A. 2007. Chondroitin sulfate glycosaminoglycans control proliferation, radial glia cell differentiation and neurogenesis in neural stem/progenitor cells. *Development* **134**: 2727–2738.
 39. Smith, G. M. and Strunz, C. 2005. Growth factor and cytokine regulation of chondroitin sulfate proteoglycans by astrocytes. *Glia* **52**: 209–218.
 40. Snow, D. M. and Letourneau, P. C. 1992. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *J. Neurobiol.* **23**: 322–336.
 41. Snow, D. M., Watanabe, M., Letourneau, P. C. and Silver, J. 1992. A chondroitin sulfate proteoglycan may influence the retinal ganglion cell outgrowth. *Development* **113**: 1473–1485.
 42. Tawara, A. and Hollyfield, J. G. 1990. Proteoglycans in the mouse interphotoreceptor matrix. III. Changes during photoreceptor development and degeneration in the rds mutant. *Exp. Eye Res.* **51**: 301–315.
 43. Taylor, K. R. and Gallo, R. L. 2006. Glycosaminoglycans and their proteoglycans: host associated molecular patterns for initiation and modulation of inflammation. *FASEB J.* **20**: 9–22.
 44. Uemura, A., Kusuhara, S., Katsuta, H. and Nishikawa, S. 2006. Angiogenesis in the mouse retina: a model system for experimental manipulation. *Exp. Cell Res.* **312**: 676–683.
 45. Zako, M., Shinomura, T., Miyaishi, O., Iwaki, M. and Kimata, K. 1997. Transient expression of PG-M/versican, a large chondroitin sulfate proteoglycan in developing chicken retina. *J. Neurochem.* **69**: 2155–2161.