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Even though much is known about the distribution and function of proteoglycans, most of what is known about function comes from studies on artificial substrates. The basal lamina of neural retina (inner limiting membrane) is used here as a natural system to study the function of proteoglycans during morphogenesis.

In this thesis, distribution and function of both heparan sulfate proteoglycans and chondroitin sulfate proteoglycans were examined in developing embryonic chicken neural retina, particularly in the inner limiting membrane.

Autoradiographic studies using ^{35}S-sulfate, revealed that proteoglycans were mainly associated with the inner limiting membrane and pigmented epithelium, with an increase in inner and outer plexiform layers between 7- and 10-days. When combined with selective enzymatic digestion of cryosections, autoradiographic studies showed that heparan sulfate proteoglycans were mainly

associated with the inner limiting membrane in 7-day old retina and associated with both the inner limiting membrane and inner and outer plexiform layers in 10-day old retina. Chondroitin sulfate proteoglycans were evenly associated with retinas of both ages.

Immunohistochemical studies showed that inner limiting membrane heparan sulfate proteoglycans were present from day 5 to day 14 and that the core proteins of these proteoglycans were distinct from those in the remaining retina.

A combination of enzymatic and chemical digestion and chromatographic separation showed that, compared to its total volume in the retina, the inner limiting membrane was enriched in total proteoglycans and in the relative amount of heparan sulfate proteoglycans.

Functional studies demonstrated that when used as a natural substrate, the heparitinase-treated, but not chondroitinase-treated, inner limiting membrane inhibited both axonal growth rate and density of retinal ganglionic cells in an explant of retina placed on the membrane. Heparitinase treatment did not act by blocking the adhesion of isolated neuronal cells to the inner limiting membrane or by causing their death, but it did reduce the numbers of neurite-bearing cells and the length of cell neurites. These results support the hypothesis that heparan sulfate proteoglycans, but not chondroitin sulfate proteoglycans, in the inner limiting membrane play an important role in modulating neurite extension and promoting neuronal cell sprouting.

Proteoglycans in the Inner Limiting Membrane and Their Influence on Axonal Behavior in Embryonic Chicken Retina

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CHAPTER I. GENERAL INTRODUCTION

Correct connection of neurons to their targets during embryonic development is critical for normal function of the vertebrate nervous system. Basic questions faced by embryologists and neurobiologists are how do neurons detect their target tissues, and then how do they send out their axons following correct pathways to connect with their targets? During early embryogenesis the newly-differentiated neurons are exposed to an array of specific microenvironmental cues. In response they send out their axons to their correct target cells or tissues in a complex but highly ordered way. These specific cues consist of molecules along the migratory pathways of the neuronal axon. What are these guidance cues? And how do neurons recognize them along their pathways to reach their definitive target tissues during early development? More than a century ago (1890), Ramon y Cajal discovered the elongating tips of developing nerve fibers, growth cone, of neuronal cells and first proposed that neuronal cells of a developing nervous system are recognizing one or more molecules, later called chemoattractants, such as signaling molecules on neighboring cells or local gradients of extracellular matrix (ECM), as their guidance cues. These signals instruct or limit the nascent cell to choose a particular destination.

This pathway-finding process has also been called the 'chemoaffinity hypothesis', which suggests that neurons may carry some kind of individual identification tags, presumably cytochemical in nature, by which they are distinguished one from another, and further that each axon links only with neurons to which it becomes selectively attached by specific chemical affinities (Sperry, 1963). This hypothesis has attracted a number of cell biologists to investigate and study the molecules that might be involved in guidance of axons to their final destination (Keynes & Cook, 1990, 1992; Liesi, 1990; Tessier-Lavigne & Placzek, 1991; Harrelson, 1992; Tessier-Lavigne, 1992).

Interaction between the ECM and cell surface receptors are thought to play pivotal roles in cell movement and axon guidance. Pathfinding processes involve cell-cell and cell-substrate interactions. For many years, a number of investigators have been studying the mechanisms of cell-cell and cell-substrate recognition, adhesion, and interaction between neuronal cells and between neuronal and glial cells and their environmental molecules that might play major roles in pathfinding processes (for review see Lander 1989). Among the best studied candidate molecules are the cell membrane-bound neuronal cell adhesion molecules (NCAM) (Rutishauser et al., 1988; Edelman & Crossin, 1991) and cadherins (Tomaselli & Reichardt, 1989), and the integrins (Hynes, 1987; Alberda & Buck, 1990) and their interaction with basal lamina-associated laminin (Beck et al., 1990; Mecham, 1991) and ECM molecules, e.g. fibronectin (Sanes, 1989; Reichardt & Tomaselli, 1991).

There is yet an another kind of molecule, the proteoglycans, which might also be playing very important roles during these processes because of their presence on the cell surface and in the basal lamina and in ECM. The term 'proteoglycan' was first introduced in 1967 (Balazs, 1970) to describe the family of molecules in which glycosaminoglycan chains are linked to protein. Proteoglycans are found in a wide variety of tissues in both embryonic and adult animals. Although the functional roles in nervous systems of many glycoprotein molecules such as N-CAM on cell surfaces and laminin in ECM have been well characterized, relatively little is known about the role of the more heterogeneous proteoglycans. The most-well studied proteoglycans of nervous systems are those in rat brain (Margolis & Margolis, 1989) and embryonic chicken retina (Morris, 1976; Morris et al., 1987). Even though both their biochemical characterization and biosynthesis have been investigated, their tissue distribution has not been studied. In order to study the function of the proteoglycans in nervous tissue, it is important to investigate how the proteoglycans are localized in this tissue.

Structures of proteoglycans and glycosaminoglycans

Proteoglycans are polyanionic macromolecules found on many cell surfaces and in ECM. Each proteoglycan has a protein core (hyaluronic acid is an exception, not attached to a protein core) to which one or more polysaccharide chains, named glycosaminoglycans, are bound covalently. These glycosaminoglycans are long unbranched repeating sulfated (hyaluronic acid chain

is not sulfated) disaccharide unit chains. Due, in part, to different disaccharides bound to a protein core, proteoglycans may have different characteristics. Glycosaminoglycans exist in four main forms: chondroitin sulfate and dermatan sulfate, heparan sulfate and heparin, keratan sulfate, and hyaluronic acid. A chondroitin sulfate proteoglycan contains a protein core to which a few to hundreds of N-acetylgalactosamine and D-glucuronic acid repeating units are attached to by serine-xylose-galactose-galactose linkages; sulfation generally occurs in either the 4- or 6- position of the N-acetylgalactosamine residue (Roden, 1980; Hook et al., 1984). Dermatan sulfate is similar to chondroitin 4-sulfate but contains the epimere L-iduronic acid rather than D-glucuronic acid (Margolis & Margolis, 1989; Margolis & Margolis, 1989). Mannose-linked keratan sulfate chains are present as minor components of chondroitin sulfate proteoglycans (CSPGs). The disaccharide repeating unit of a heparan sulfate proteoglycan (HSPG) is composed of N-acetylglucosamine and glucuronic acid. Glycosaminoglycan chains of heparan sulfate attach to their protein core by the same molecule sequence as those for CSPGs (Roden, 1980). Most of the amino groups of the glucosamine residues are sulfated, rendering a structure that is unique to heparan sulfate or heparin. Ester sulfate (O-sulfate) groups are present on C-6 of the glucosamine residues and perhaps to some extent on C-3. O-sulfate groups are also present on C-2 of many of the iduronic acid units. However, glucuronic acid residues are rarely sulfated. Variability within each proteoglycan family of molecules involves core protein characteristics, number and length of the glycosaminoglycan chains, the extent to

which glucuronic acid residues are epimerised to iduronic acid, and the extent and position of sulfation (Roden, 1980; Hascall, 1981). Specific forms of proteoglycans are found in different tissue matrices or plasma membranes of different cell types. A number of proteoglycans, such as aggrecan, syndecan, and betaglycan, carry two types of side chains, heparan sulfate and chondroitin sulfate (Roden, 1980; Couchman et al., 1984).

Localization (association) of proteoglycans

Proteoglycans are widely distributed in both embryonic and adult animal tissues. They are either associated with cell surfaces or located in ECM. CSPGs are usually found in the ECM whereas HSPGs are associated with cell membranes or as a component of the basement membrane.

Basement membranes are specialized extracellular matrices with support, sieving, and cell regulatory functions. The molecular architectures of these matrices are created through specific binding interactions between their unique glycoprotein and proteoglycan constituents. Heparan sulfate is a major glycosaminoglycan which is associated with the epithelial or muscle basal lamina of many different tissues (Laurie et al., 1982, 1983, 1988; Stow et al., 1985; Grant & Leblond, 1988; Lin, 1990). HSPGs are also found associated with cell membrane (Zaremba et al., 1989; David et al., 1992), and even with cytoplasmic compartments (Pacifici et al., 1983; Morris et al., 1987).

In the retina CSPGs are found predominantly as ECM molecules (Morris et al., 1987), as in the cone sheath of primate retina (Hageman & Johnson, 1987), in the regions of the outer and inner photoreceptor segments of rats (Porrello & LaVail, 1986), with minor amounts in basement membrane (Aquino et al., 1984a, b; McCarthy et al., 1989; Faassen et al., 1992), or on cell surfaces (Iida et al., 1992).

General functions of proteoglycans in non-neuronal tissues

Glycosaminoglycans of proteoglycans usually are highly sulfated, which renders them highly negatively charged. Such glycosaminoglycans play important roles in many systems of adult animals as well as in developing embryos. The functions of proteoglycans in animal tissues can be roughly separated into two categories: space-filling and specific interactions (Muir, 1983). The space-filling role of proteoglycans is important for adult animals to maintain the structural integrity of various connective tissues (Goetinck et al., 1990). They are involved in shaping and modeling of such tissues during early development. Proteoglycans can also interact with other molecules in animal tissues to play vital biological functions. Glycosaminoglycan chains as well as the protein core of proteoglycans have the ability to bind to other macromolecules (Kjellen and Lindahl, 1991). For instance, proteoglycans can (1) bind enzymes to maintain them in interactive form for intracellular storage (Stevens and Austen, 1989), modulate enzyme activity (Le Trong et al., 1987), anchor enzymes at the cell surface (Hjalmarsson et al., 1987)

or in ECM (Adachi and Marklund, 1989), regulate enzyme expression, or promote enzyme inhibition (Redini et al., 1988); (2) bind serine protease inhibitors to promote inactivation of thrombin (Lindahl, 1989), inactivate coagulation enzymes at vascular endothelium (Maimone and Tollefsen,1990), and inactivate serine proteases in the ECM (Farrel and Cunningham, 1986); (3) bind ECM to modulate coagulation and complement processes (Lane et al., 1987), mediate and modulate cell-substrate interactions (Martin and Timpl, 1987), organize basement membranes (Yurchenco & Schittny, 1990), and regulate the permeability of these membranes to small molecules (Kashihara et al., 1989; Kanwar et al., 1992); and (4) bind growth factors to protect them from being degraded by enzymes in the ECM (Fayein et al., 1990). Proteoglycans also (5) influence cell differentiation and morphogenesis (for review see Goetinck, 1991).

Proteoglycans in nervous systems and their biosynthesis and regulation

Proteoglycans are not restricted to connective tissues such as cartilage and bones (Hardingham, 1981; Muir, 1983; Hardingham & Fosang, 1992), but found in other tissues as well, including central nervous tissues, such as brain (Margolis, et al., 1975; Aquino et al., 1984b) and retinas (Cole et al., 1985; Morris et al., 1987). Their distribution in the ECM varies spatially and temporally during avian neural crest development (Perris et al., 1991). Proteoglycans have been found in embryonic brains (Kiang et al., 1981; Oohira et al., 1988) and retinas (Morris et al., 1977), and their biosynthesis seems to be regulated during embryogenesis. In

rat brain, the levels of hyaluronic acid, chondroitin sulfate, and heparan sulfate all increase post-natally to reach a peak at 7 day, after which they decline steadily, attaining by 30 day concentrations within 10% of those present in adult brain (Margolis, et al., 1975). In 7 day old rat brain the CSPGs are present in the extracellular space and in the astroglial cytoplasm. There is very little associated with neuronal cell bodies or axons; by 10 days, the proteoglycans associated with astroglial cells have increased and those located in the extracellular space significantly decreased (Aquino et al., 1984a). In embryonic chicken retinas, proteoglycans are also actively synthesized (Morris, 1984; Morris et al., 1984). In retina tissue preparations different proteoglycan have been isolated and characterized, including a large chondroitin sulfate/dermatan sulfate proteoglycan (CS/DSPG) from the culture medium, a large HSPG from the urea-detergent extract, and two smaller and possibly related HSPGs (Morris, 1984). Between 5 and 14 days of development the proportion of heparan sulfate in the chicken retina decreases from 89% to 61% of the total ^{35}S-sulfate-labeled glycosaminoglycans, and chondroitin sulfate increases from 7% to 34% (Morris et al., 1977). The ratio of the chondroitin 4-sulfate to chondroitin 6-sulfate increases sixfold (Morris et al., 1977). By using different extraction methods, compartmentalization of the proteoglycans may be investigated (Morris et al., 1987). Most, if not all, CSPGs are found in saline-buffered extracts, whereas HSPGs are exclusively present in guanidine hydrochloride-detergent buffer extracts. Therefore, CSPGs may be

entirely present in matrix or loosely associated with cell surfaces, whereas HSPGs may be located in regions of synaptogenesis, or associated with cell membranes.

Recent studies using pure cell culture techniques indicate that ECM and cell membrane-associated proteoglycans from cultures of isolated neuronal and photoreceptor cells (Needham et al., 1988) are entirely HSPGs while those from glial-like cells (Threlkeld et al., 1989) are both chondroitin sulfate/dermatan sulfate proteoglycans (CS/DSPGs) and HSPGs, with HSPGs being the major component.

An HSPG has been identified in the neuronal cell line PC12 by immunopurification methods (Matthew et al., 1985). This HSPG represents both cell surface-associated and PC12 medium forms.

Interaction between proteoglycans and other extracellular matrix molecules and cell adhesion molecules in the nervous system

Axonal guidance involves many cell functions and cell behaviors, such as cell-cell and cell-substrate recognition, adhesion, and interaction. These processes occur concurrently. Cells usually interact with many different molecules that may form specific arrays with which cells interact. Embryonic chick neural retina cells in culture release complexes of a 170,000 MW protein and glycosaminoglycans, termed adherons, which enhance cell-substratum adhesion when adsorbed to nonadhesive surfaces. An HSPG has been found to participate in retinal cell-adheron binding (Schubert & LaCorbiere, 1985a, b). Adhesion of retina neural cells to a substrate coated with this 170,000 MW protein can be inhibited by

adding heparan sulfate to the culture (Cole et al., 1985). Laminin has been found to be the major active molecule in neurite outgrowth-promoting media produced by a variety of cell types. The laminin usually exists as a complex associated with HSPGs (Lander, et al., 1985; Lander, et al., 1985). The complex of laminin and HSPGs has been found to be a more active neurite-promoting factor than laminin alone (Chiu et al., 1986). Antibodies made against laminin can block laminin-promoted neurite growth but can not block outgrowth induced by an HSPG-laminin complex (Lander, et al., 1982; Manthorpe et al., 1983; Edgar et al., 1984, 1988). CSPGs are also found to form a complex with laminin (Chiquet & Fambrough, 1984; Hoffman & Edelman, 1987; Perris & Johansson, 1987).

A chondroitin sulfate proteoglycan has been found to interact with the ECM component tenascin, which has cell-binding properties (Grumet et al., 1985; Chiquet-Ehrismann et al., 1986; Hoffman & Edelman, 1987). The CSPG-tenascin complex can block the binding of neurons to tenascin-coated beads (Hoffman & Edelman, 1987).

Proteoglycan functions in nervous systems

Proteoglycans may play key roles in many biological activities in nervous systems, such as anchorage of acetylcholinesterase to the neuromuscular junction (Brandan & Inestrosa, 1986; von Bernhardi and Inestrosa; 1990), or stabilization of acetylcholine receptors (Bayne et al., 1984). Proteoglycans also direct neurite orientation (Snow et al., 1990) and play important roles in cell-substratum (Cole

et al., 1985) and cell-cell adhesion (Cole et al., 1986) of neuronal cells, neurite formation (Dow et al., 1988; Lafont et al., 1992), and neurite extension and outgrowth (Matthew et al., 1985).

Proteoglycans as neurite outgrowth blockers (inhibitors)

Some proteoglycans may function as blocking molecules to direct neuronal migration in one direction but not the other in early rat embryonic development when the roof plate, located along the dorsal midline of the developing spinal cord, is a putative axon barrier. Both ventral commissural and dorsal root ganglia migrate along but do not cross the roof plate to reach their potential target in the contralateral side of the spinal cord. A unique keratan sulfate proteoglycan found along the roof plate may be responsible for the inhibition of axon elongation through the roof plate in the embryonic spinal cord (Snow et al., 1990). Other reports have shown that the posterior sclerotome in chicken embryos, which acts as a barrier to axon growth, contains a high concentration of glycosaminoglycans (Tosney & Landermesser, 1985). In the developing retina, retinal ganglion cells send their axons toward the optic fissure through which the axons reach their final destination, the optic tectum, in the brain. CSPGs are found to repel elongating ganglion cell axons in rat retina tissue culture (Snow et al., 1991), suggesting that CSPGs may direct the orientation of growing neurons by repelling their axons in order to allow the neurite to extend in one direction but not the other (Snow et al., 1990). CSPGs in ECM are found to regress gradually while ganglion cells

differentiate and progressively send out their axons during early rat retina development (Brittis et al., 1992). These results suggest that proteoglycans may play a regulatory role in directing the migrating neuronal cell axons by repelling extension of the axons or helping control the onset of ganglion cell differentiation by influencing the direction of their axons.

Concentrations of proteoglycans relative to those of other molecules, such as laminin and collagen, may affect neuronal cell behavior differently. Cartilage proteoglycan may inhibit neurite elongation from dorsal root ganglion neurons according to Snow et al. (1990), but Oohira et al. (1991) were unable to demonstrate this inhibition of neurite outgrowth. One difference between these two reports is the amount of the CSPG used in their cell cultures. Even the same core protein of CSPG isolated from 10-day-old Sprague-Dawley may promote neurite outgrowth at 2.5-2500 ng protein/ml (Iijima et al., 1991) and inhibit neurite outgrowth at 250-25,000 pg protein/ml (Oohira et al., 1991).

Proteoglycans influence neurite polarity

Proteoglycans and glycosaminoglycans also influence neuronal polarity. Chondroitin sulfate and heparan sulfate enhance axonal growth, whereas dermatan sulfate increases dendrite growth (Lafont et al., 1992). The authors suggest that proteoglycans and glycosaminoglycans may influence neuronal migration, either permissively or repulsively, by changing the morphology of the neurons.

Proteoglycans as neuronal cell adhesion molecules

HSPGs released from retina cell cultures promote cell adhesion to dishes (Cole et al., 1985; Schubert & Lacorbiere, 1985b) and neuronal cell-cell adhesion (Cole et al., 1986). Chondroitin sulfate glycosaminoglycans, on the other hand, also enhance cell-cell adhesion of retina cells (Morris, 1976, 1979), but probably by a different mechanism, since the amount of chondroitin sulfate used to promote cell-cell adhesion is several magnitudes more than that needed for HSPGs. HSPGs but not CSPGs are involved in heterophilic-binding to N-CAM molecules to assist cell-cell adhesion of neural retina cells (Schubert et al., 1983; Cole et al., 1986; Cole & Akeson, 1989). On the other hand, the promotion of cell-cell adhesion of chondroitin sulfate may be by steric exclusion (Morris, 1993).

Proteoglycans as neurite promoters

The structural properties of proteoglycans are responsible for their biological characteristics. CSPGs are usually highly sulfated with strong negative charges. These highly charged molecules tend to bind to water in tissues, and during early development, the extracellular hyaluronic acid and CSPGs in brain tissue may provide a highly hydrated and penetrable matrix which allows neurons to migrate (Margolis & Margolis, 1989; Margolis & Margolis, 1989).

HSPGs isolated from bovine endothelial cells were first shown to induce elongation of rat sympathetic neurites by Lander et al., (1982). Since then, the accumulated literature has shown that these proteoglycans promote neurite

outgrowth in a variety of neuronal cells. HSPG secreted into the medium of PC12 cells and nonneuronal dorsal root ganglion cells, the HSPG appears to be derived from a cell surface (Matthew et al., 1985), induces rapid neurite outgrowth by primary sympathetic neurons after adsorption onto polylysine-coated dishes. The promoting effect is heparitinase sensitive, leading the authors to conclude that the glycosaminoglycans side chains of the HSPG are responsible for promoting neurite outgrowth. On the other hand, the core protein, not glycosaminoglycan side chains, of CSPGs from rat brain promotes neurite outgrowth from neocortical neurons in culture (Iijima et al., 1991).

HSPGs, isolated from Engelbreth-Holm-Swarm tumor cells, mediate a different type of neurite outgrowth than laminin does (Hantaz-Ambroise et al., 1987). HSPG promotes neurite elongation whereas laminin enhances neurite branching. The promoting effect of the HSPG can be blocked by an anti-HSPG polyclonal antibody, but the authors have not shown whether the antibody recognizes the core protein or the carbohydrate moiety of the HSPG.

Proteoglycans as guidance molecules

Proteoglycans have been shown to have either inhibitory effects (most CSPG and keratan sulfate proteoglycans (KSPG)) or promoting effects (most HSPGs) on neurite outgrowth. Little is known about proteoglycans as guidance molecules and the research on this subject is so far indirect. Only one report (Wang & Denburg, 1992) suggests that heparan sulfate or heparin may act as a

neuronal guidance molecule. In this study, the authors used exogenous glycosaminoglycans to compete with GAG-binding sites. Of all glycosaminoglycans tested, only heparin and heparan sulfate altered (perturbed) the normal pathways of pioneer axons of cockroach embryos in culture. Heparinase II and heparitinase have also been shown to have similar perturbing effects.

Proteoglycans as growth factor receptors and modulators

The most recent and intriguing findings on the functions of proteoglycans implicate them as modulators for growth factors. Both HSPGs and heparin have been found to act as modulators of growth factors through their glycosaminoglycans. Basic fibroblast growth factor (bFGF), also called heparin-binding growth factor, binds to heparin or heparan sulfate chains of proteoglycans which appear to protect the factors from protease degradation (Burgess & Maciag, 1989). Moreover, proteoglycan binding may be important in providing a matrix-bound or cell surface-bound reservoir of bFGF (Ruoslhti & Yamaguchi, 1991). The most recent study has shown that it is necessary for bFGF molecules to bind heparan sulfate prior to binding to their cell membrane receptors (Yayon et al., 1991). It is likely that the glycosaminoglycan-basic FGF complexes change the conformation of the growth factors as a prerequisite for binding to their receptors. Basic FGF signalling requires its interaction with at least two receptors, an HSPG and a tyrosine kinase (Olwin & Rapraeger, 1992). Treatment of skeletal muscle myoblasts with cholate, a reversible inhibitor of glycosaminoglycan sulfation,

reduces glycosaminoglycan sulfation by 90% and binding of fibroblast growth factor to the high affinity sites, tyrosine kinase, by 80%. This treatment of the myoblasts results in termination of differentiation. Cholate inhibition of bFGF signalling can be reversed by simultaneous addition of sodium sulfate or heparin. Treatment with a selective enzyme, heparitinase, abolishes the activity of FGF, showing that the binding function belongs to the sugar chains. Heparin can also modulate the neurotropic effects of basic as well as acidic fibroblast growth factors and nerve growth factor on PC12 cells (Neufeld et al., 1987).

Syndecan, a cell membrane-bound proteoglycan that has both heparan sulfate and chondroitin sulfate chains, has also been found to be able to bind basic fibroblast growth factors (Vainio et al., 1989). HSPGs in ECM also bind to acidic fibroblast growth factors and modulate the mitogenic effects of the growth factor (Gordon et al., 1989).

The binding of growth factors with proteoglycans also can be mediated through the protein core of the proteoglycans. Transforming growth factor-ß has been found to bind the protein core of an HSPG but not its glycosaminoglycan chains (Andres et al., 1989; Cheifetz & Massague, 1989).

Visual system as a model to investigate pathfinding

The visual system of vertebrates appears to be a perfect model to study pathfinding process. Avian retinas are used for conducting such investigation because of their stratified organization (Fig. I-1). Avian retinas do not contain

blood vessels and connective tissues. They consist of only two types of cells: neuronal cells and glial cells (Müller cells). In this study, we have used embryonic chick neural retina because the ganglionic cells and their axons are in close contact with retinal basement membrane, inner limiting membrane, which is composed of extracellular matrix molecules which might play important roles in organization and behavior of the ganglionic cells and their axons.

Rationale for thesis

Proteoglycans influence the behavior of cells. In the case of neuron axonal guidance, proteoglycans may be involved in directing neurons by either repelling or inhibiting their growing axons in one direction and permitting the other or just simply promoting or stimulating their outgrowth. How do neurons recognize or sense targets that are far remote from their original 'birthplaces', and then correctly send out their axons to contact their definite target tissue (for example, ganglion cells send their axons from retina to tectum in brain)? Most research on this subject has been done with *in vitro* systems, employing one or two putative molecules attached to a dish to investigate their possible roles in guiding axons. The difficulty with such systems is that (1) the native conformation of molecules and their interaction with other molecules may be critical, and (2) *in vitro* systems typically overlook gradients and microenvironmental changes. If there are gradients or specific guidance molecules in the ECM surrounding the neuronal cells or on the neighboring cell membranes, then it may be necessary for the

constant production of 'guidance molecules' to maintain the 'gradient'. However, in *in vitro* systems it is difficult to maintain a constant gradient or manipulate or mimic *in vivo* situations. A step gradient has been exploited *in vitro* to mimic the *in vivo* situation (Snow & Letourneau, 1991). Neuronal cells usually stop extending their axons when they encounter a high concentration of proteoglycan. If, however, the neuronal cells were placed onto this high concentration on the step gradient, the cell bodies *did* extend their processes. This paradox may be due to the fact that the step gradient is still an artificial system upon which, instead of through which, the processes extended and failed to include adhesive substrates such as laminin found *in vivo*.

This thesis attempts to gain the advantage of control offered by an *in vitro* system, while at the same time maintaining the structure and molecular interactions of an *in vivo* system, by studying retina axonal outgrowth on a preparation of isolated inner limiting membrane. This first chapter has attempted to provide the historical background to proteoglycans in nervous tissue. Chapter II localizes proteoglycans within neural retina immunochemically and autoradiographically. Chapter III uses radioisotopes to determine the quantity of proteoglycans in both retina and isolated inner limiting membrane, a special basal lamina which underlies the retina ganglion cells. Chapter IV examines the influence of inner limiting membrane proteoglycans on retina explants and isolated cells.

Fig. I-1. Organization of a 10-day old embryonic chicken neural retina. (A) Cross-section of a 10-day old neural retina. The retina contains three nuclear layers and two synaptic layers. Three nuclear layers are outer nuclear layer (ONL), inner nuclear layer (INL), and ganglionic cell layer (GCL). Two synaptic layers between nuclear layers are outer plexiform layer (OPL), and inner plexiform layer (IPL). Inner limiting membrane (ILM, about 1-2μm thick in chick) is closely associated with ganglionic cell layer and ganglionic cell axons (ONF). (B) Diagrammatic drawing of a 10-day old retina organization. R & C = Rods and cones; OLM = Outer limiting membrane. Bar = 100 μm.

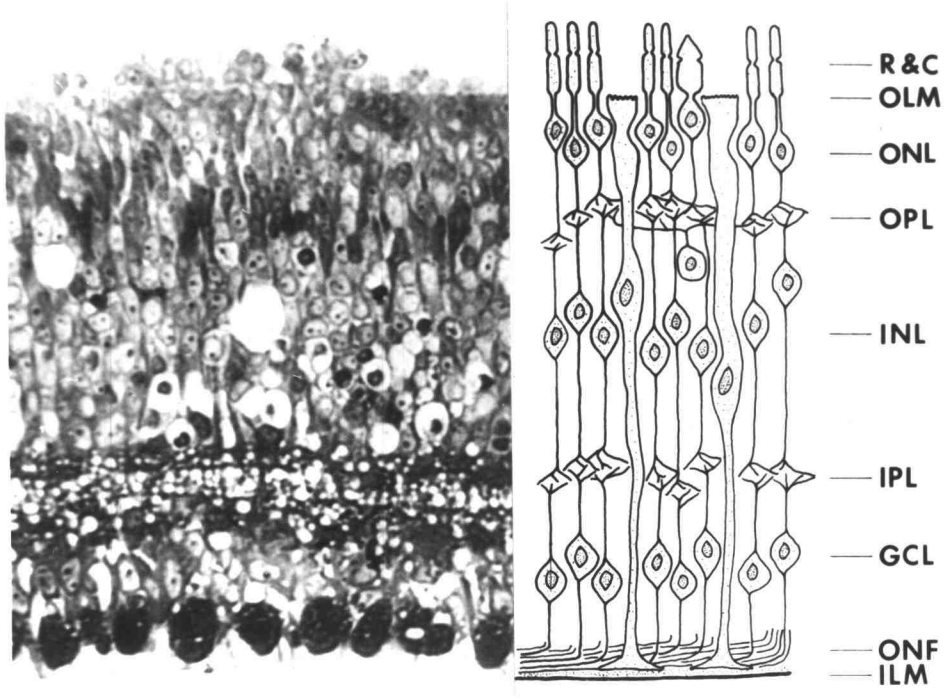


Fig. I-1 (Cont.)

CHAPTER II. HEPARAN SULFATE PROTEOGLYCAN BECOMES CONCENTRATED IN THE PLEXIFORM LAYERS AND INNER LIMITING MEMBRANE DURING DEVELOPMENT OF EMBRYONIC CHICKEN NEURAL RETINA

Abstract

Understanding the ability of chondroitin sulfate/dermatan sulfate proteoglycans (CS/DSPG) and heparan sulfate proteoglycans (HSPGs) to influence neural development depends upon understanding their distribution in the tissue. In this study, we have used both autoradiographic and immunohistochemical methods to study the distribution of these proteoglycans in embryonic chicken neural retina. Autoradiographs of enzymatically digested cryosections of radiolabeled fresh frozen neural retinas revealed that HSPGs are associated mainly with the inner limiting membrane (ILM) in 7-day embryos and with the ILM as well as outer and inner plexiform layers in 10-day embryos. On the other hand, the CS/DSPGs are distributed across the retina in both young and old embryos. A monoclonal antibody that recognizes basal lamina HSPG core protein stained only the ILM. Moreover, this antibody stained the ILM specifically in retinas of all ages examined (4 days through 14 days). These results support the idea that HSPGs in the ILM, because of their early localization and distinct structure, may guide the developing ganglion cell axons.

Introduction

Proteoglycans are macromolecules consisting of a core protein to which negatively charged glycosaminoglycan side chains are O-linked to serine or threonine (Roden, 1980). In addition to their wide distribution in nonneuronal tissues (mainly in connective tissues), proteoglycans occur also in both embryonic and adult nervous systems of all vertebrate classes (reviewed by Margolis and Margolis, 1989) and at least some invertebrates (Brower et al., 1987; Campbell et al., 1987; Meyer et al., 1988; Wang & Denburg, 1992). Proteoglycans in the nervous system are particularly interesting because of their capacity to influence neuronal behavior such as neurite formation (Metthew et al., 1985) and neurite outgrowth (Chernoff, 1988; Dow et al., 1988).

To understand the roles of proteoglycans in central nervous systems during early development, retinas of vertebrates have been investigated in numerous studies because of their highly stratified organization and ease of isolation and culture free of contaminating connective tissue. In earlier studies, cytochemical and autoradiographic methods revealed mucopolysaccharides (old name for proteoglycans) and ^{35}S-sulfate associated with the inter-photoreceptor cell layer, inner and outer plexiform layers, and inner and outer limiting membrane layers of retinas from several vertebrate species (Zimmerman & Eastham, 1959; Fine & Zimmerman, 1963; Hall, M. O. et al., 1965; Ocumpaugh & Young, 1966; Röhlich, 1970). More recently, immunohistochemical studies have revealed

CSPGs associated with horizontal cells, ganglion cells, Müller cells, and ILM but not with optic nerve axons of rat retina (Aquino et al., 1984a). They are also dominant components of cone photoreceptor matrix sheaths of primates (Hageman & Johnson, 1987). In embryonic chicken retina, CS/DSPG and HSPG are actively synthesized (Morris, 1984; Morris et al., 1984, 1987) and undergo a progressive transition from predominantly HSPG at earlier developmental stages (5 days) to predominantly CS/DSPG at later stages (14 days) (Morris et al., 1977). Biochemical study indicates that HSPGs are mainly associated with cell surfaces, whereas CS/DSPGs are localized in extracellular matrix (Morris et al., 1987). In purified retina cell cultures, both neuronal cells (Needham et al., 1988) and glial cells (Threlkeld et al., 1989) have been found to produce CS/DSPG and HSPG, with HSPG being the predominant proteoglycan type. Despite evidence of biosynthesis of proteoglycans, little is known about how different proteoglycans are localized in the stratified retina tissues. In this work we examine the distribution of proteoglycans in embryonic chicken neural retinas. By using autoradiographic and immunohistochemical methods we directly demonstrate the relative enrichment of HSPG in both the inner and outer plexiform layers, and the ILM. We show that the HSPG of the ILM is a typical basal lamina HSPG, consistent with its basal lamina origin, and is distinct from that associated with the inner plexiform layer, the layer of synaptic contacts between ganglion and bipolar cells. We also demonstrate that the distribution of the HSPGs in the retina is developmentally regulated, consistent with a role in neurogenesis.

Materials and methods

Autoradiography

Seven- and ten-day old chick embryos were labeled with ^{35}S-sulfate (200μCi/egg) in vivo overnight by applying the radioisotope to the shell membrane which had been torn by syringe needle in order to facilitate passage of the radioisotope onto the chorioallantoic membrane. After 24 hr retinas were dissected from the embryos, placed on nitrocellulose membrane filters, washed, and either embedded directly in Tissue-Tek O.C.T. compound (Miles) without fixation for enzyme digestion, or they were fixed in glutaraldehyde, dehydrated, and embedded in paraffin. Ten μm fresh-frozen sections were incubated on glass slides with 150 units chondroitinase ABC in Tris Acetate buffer (0.1 tris-acetate, pH7.4, 5 mM phenylmethylsulfonylfluoride (PMSF), 0.75 mM pepstatin A, 10 mM N-ethylmalemide, 10 mM EDTA) or 3.75 units heparitinase in Tris buffer (0.1 M tris-acetate, pH 7.4, 2 mM PMSF, 0.15 mM pepstatin A, 10 mM calcium acetate), or tris-acetate buffer alone for 1 hr at 37°C. The reaction was stopped by adding 4% paraformaldehyde in 0.1 M phosphate buffer plus 0.05% ruthenium red for 10 min, followed by washing in 0.1 M phosphate buffer. Both fresh-frozen and fixed, paraffin-embedded retinal sections were coated with emulsion (Kodak NTB-3) and kept in the dark at 4°C for two to three weeks. The emulsion was developed, and the sections were observed with bright field and dark field microscopy.

Immunohistochemistry

Four-, seven-, ten and thirteen-day old chick retinas were dissected, mounted on a nitrocellulose membrane filter, embedded in O.C.T., frozen in liquid nitrogen, and sectioned with a cryostat, or the ILM sheets were isolated from chick retinas according to Halfter et al. (1987). The fresh-frozen sections or isolated ILM sheets were incubated in phosphate buffer containing 10% horse serum for 10-20 min, and then incubated with a monoclonal antibody against embryonic chicken muscle basal lamina HSPG (antibody 33-2, Bayne et al., 1984; obtained from the Developmental Studies Hybridoma Bank, Baltimore) or Hybridoma Bank NS-1 control medium for 30-60 min. After several 5-min washes the sections were stained with the fluorescent dye Bodipy™, conjugated to goat anti-mouse IgG (Molecular Probes, Eugene, Oregon) in the same buffer for 30-60 min. After several rinses the sections were observed under a Zeiss fluorescence microscope equipped with epi-illumination.

Results

To determine the distribution of the proteoglycans in the embryonic chicken retina we radioactively labeled retinas *in vivo*. Autoradiography showed that the radioactivity was unevenly distributed across the retina and concentrated on different cell layers. In 7 day-old retina the radioactivity, shown by silver grains on film, was localized only outside the neural portion, in the ILM and in the

pigmented epithelium (Fig II-1B). In contrast, in 10 day-old retinas the major sites of radioactivity were the ILM and pigmented epithelium (not shown), as well as the inner plexiform layer of the neural retina (Fig II-2B). A smaller amount of radioactivity, when compared with those associated with inner plexiform layer and ILM, also was associated with the outer limiting membrane and outer plexiform layer(Fig II-2B). Other parts of the retinas showed relatively small amounts of radioactivity when compared with the outer and inner plexiform layers and ILM.

To determine what types of proteoglycans were associated with each layer, we used specific enzymes to digest the radioactively-labeled 7 and 10 day old retinas. Enzymatic digestion of ^{35}S-sulfate in labeled fresh frozen tissue showed that radioactivity in the inner and outer plexiform layers was relatively resistant to chondroitinase digestion but susceptible to heparitinase digestion in 10 day old retinas (Fig. II-4B). The radioactivity associated with ILM was also resistant to chondroitinase digestion and susceptible to heparitinase digestion in both 7 day old (Fig. II-3A) and 10 day old retinas (Fig. II-4A). It was not possible to demonstrate radioactivity associated with chondroitin sulfate in the outer photoreceptor layer as in adult primate retinas (Hageman & Johnson, 1987), possibly because the outer segment of the photoreceptor cells is not fully formed yet at these stages.

A monoclonal antibody against chick muscle basal lamina HSPG core protein recognized the ILM but not other retinal layers in all ages tested from 4

through 13 days of development (Fig. II-5A, II-5B, II-5C, and II-5E). Sections treated with control medium (NS-1) showed no staining (Fig. II-5F).

To confirm that the basal lamina HSPGs were associated with the ILM but not with Müller cell endfeet that adhered to the ILM, we used the antibody 33-2 to stain the isolated ILM sheets. The antibody uniformly stained the ILM sheet (Fig. II-6A) and was not restricted to or concentrated in punctate regions, which should have occurred if the HSPGs were concentrated in the Müller cell endfeet. Furthermore, when the Müller cell endfeet on the ILM (Fig. II-6D) were removed by using 2-4% Triton X-100 (Halfter et al., 1987) either before or after the antibody reaction, the staining was not affected (Fig. II-6C). Staining freshly-isolated ILM with carbocyanine dye DiI revealed the Müller cells endfeet (Fig. II-6E).

Discussion

Earlier studies have shown that embryonic chicken retinas actively synthesize both HSPG and CS/DSPG (Morris, 1984; Morris et al., 1987). Biochemical study has shown that the former is predominately associated with the cell membrane and the latter with extracellular spaces and matrix (Morris et al., 1987).

In this study we demonstrated patterns of ^{35}S incorporation into embryonic chicken retinas at two different ages: 7 and 10 days. We found that 7 day-old

retinas had a relatively uniform distribution of silver grains across the tissue, except for more silver grains in the ILM. Ten day-old retinas had three bands of silver grains, which represented inner and outer plexiform layers, and ILM. We believe that these ^{35}S-sulfate labeled materials represent mostly proteoglycans for two reasons: (1) the bands were digestible with heparitinase and chondroitinase (see below), and (2) earlier autoradiographic and histochemical studies of rat and mouse retina tissues have shown that these portions of retina contain mainly proteoglycans (Hall, et al., 1965; Ocumpaugh & Young, 1966; Zimmerman & Eastham, 1959). Since the distribution of proteoglycans in fresh frozen retinas was examined following 24 hr of labeling *in vivo*, we also believe that the ^{35}S-sulfate labeling represents the total proteoglycans in all pools and reached equilibrium in the proteoglycans. In tissue cultures, the half-time for maximum labeling and the highest incorporation rate into the chick retina proteoglycans is 6 hr (unpublished observations, Morris). By using selective enzyme digestion of radioactively-labeled retina sections we further found that, compared with the rest of the retina, these radioactively-labeled materials in the inner and outer plexiform (synaptic) layers as well as in ILM are relatively resistant to chondroitinase digestion and susceptible to heparitinase digestion. These results indicate that more HSPGs than CSPGs are associated with both synaptic layers in older retinas (10 day-old) and with ILM in both 7 day-old and 10-day old retinas. The changes from evenly-distributed proteoglycans in the younger retina to differentially-distributed proteoglycans in the older retina may reflect developmental regulation of

biosynthesis of these macromolecules. During early development of chicken embryos, the retina has long been known to undergo three different periods of changes (Weysse & Burgess, 1906). The retina tissues undergo cell multiplication from the 2nd to 8th incubation day followed by cellular readjustment from 8th to 10th incubation day. Then, from the 10th day to the end of incubation the retinas undergo final differentiation. During the 1st period of development, retina tissue starts to change from undifferentiated cell types to differentiated cell types (Coulombre, 1955). Our results show that during this period proteoglycan synthesis is relatively evenly distributed. During the 2nd period of development, the cells of retina tissue start to rearrange themselves and form a few distinguishable layers including two synaptic layers, the inner plexiform and the outer plexiform layers (Coulombre, 1955; Romanoff, 1960). During the 2nd period proteoglycan synthesis, especially HSPGs, is more active in these two synaptic layers, where they may be involved in the formation of synapses (Swenarchuk et al., 1990; Anderson et al., 1991).

Developmentally-regulated biosynthesis of proteoglycans in the nervous system has been cited in numerous studies. Chicken embryonic neural retina biosynthesizes predominately HSPGs in the younger stages and predominately CSPGs in the older stages (Morris et al., 1977). It has also been found that biosynthesis of CSPGs in the rat brain is developmentally regulated (Margolis, et al., 1975). While the developmental profile of these proteoglycans has been established, the identity of cells producing the proteoglycans has not been shown *in*

vivo. Recent studies indicate that ECM and cell membrane-associated proteoglycans from cultures of isolated neuronal and photoreceptor cells (Needham et al., 1988) are entirely HSPG while those from glial-like cells (Threlkeld et al., 1989) are both CS/DSPG and HSPG, with HSPG being the major component. At least one study has shown that, in cell culture, the source of HSPGs is sensory neurons (Dow et al., 1988).

Accumulation of HSPGs in the inner and outer plexiform layers may indicate important biological activities for these molecules in these particular areas. For example, it has been found that HSPGs may promote neurite formation (Dow et al., 1988; Lafont et al., 1992), neurite outgrowth (Chernoff, 1988; Dow et al., 1988; Cole & McCabe, 1991) and neurite extension (Matthew et al., 1985; Cole & Burg, 1989) in other nervous tissues in culture. HSPGs have also been implicated in anchorage of acetylcholinesterase to the neuromuscular junction (Brandan & Inestrosa, 1986) and stabilization of acetylcholine receptors (Bayne et al., 1984). So far, the importance of the accumulation of these particular molecules in these two synaptic areas is still unknown.

HSPGs associated with Müller cell endfeet could not explain HSPGs in the ILM. The ILM proteoglycans were not solubilized in a detergent solution even though the detergent solution removed the Müller cell endfeet. Autoradiographs revealed the labeled materials distributed evenly across the ILM and not concentrated at the cellular surface, and antibodies against chick muscle basal

lamina HSPG reacted evenly over the ILM and were unreactive to the Müller cell endfeet.

The monoclonal anti-chick muscle basal lamina HSPG antibody (Bayne et al., 1984) reacted with only the ILM, not the plexiform layers. The same antibody also demonstrates that HSPGs in the chicken muscle basal lamina are different from those in the surrounding connective tissues (Bayne et al., 1984). These results indicate that the core protein of the HSPGs in ILM may contain a distinct domain that is not present in HSPGs from other parts of the retina tissue. Mouse mammary epithelial cells have been found to produce two HSPGs with distinct core proteins (Jalkanen et al., 1988). One HSPG is found only at cell surfaces and the other only in the basement membrane. This result suggested the possibility, that despite HSPGs having similar glycosaminoglycan chains, they may be sorted by the cells to deposit in the different regions according to the differences of their core proteins.

Even though HSPGs may have a similar or identical glycosaminoglycans chains they may play different roles during embryonic development at different regions. For instance, in both embryonic chicken (Cirillo et al., 1990) and mouse (Fayein et al., 1990a) retinas it has been found that the bFGF binding sites are located in outer and inner plexiform layers, and in the ILM. By using a combination of radioactive (^{125}I)-labeled bFGF binding assay and enzymatic degradation, these authors found that the binding of bFGF to receptors in the ILM but not in the plexiform layers is highly sensitive to heparitinase digestion and is

sensitive to competition by excess exogenous heparin in both mouse and chick embryos. These results indicate that glycosaminoglycans of the HSPGs in the ILM have different functions.

HSPGs are now recognized to be one of the major components of basal lamina, which also includes type IV collagen and laminin. Although the functions of HSPGs are not fully understood, some are involved in cell attachment (Laterra et al., 1983; Robinson et al., 1984) and in regulating cellular differentiation (Cohn et al., 1977; Bernfield et al., 1984; Li et al., 1987) in nonneuronal tissues. Others function in the basal lamina as a structural component (Fujiwara et al., 1984), in being involved in its formation (Martin et al., 1984; Laurie et al., 1986; Paulsson et al., 1986), in establishing a charge barrier for filtration (Rennke et al., 1975; Brenner et al., 1978; Farquhar, 1981; Stow et al., 1985; Stow et al., 1989), and by sequestrating growth factors (Hageman et al., 1991) and extracellular ions (Arnott & Mitra, 1984; Lerner & Torchia, 1986).

In conclusion, the proteoglycans, particularly HSPGs, in retinas of embryonic chickens show patterns of progressive localization in synaptic regions between 7 and 10 days of incubation. The change of HSPG pattern indicates that the biosynthesis of these neuronal proteoglycans is developmentally regulated. The HSPGs in the ILM were present at all developmental stages we tested, from 4 through 13 days of incubation. The basal lamina HSPGs that were recognized by the monoclonal antibody were not found in other parts of retinas. This suggests that the core protein of the basal lamina HSPGs has at least one epitope that is

different from those in the synaptic layers. This result also suggests that the HSPGs of the ILM and of the plexiform layers are playing different roles in the development and/or functions of the retina.

Fig. II-1. Autoradiograph of ^{35}S-sulfate labeled glutaraldehyde-fixed 7-day old embryonic chick retina. ^{35}S-sulfate labeled materials were mainly associated with the inner limiting membrane and pigmented epithelium of the retina. A. bright field; B. dark field. ILM: Inner limiting membrane. PE: Pigmented Epithelium. Bar=100μm.

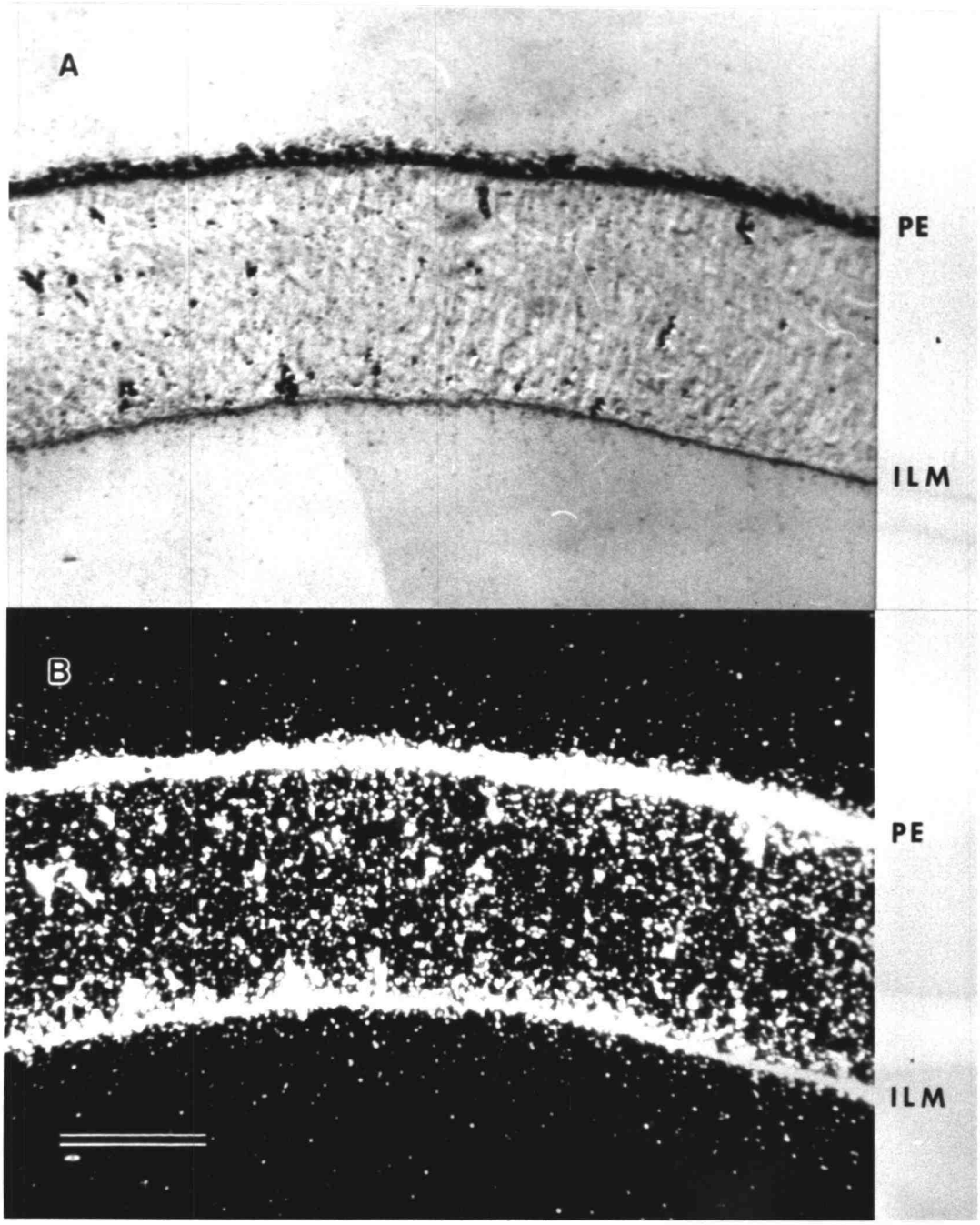


Fig. II-1 (Cont.)

Fig. II-2. Autoradiograph of ^{35}S-sulfate labeled glutaraldehyde-fixed 10-day old embryonic chick retina. ^{35}S-sulfate labeled materials were mainly associated with the outer plexiform layer, the inner plexiform layer, and inner limiting membrane of the retina. The heavily-labeled pigmented epithelium was removed during dissection. Arrow heads in A and B indicate the inner limiting membrane peeled away from the rest of the retina. A. bright field; B. dark field. OLM: Outer limiting membrane (zonula occludes of photoreceptor cells); ONL: Outer nuclear layer of photoreceptor cells; OPL: Outer plexiform layer: INL: Inner nuclear layer; IPL: Inner plexiform layer; GCL: Ganglion cell layer; OFL: Optic fiber layer; ILM: Inner limiting membrane. Bar=100μm

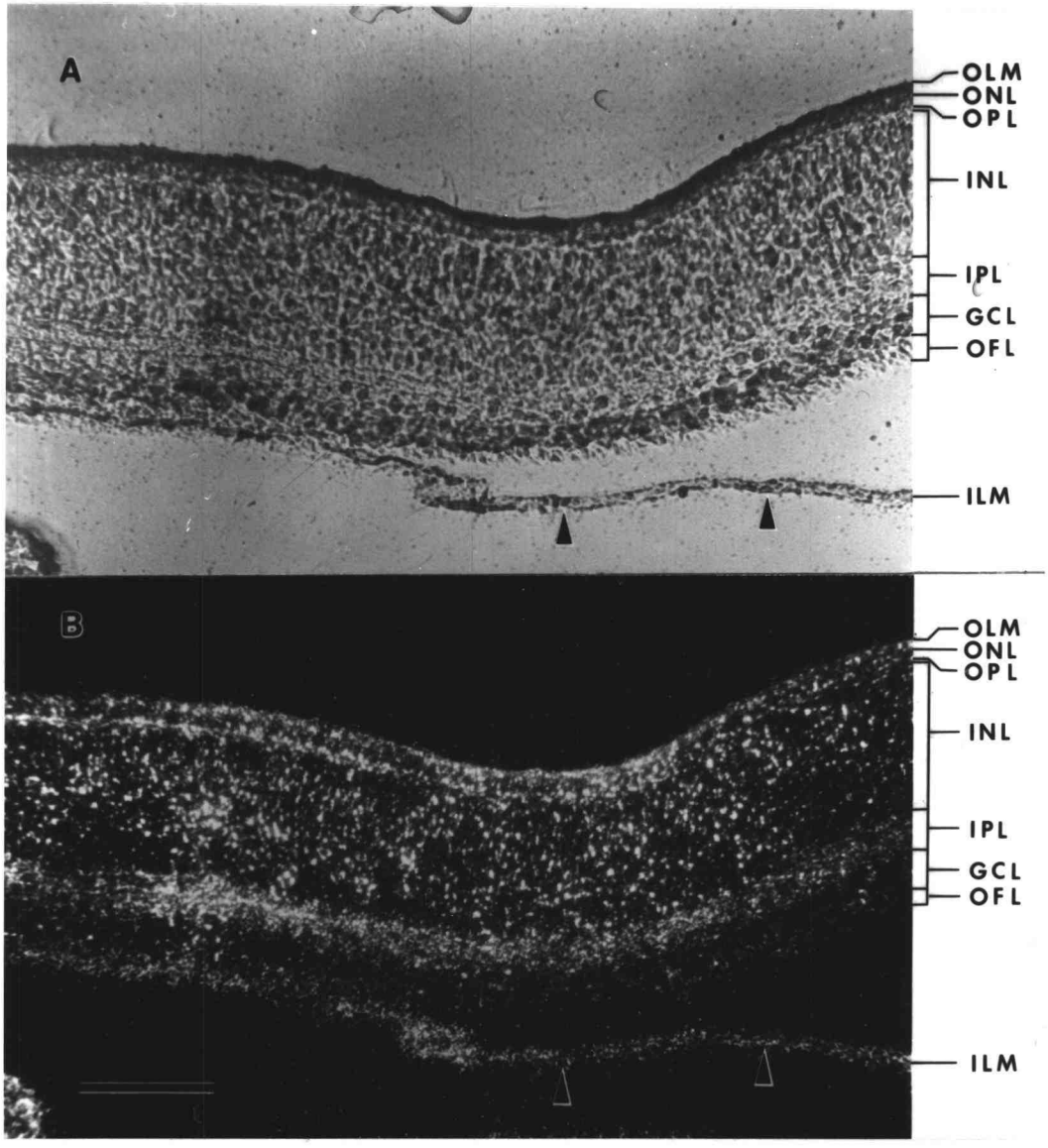

A
OLM
ONL
OPL
INL
IPL
GCL
OFL
ILM
B
OLM
ONL
OPL
INL
IPL
GCL
OFL
ILM

Fig. II-2 (Cont.)

Fig. II-3. Autoradiographs of enzymatically digested fresh frozen 7-day old chick retina tissue viewed with dark field microscopy. ^{35}S-sulfate labeled retina tissue digested with chondroitinase ABC (A), digested with heparitinase (B), or treated with buffer alone (C). ILM: inner limiting membrane; PE: pigmented epithelium. Bar=100μm.

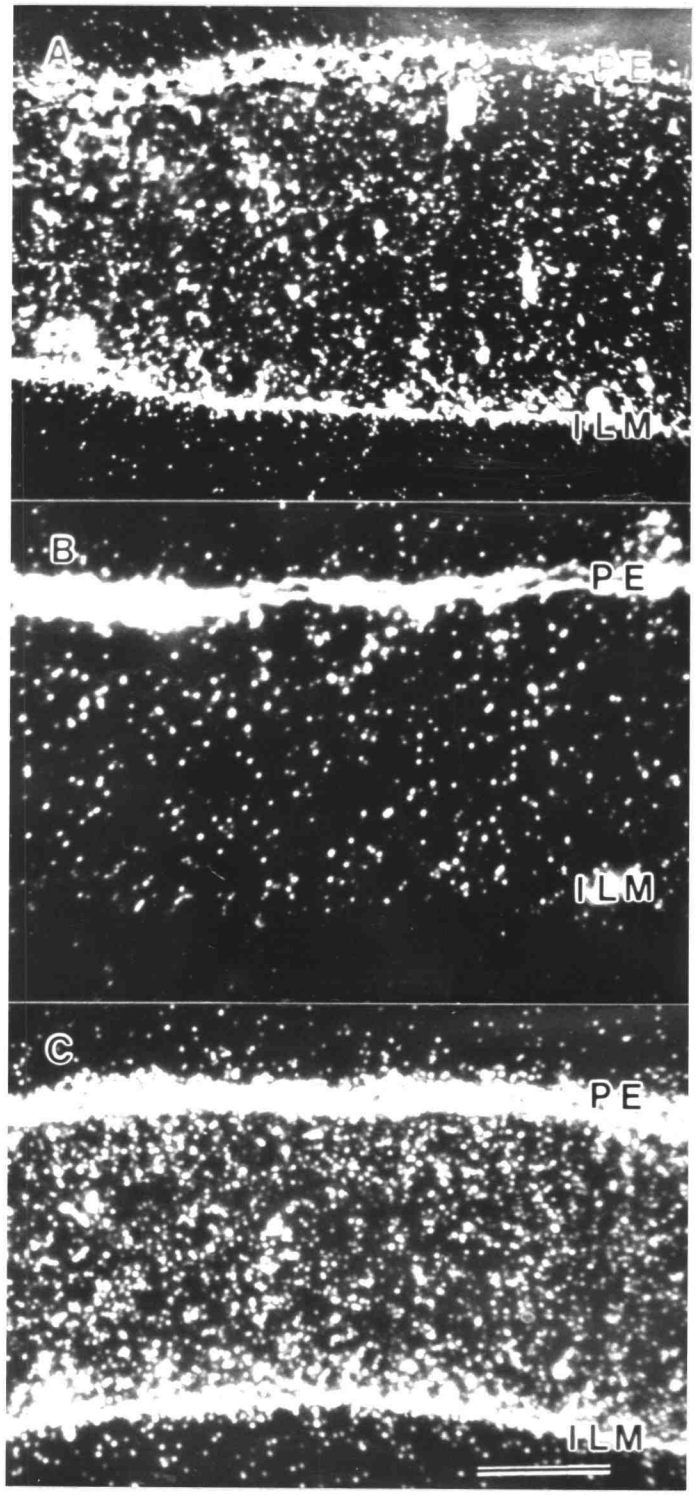


Fig. II-3 (Cont.)

Fig. II-4. Autoradiographs of enzymatically digested fresh frozen 10-day old chick retina tissue viewed with dark field microscopy. ^{35}S-sulfate labeled retina tissue digested with chondroitinase ABC (A), digested with heparitinase (B), or treated with buffer alone (C). Arrow heads in (A) and (B) mark the edge of a nitrocellulose membrane filter that was used to support the retina for O.C.T. embedding. The tissue has been partially separated from the membrane filter in (A); the membrane filter in (C) was completely dislodged during the wash procedures. ILM: inner limiting membrane; IPL: inner plexiform layer, OPL: outer plexiform layer, PE: pigmented epithelium. Bar=100μm.

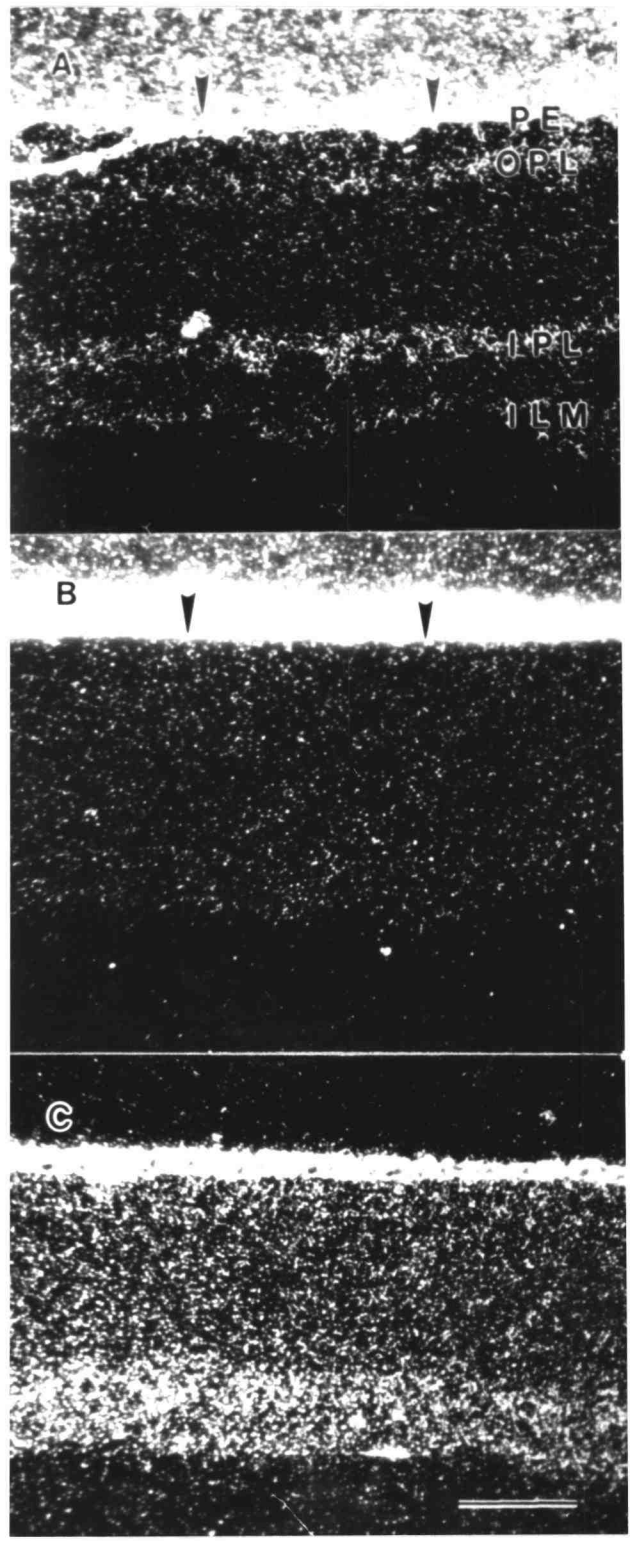


Fig. II-4 (Cont.)

Fig. II-5. Micrographs of embryonic chicken retina incubated with anti-basal lamina heparan sulfate proteoglycan antibody 33-2. The antibody was recognized with a fluorescent (Bodipy™ conjugated) secondary antibody. The anti-basal lamina HSPG recognized inner limiting membrane of all stages tested. (A) four-day old retina (and five-day old retina, not shown); (B) seven-day old retina; (C) ten-day old retina; and (E) thirteen-day old retina. The antibody also stained the pigmented epithelium. Other layers of the retina failed to bind antibody. (D) Phase contrast micrograph of same section as in (C). The arrow heads in (A), (C), (D), and (F) indicate the edge of nitrocellulose membrane filter which was used to support the retina for O.C.T. embedding. The membrane filters with pigmented epithelium in (B), and (E) were completely dislodged during the wash procedures. (F) Neither inner limiting membrane nor pigmented epithelium fluoresced after treatment with control hybridoma supernatant although the membrane filter did bind antibody and/or dye nonspecifically. ILM: inner limiting membrane; PE: pigmented epithelium. Bar=50μm in (A); =100μm in (B) to (F).

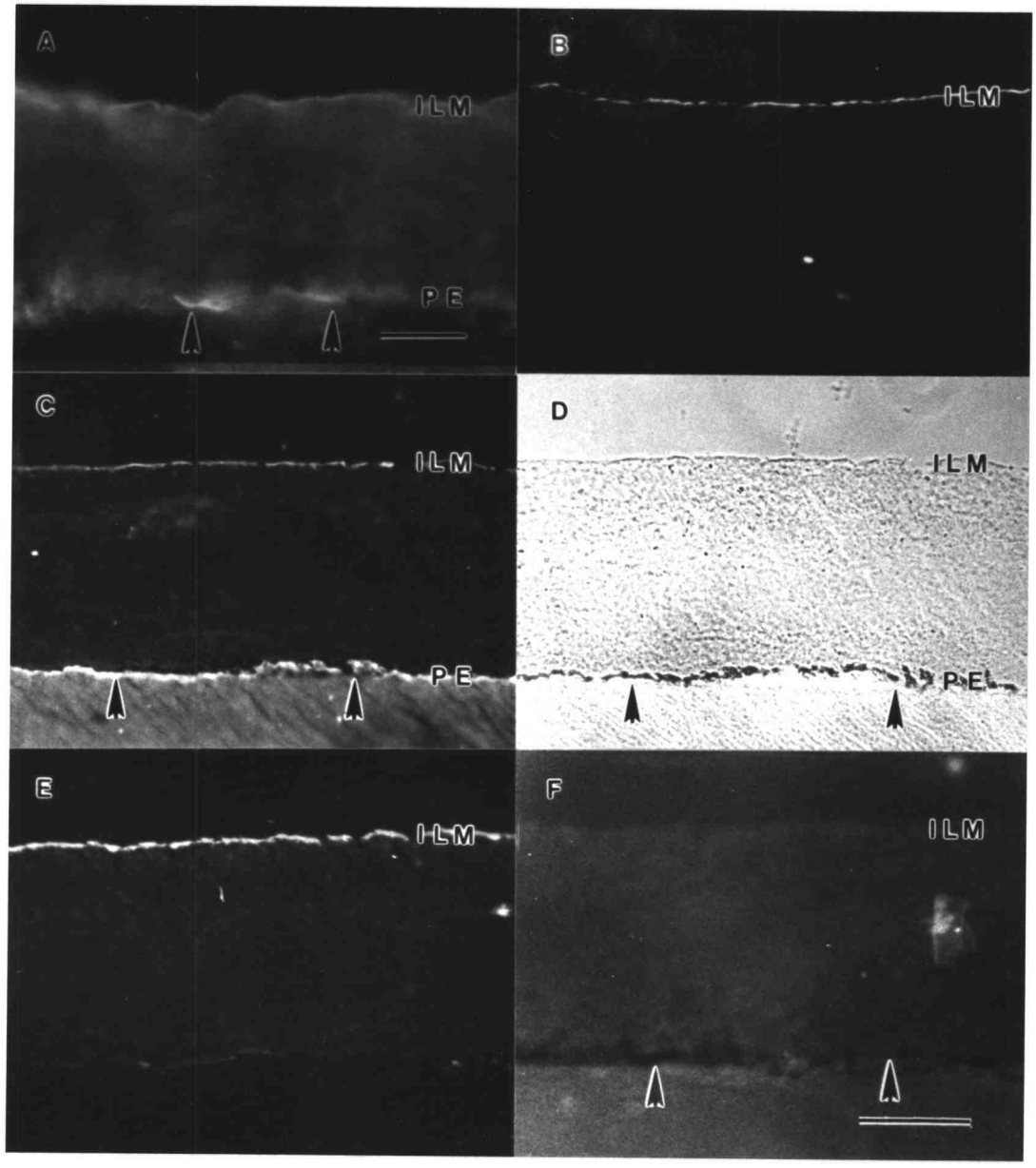


Fig. II-5 (Cont.)

Fig. II-6. Micrographs of isolated inner limiting membrane incubated with anti-basal lamina heparan sulfate proteoglycan monoclonal antibody. Fluorescence (A) and (C) and phase contrast (B) and (D) micrographs of immunohistochemical staining of isolated inner limiting membrane. Monoclonal anti-basal lamina HSPG antibody bound evenly to the surface of the inner limiting membrane sheet (A) and was not restricted to the punctate Müller cell end feet (arrows in B). The antibody still was bound to the inner limiting membrane (C) even after the inner limiting membrane was treated with 2% Triton-X 100 to remove Müller cell endfeet. No Müller cell endfeet can be detected under the microscope (D). If a freshly-isolated inner limiting membrane was stained with DiI (a fluorescent dye to stain cell membrane), the ILM would show a punctuated pattern (E). (F) Bright field of (E). Arrows in (B) and (E) indicate Müller cell endfeet. Bar=20μm in A to E

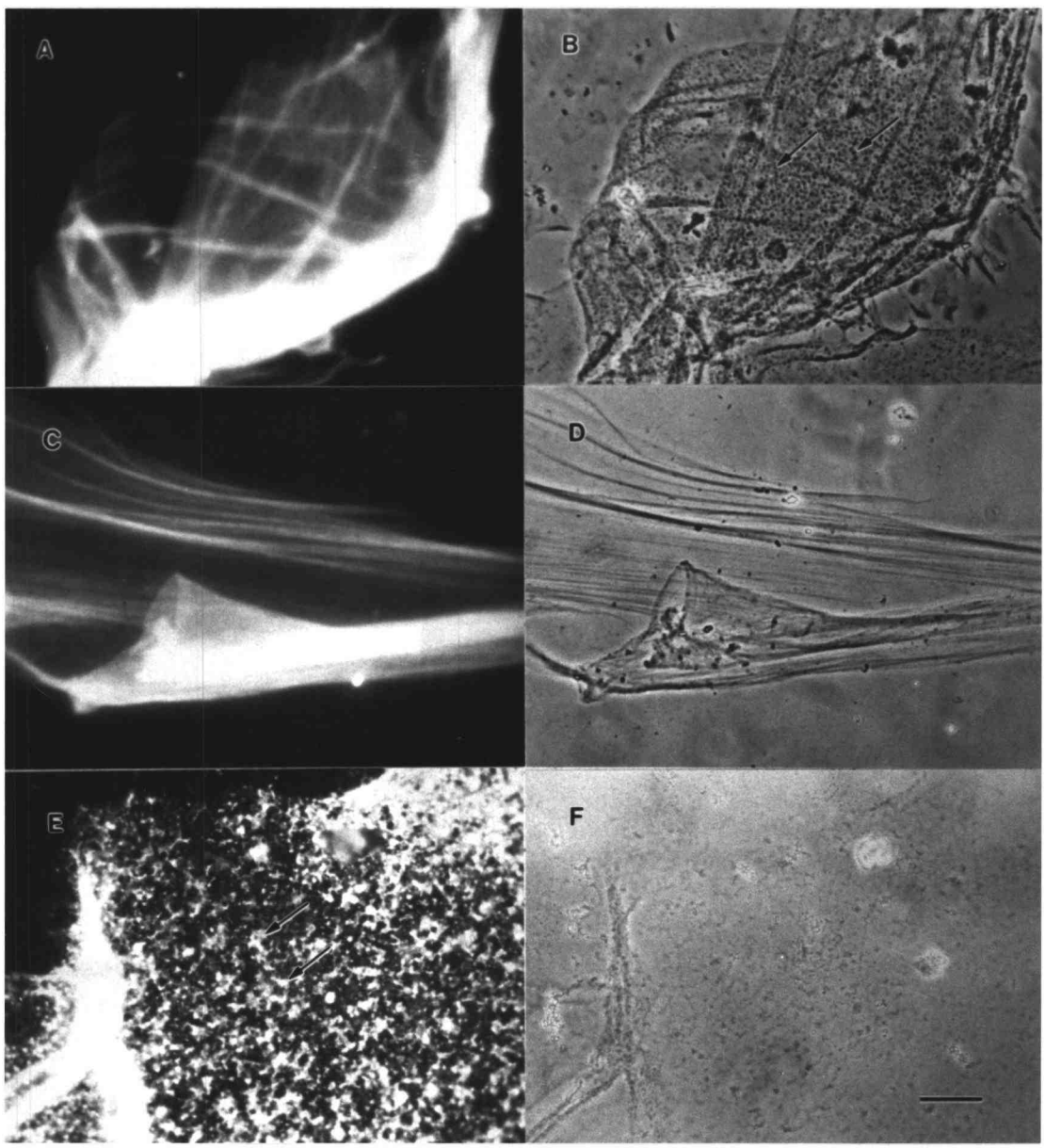


Fig. II-6 (Cont.)

CHAPTER III. HEPARAN SULFATE IS A MAJOR GLYCOSAMINOGLYCAN COMPONENT IN THE INNER LIMITING MEMBRANE OF EMBRYONIC CHICKEN NEURAL RETINA

Abstract

Immunocytochemical and autoradiographic studies show that heparan sulfate proteoglycans (HSPGs) of the neural retina are mainly associated with outer and inner plexiform layers, and inner limiting membrane (ILM) whereas the chondroitin sulfate proteoglycans (CSPGs) are evenly distributed across the retina. In this study, we have isolated the ILM to compare its proteoglycans directly with those from the rest of the retina. Ten- and eleven-day retinas were labeled with ^{35}S-sulfate, and proteoglycans were isolated from extracts of the dissected ILM of the labeled retina and remaining retina by gel filtration methods. Chemical degradation of HSPG and specific lyase digestion of CS/DSPG showed that the ILM on average contained about three fourths of its ^{35}S in HSPG and one fourth in CS/DSPG, whereas the remaining retina contained approximately equal amounts of its ^{35}S in the two proteoglycans. The enrichment of HSPG in the ILM is of particular interest, since HSPGs have been implicated in retinal neurite extension and formation. Our results, therefore, support the idea that these molecules are not only structural components in basal lamina as suggested by some other studies, but play critical roles in the extension of the ganglion cell axons.

Introduction

In biochemical studies of whole retinas, HSPGs have been demonstrated to be tightly associated with a fraction extractable only with detergent and/or with high ionic strength buffers, such as those represented by cell membranes and basal lamina biochemically (Morris et al., 1987). In the previous study (chapter II), we demonstrated by both immunohistochemical and autoradiographic techniques that the retinas contain an abundance of HSPGs in their ILM (i.e. the retinal Müller cell basal lamina), indicating that these molecules may play an important role in ganglion cell axon extension and migration. In order to understand if and how proteoglycan molecules in the ILM participate in normal embryonic development in retina, it is necessary to establish the relative amount of each proteoglycan in this structure. However, characterization of the HSPG in the basal lamina is now still limited to qualitative immunohistochemical localization (Rada & Carlson, 1991; Vila-Porcile et al., 1992; Vila et al, 1992; Rada and Carlson, 1992; Rucosuec, 1992; Sudey et al, 1992; Hinsch, et al, 1992) or semi-quantitative histochemical studies, using cationic dyes, such as ruthenium red or cupromeronic blue (Call & Hollyfield, 1990), which stain the negatively charged sulfate groups of the glycosaminoglycan side chains. Quantitative characterization of basal lamina HSPGs in other tissues has not been reported, probably due to the difficulty of isolating the lamina from between sandwiching cell layers (e.g. between epithelial and mesenchymal interfaces of tissues). In this study, we took advantage of the

location of ILM (between neuroepithelium and vitreous body of the eye) for its relative ease of isolation. We labeled embryonic chick neural retinas with ^{35}S-sulfate in culture, separated the ILM mechanically from the rest of the retina, and extracted the proteoglycans from it and the remaining retina. We directly demonstrate the relative enrichment of HSPG in the ILM, consistent with its basal lamina composition and also show that it is a different HSPG than that associated with the inner plexiform layer, the layer of synaptic contacts between ganglion and bipolar cells.

Materials and methods

Tissue culture and radioisotopic labeling

Four retinas from 10-day or 11-day white leghorn chicken embryos were dissected free from sclera and pigmented epithelium and incubated in 8 ml of Eagle's minimum essential medium (MEM) supplemented with 10% bovine calf serum (BCS), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). After 1 hour at 37°C on a gyrator shaker in 50-ml Erlenmeyer flasks, the retinas were incubated in sulfate-free Hanks' balanced salt solution (sfBSS) for 30 min. They were then labeled for 6 hours in fresh sulfate-free BSS containing 200 μCi/ml of ^{35}S-sulfate and 10% BCS, but no antibiotics. The BCS provided an essential minimum level of non-radioactive sulfate.

Isolation of inner limiting membrane

The labeled retinas were washed three times with Eagle's minimum essential medium and placed on ice in a Maximov depression slide. The ILM was carefully separated from the retina mechanically, using glass microelectrodes as dissecting needles. The ILM and remaining retina tissues were then washed three times in balanced saline solution by gentle centrifugation.

Isolation of proteoglycans

Proteoglycans were extracted from the ILM and remaining retina tissues with 4 M guanidine hydrochloride in 50 mM sodium acetate buffer (pH 5.8, containing a mixture of protease inhibitors) plus 2% Triton X-100 as before (Morris et al., 1987). Unincorporated isotope and low molecular weight substances were removed and the extracts transferred into the same buffer plus 0.15 M NaCl and 0.5% CHAPS (3-[(3-cholamindopropyl)dimethylaminonio]-1-propansulfonate) on Sephadex G-50 columns. Proteoglycans were then eluted with 0.6 M NaCl and 0.5% CHAPS from a DEAE Sephacel column following a wash of the G-50 column with 0.3 M NaCl with 0.5% CHAPS. Samples to which 0.5 mg each of chondroitin 6-sulfate and heparin were added as carriers were dialyzed against deionized-distilled H_2O for two days at 4°C and lyophilized.

Analysis of glycosaminoglycans

Aliquots were digested with chondroitinase ABC for determination of CS/DSPG (Oike et al., 1980) or degraded with nitrous acid for determination of HSPG (Shively & Conrad, 1976). Treated samples and untreated controls were applied to Sephadex G-50 chromatography columns to separate the digested from undigested samples.

Scanning electron and light microscopy

Washed retinas were mounted on DEAE paper in BSS. The ILM was partially peeled either from freshly dissected retinas or from retinas which had been cultured in sulfate-free BSS plus 10% bovine calf serum (BCS) for 6 hr. Retinas were then fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 1 hour, washed twice with phosphate buffer and postfixed with 1% OsO_4 in 0.1 M phosphate buffer for 1 hour. They were dehydrated and critical point dried for observation with an Amray AM-1000 scanning electron microscope. For light microscopy, the ILM was separated from the retina and fixed in 100% methanol to prevent it from rolling up during observation.

Results

In order to quantify the relative amount of proteoglycans in the ILM we mechanically isolated the ILM from retinas. Examination of the isolated ILM with

the scanning electron microscope failed to reveal contaminating intact cells or ganglionic cell axons (Fig.III-1). Small membranous vesicles on the ILM probably were Müller cell endfeet (Halfter et al., 1987; Schlosshauer et al., 1991).

Approximately 5-10% of total radioactivity in retinal proteoglycans was associated with the ILM and 90-95% of the radioactivity with the remaining retina, while the volume occupied by the ILM relative to the remaining retina was only about 1-2% (Table III-1). The types of the proteoglycan present in the ILM and the rest of the retina as well as in the intact retina were determined by chromatography on Sephadex G-50 before and after chondroitinase ABC digestion and nitrous acid degradation. The untreated proteoglycan fractions from all samples eluted from G-50 as single peaks of high-molecular-weight macromolecules (Fig. III-2C). On the basis of susceptibility to nitrous acid degradation (Fig. III-2A) and selective enzyme digestion (Fig. III-2B), the amount of the ^{35}S-sulfate in HSPG was about three times that in CS/DSPG in the ILM; while in the remaining retina and in the intact retina the amounts were nearly equal (Table III-2). Approximately 2-4% of ^{35}S-sulfate labeled materials from both fractions that bound to DEAE was resistant to both chondroitinase and nitrous acid. This material, which was not investigated further, probably was keratan sulfate (Morris, 1984).

Discussion

In this study we separated ILM from embryonic chicken retina mechanically. For biochemical characterization this technique has advantages over the one used by Halfter (1983), in which he allowed the ILM side of the retina to attach to a polylysine-coated surface and then removed the rest of the retina. Examination of the mechanically-isolated ILM under the scanning electron microscope failed to reveal contaminating intact cells or ganglion cell axons (Fig III-1). Small membranous vesicles on the ILM probably were Müller cell endfeet (Halfter et al., 1987; Schlosshauer et al., 1991).

Using a biochemical approach heparan sulfate was found to be the major glycosaminoglycan of the ILM of chicken retina. Significant amounts of chondroitin sulfate were also found. The amount of heparan sulfate was about 2- to 3-fold more than that of chondroitin sulfate. The rest of the retina (retina with ILM removed) contained about equal amounts of both heparan sulfate and chondroitin sulfate glycosaminoglycans. The amount of glycosaminoglycans in the ILM constituted about 5 to 10% of total glycosaminoglycans in the whole retina.

Embryonic chicken retinas actively synthesize CS/DSPG and HSPG (Morris, 1984). The former is predominantly localized in the saline extractable extracellular matrix, and the latter is associated with the detergent and high ionic strength-extractable cell membranes and basal lamina (Morris et al., 1987). It is likely that HSPG and CS/DSPG in the ILM of chick retina are synthesized only by

Müller (glial) cells. Recent studies indicate that ECM and cell membrane-associated proteoglycans from cultures of isolated neuronal and photoreceptor cells (Needham et al., 1988) are entirely HSPG while those from glial-like cells (Threlkeld et al., 1989) are both CS/DSPG and HSPG, with HSPG being the major component. In the previous study, we show that the HSPGs of the ILM and the neural cells are immunochemically distinct. It is probable that the proteoglycans isolated from the ILM contain only minor contributions from Müller cell endfeet that remain attached to it. Autoradiographs revealed the label distributed evenly across the ILM (see Chapter II Figure II-2) and not concentrated at the cellular surface of 10 day old retina. And antibodies against chick muscle basal lamina HSPG reacted evenly over the ILM and were unreactive to the contaminating Müller cell endfeet (see Chapter II Figures II-3 and 4). To avoid the possibility of extracting proteoglycans from the ILM, we did not remove the endfeet with detergent (chapter II and Halfter, 1987).

We also found that CS/DSPG is one component of the ILM. The amount of CS/DSPG is about one-third to one-half of that of HSPG in the ILM. CS/DSPGs are not commonly associated with basal lamina (Yurchenco & Schittny, 1990), even though it has been demonstrated immunocytochemically in specialized basement membranes of some vertebrates, such as in Bruch's membrane (basement membrane of pigmented epithelium) of rat retina (Lin et al., 1992), of human eye (Call & Hollyfield, 1990), dermal-epidermal junctions of adult rat skin (McCarthy et al., 1989, 1990), basement membrane around neural

tube, notochord and pronephros of embryonic chicken (Shinomura et al., 1990), Reichert's membrane and kidney of rat (McCarthy & Couchman, 1990) (also for review see Yurchenco and Schittny, 1990). However, CSPGs are not found in the external limiting membrane of embryonic chicken optic tectum by immunohistochemical methods (Kroger & Niehorster, 1990). In this study, we have not been able to establish whether the CS/DSPG is one component of the macromolecules of the ILM or a contaminant from the remaining retina (i.e. Müller cell endfeet). However, CSPG has been demonstrated immunocytochemically in the ILM of rat retina (Aquino et al., 1984b; Morriss-Kay & Tuchett, 1989). The function of the CS/DSPG in basal lamina or basement membrane is still not clear (reviewed by Yurchenco and Schittny, 1990). So far only one larger CSPG complex (MW 1000 kD) has been found in the electric organ basement membrane of electric fish (Iwata & Carlson, 1991), where it is suggested to help create extracellular aqueous space due to its larger size and bottlebrush-like glycosaminoglycan chains (cartilage CSPG like) and its disulfide-stabilization with the complex of one small putative proteoglycan and three other non-proteoglycan proteins (Iwata & Carlson, 1991). However, embryonic chicken retina CSPGs are unlike cartilage proteoglycans in their smaller size and small number of glycosaminoglycan chains (Morris et al., 1987). It is likely that the CSPGs in the ILM are structural components since our functional studies (chapter IV) failed to demonstrate that this molecule (CSPGs) affected neuronal behavior.

The amount of proteoglycan in the ILM (5-10% of total) was on average about 2.5 to 5 times the amount predicted solely from the volume of total tissue represented by the ILM, as estimated by measurements on fixed tissue sections (Table III-1). Because of the large amount of ILM that would have been required to analyze proteoglycans biochemically, we used ^{35}S-sulfate incorporation to estimate the relative amounts of HSPG and CS/DSPG. Under our conditions ^{35}S-sulfate probably had saturated all pools and had reached equilibrium in the proteoglycans. We labeled the retina tissues in vitro for 6 hrs, which is the time of half maximum labeling and the time of highest incorporation rate into retina proteoglycans in culture (unpublished observations by JEM).

In this study we confirmed that HSPGs are the major proteoglycans in the retina tissue overall. This finding is in good agreement with previous studies of extracted retina tissue (Morris, 1984; Morris et al., 1987), retina cell cultures (Burg & Cole, 1990) and purified cell cultures (Needham et al., 1988; Threlkeld et al., 1989). In addition we showed that HSPGs were the predominant proteoglycans in the ILM. The abundant HSPGs in the ILM may contribute to neurite outgrowth and orientation, since HSPGs have been implicated in retinal neurite extension (Matthew et al., 1985) and formation (Dow et al., 1988), and in retina cell-cell and cell-substrate adhesion (Cole & Glaser, 1986). In the latter instance, HSPGs may act in association with the neural cell adhesion molecule, which binds both HSPGs and laminin, constituents of the basal lamina (Cole et al., 1985; Cole & Glaser, 1986; Cole & Burg, 1989). Interactions between migrating

ganglion cell axons and experimentally modified ILM are being investigated to distinguish between these alternatives.

Fig. III-1. Scanning electron micrographs of a 11-day old chick neural retina with its inner limiting membrane partially lifted. A. Inner limiting membrane has been loosened and partially lifted. Arrow heads indicate the Müller cell endfeet and arrows indicate the stretched Müller cell fibers. B. Inner limiting membrane has been peeled away revealing the fasciculated ganglionic cell axons (GCA). Arrow heads indicate the Müller cell endfeet vesicles on the inner limiting membrane. IF, inner face of ILM; OF, outer face of ILM. C. Light micrograph of an isolated half ILM, fixed in methanol to prevent rolling. Bar=10μm in A and B; Bar=1 mm in C.

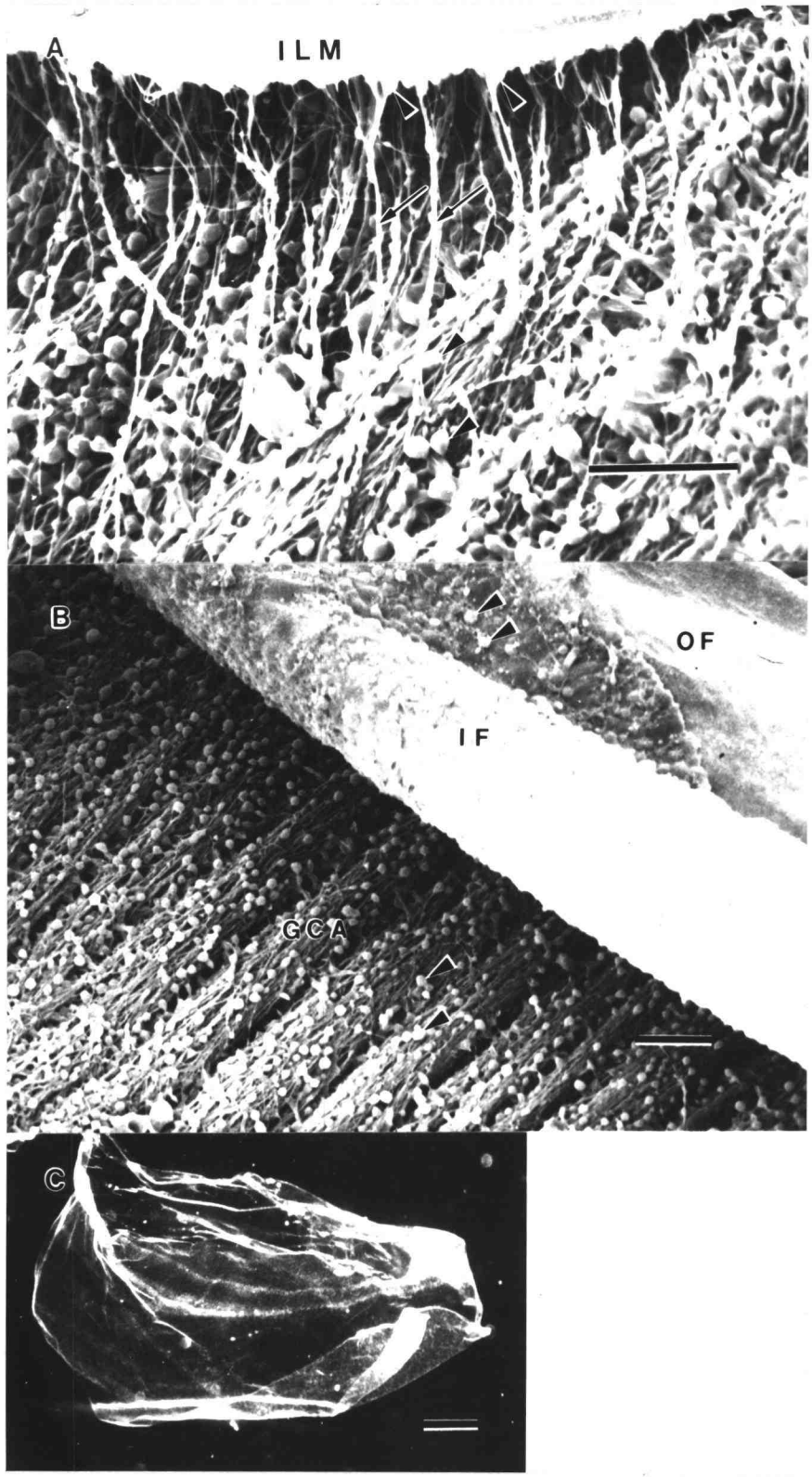


Fig. III-1 (Cont.)

Fig. III-2. Sephadex G-50 chromatography profiles of ^{35}S-sulfate labeled proteoglycans. Proteoglycans treated with nitrous acid (A) or chondroitinase ABC (B) before chromatography to digest, respectively, heparan sulfate or chondroitin/dermatan sulfate. A relatively greater proportion of the nitrous acid degradation product (included volume) is shown in the ILM (summarized in Table 1). (C) Untreated controls eluted entirely in the excluded volume. R-ILM, retina minus inner limiting membrane; WR, intact retina.

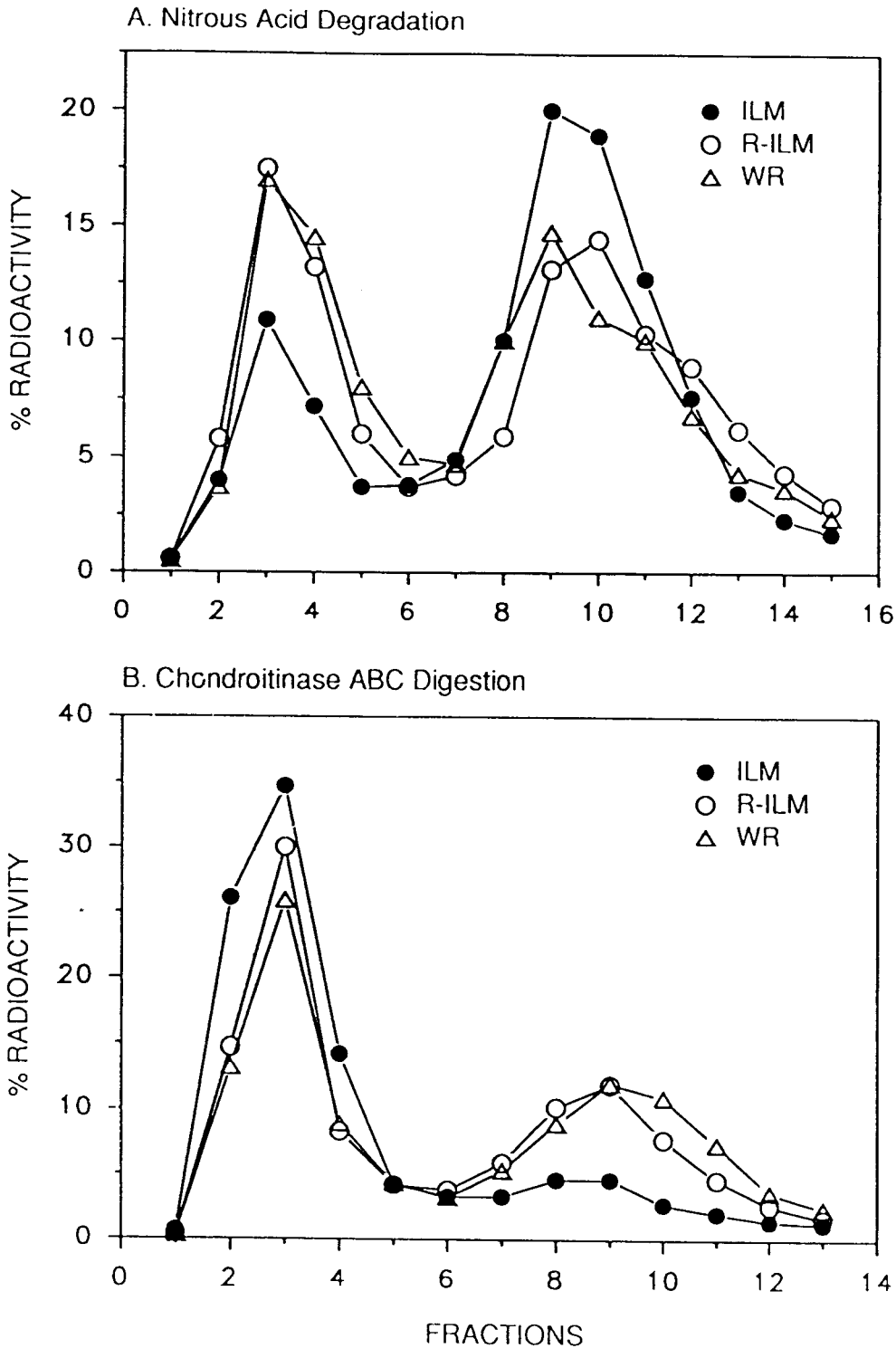


Fig. III-2 (Cont.)

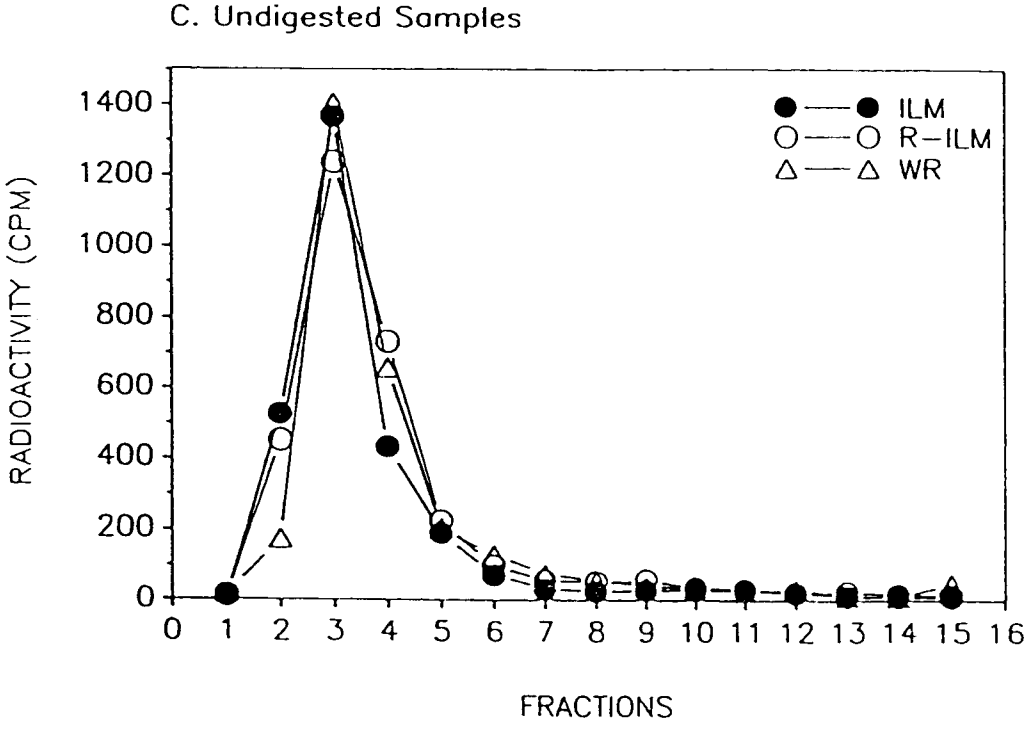


Fig. III-2 (Cont.)

Table III-1 Comparison of proteoglycans and volume occupied in each compartment

Subfraction	Amount of PGs (%)	Volume of tissue (%)*
ILM	5-10	1-2
WR	90-95	> 98

* The tissue volume was calculated from measurements of thin sections of the 10-day neural retina on a computer equipped with image analysis software (Brain™ from Drexel University). The plastic-sections were stained with methylene blue and photographed. Measurements were made on the images of the ILM and surrounding spaces between ganglion cell fascicles (the surrounding spaces represent the Müller cell endfeet) and of the rest of retina. Both measurements (ILM and rest of the retina) were then converted into percentages.

Table III-2. Distribution of Proteoglycans

	% of Total Proteoglycans*	
Subfraction	HSPG	CS/DSPG
ILM	64 - 78	20 - 35
R-ILM	50 - 56	42 - 48
WR	54 - 56	42 - 44

* After DEAE chromatography the isolated proteoglycans from the inner limiting membrane (ILM) fraction, remaining retina (R-ILM) and intact retina (WR) were either digested with chondroitinase ABC or hydrolysed with nitrous acid. After enzymatic digestion and chemical hydrolysis the samples were applied to G-50 chromatography columns to separate the digested products. Untreated aliquots of the proteoglycans (data not shown) were also chromatographed to establish a baseline for the chondroitinase and nitrous acid digests.

The percent of ^{35}S activity was calculated for three experiments. The total actual counts were $2.4x10^5$, $3.6x10^5$ and $9.6x10^4$ dpm before enzymatic digestion and chemical degradation.

CHAPTER IV. HEPARAN SULFATE IN THE INNER LIMITING MEMBRANE PROMOTES AXONAL OUTGROWTH OF EMBRYONIC CHICKEN NEURAL RETINA

Abstract

Proteoglycans are important extracellular molecules in enhancing neuronal cell adhesion, promoting neurite outgrowth (heparan sulfate proteoglycans), and inhibiting or repelling (chondroitin sulfate proteoglycans) neurite migration in *in vitro* systems. However, their functions *in vivo* are still not clear. In the present study, the inner limiting membrane (ILM) from embryonic chicken retinas was evaluated as a natural substrate on which to investigate the possible functions of these glycosaminoglycans on ganglion cell axon outgrowth. The ILM was modified by removal of specific glycosaminoglycans with enzymes. Neuronal cells or tissue explants from chicken embryonic retina were then allowed to grow on these modified substrata. We found that both growth rate and distribution density of the ganglion cell axons on the ILM was greatly inhibited by pre-treatment with heparitinase but not with chondroitinase ABC. This indicates that the neurite outgrowth promoting activity of the ILM depends on the heparan sulfate glycosaminoglycan side chains of the proteoglycans. Analysis of radioactively-labeled proteoglycans in the culture system confirmed that the action of heparitinase was on the ILM not the explant cells. Heparitinase acted on the

ability of the ILM to influence axonal behavior without apparent inhibition of cell adhesion. These results indicate that the neurite growth promoting activity depends on the heparan sulfate glycosaminoglycan side chains. The results using the natural substrate for neuronal cell neurite outgrowth support the conclusion of others using plastic or glass substrata that heparan sulfate proteoglycans (HSPGs) promote retinal neurite extension. We conclude that these molecules have a key role in normal axonal migration in early development.

Introduction

The developing nervous system is exquisitely dependent on environmental cues that guide neurons to their definitive positions. Among the best understood cues are cell-to-cell adhesion molecules (Edelman & Crossin, 1991) and laminin (Beck et al., 1990). Proteoglycans may also be involved in organization of the nervous system, but so far the evidence is more indirect. Proteoglycans play important roles in neuronal behavior during neuronal development in both central nervous system and peripheral nervous systems. HSPGs have been implicated in promoting cell proliferation (Ratner et al., 1988), neural cell adhesion to extracellular matrix constituents (Werz & Schachner, 1988), neurite extension (Matthew et al., 1985; Dow et al., 1988; Margolis, et al., 1991) and may form a complex with neural adhesion molecules (Cole & Glaser, 1986). Keratan sulfate proteoglycans (KSPGs) may block axonal migration specifically in the developing

neural tube (Snow et al., 1990) and chondroitin sulfate proteoglycans (CSPGs) may inhibit the outgrowth of the embryonic rat retina ganglion cell axons (Snow et al., 1991) and regulate neuronal patterning in the retina (Brittis et al., 1992). Proteoglycans and glycosaminoglycans may also control the polarity of neural cells of rat mesencephalon (Lafont et al., 1992).

The function of the proteoglycans has been studied mostly by applying proteoglycans on artificial, non-biological substrates, usually in culture ware or supplemented in the culture. This technique has certain advantages, for example, by using a single molecule in cultures, cell behavior can be studied without influences from other molecules. However, in nature, the environment surrounding cells is far more complex and cells usually encounter complex combinations of many molecules in their environment. Neuronal cells can send out more neurites and longer axons on a laminin substratum when HSPG is present than on laminin alone (Chiu et al., 1986). In no studies with basal lamina or matrix *in vitro* has it been possible to displace *in vivo* structural organization of these components (Kleinman et al., 1986). In order to determine whether the native organization of basal lamina components influences axonal outgrowth, we have explored a technique using a natural substrate, inner limiting membrane, from embryonic chicken retinas. In this study, we investigated the influences of both HSPGs and CSPGs on ganglion cell axon outgrowth.

In previous studies we used embryonic chicken retinas to quantitatively and qualitatively characterize retina proteoglycans (Chai et al., 1990). We found that

ILM from chicken retinas contains HSPGs as major components of glycosaminoglycans and chondroitin sulfates as minor glycosaminoglycans. In the present study, we used enzymatically-modified ILM as a substrate to investigate the influence of the proteoglycans on ganglion cell behavior. We found that both growth rate and density (neurites per unit area) of the ganglion cell axons on the ILM were greatly affected by heparitinase (HSase) but not chondroitinase (CSase). Our results suggest that the HSPGs but not CSPGs in the ILM are responsible for growth rate and density of GCA. Thus HSPGs appear to promote axonal extension rather than cell adhesion.

Materials and Methods

Materials

Polylysine was obtained from Sigma, and chondroitinase ABC (CSase) and heparitinase (HSase) were obtained from Sigma Chemical Co.. White horn and rode island red cross chicken embryos were obtained from the Poultry Science Division of the Animal Science Department at Oregon State University.

Tissue culture

Glass coverslips were scratched in the middle with a diamond pencil and sterilized. They were then immersed overnight in 0.5mg/ml of polylysine in distilled-deionized water and washed twice with balanced saline solution (BSS).

Retinas from chicken embryos were dissected free from pigmented epithelium and sclera and anchored on membrane filters with the vitreous side up. The vitreous body was rolled over the retinas to flatten them on the filter. Individual retinas were put on single polylysine-coated coverslips with inner limiting membranes (ILM) contacting the polylysine-coated surfaces (Halfter et al., 1987). Double-zero flask-rubber stoppers were placed on the filters to press retinas onto the polylysine-coated surface tightly. After 20 min the membrane filters with retinas were removed and the ILMs remained adhered to the coverslips. Any residual retina tissues were removed by a stream of BSS. The ILMs were treated with 2% Triton X-100 in water for 45-60 min, washed five times with BSS, and transferred to a new dish containing BSS for an additional 2-2.5 hr. The coverslips were then washed three more times and broken into two pieces by applying pressure to the scratches. One half was treated with specific enzymes or with monoclonal antibodies. The other half was placed in the same medium without any treatment as control. After treatment, the coverslips with inner limiting membranes were extensively washed and the two halves of the coverslip were recombined in a 35 mm plastic petri-dish as illustrated in Fig. IV-1. In order to assure that the two halves were flat, another half coverslip was put beneath these two half pieces. The inner limiting membrane-attached coverslips were immobilized by vacuum grease (VWR) on a petri dish. Six-, seven-, eight-, ten-, and thirteen-day old embryonic chicken retinas were dissected as above and then placed on 5μm PVDF membrane filter (Millipore), and then cut into 0.3 mm strips parallel with the optic fissure.

To facilitate comparison the retina explants used were from only 3 strips closest to the optic fissure . The strips were placed on the ILM across two halves of the coverslip with an amount of medium (D-MEM supplemented with 10% FBS and 100 U/ml penicillin and streptomycin) just sufficient to allow the meniscus to wet the inner limiting membrane. The explants were allowed to bind to the ILM for 2 hr and 2 ml more medium was carefully added. The retina strips were cultured in 5% CO_2 and 95% air environment for 24 hr, unless otherwise indicated in the text. Axonal outgrowth was compared between two halves of the coverslip (described below).

Enzyme digestion

Heparitinase Digestion: the ILM on the coverslip was incubated in heparitinase in BSS supplemented with 0.05 M sodium acetate with protease inhibitors (protease inhibitors used as before, see Chapter II) for 60 min at 37°C followed by extensive washes to remove excess enzymes.

Chondroitinase ABC Digestion: the ILM on the coverslip was incubated with 0.1 unit chondroitinase ABC in BSS with protease inhibitors (protease inhibitors used as before, see Chapter II) for 45-60 min at 37°C followed by extensive washes to remove excess enzymes.

Collagenase Digestion: the ILM was incubated in 0.25mg/ml collagenase in CMF (no protease inhibitors added) for 30 min at 37°C followed by extensive washes.

Trypsin Digestion: ILM was incubated in 0.25 mg/ml crystalline trypsin in CMF (no protease inhibitors added) for 30 min at 37°C followed by extensive washes.

Cell culture

Single cells were prepared essentially as before (Morris, 1976). Six or seven day-old (E6 or E7) embryonic chicken retinas were dissected free from pigmented epithelium. They were preincubated in CMF/Mops (Mops buffered calcium and magnesium free buffer) at 37°C on a shaker at 50-75 rpm for 10 min and trypsinized in CMF at a concentration of 2.5 mg/ml at above condition for 10 min. The retinas were then washed three times with D-MEM supplemented with 10% FBS and 100 U/ml penicillin and streptomycin, and transferred to F12 nutrient mixture supplemented with insulin (5mg/ml), sodium selenite (30 nM), iron-saturated conalbumin (25 μg/ml), and 100 U/ml penicillin and streptomycin (Hall, D. E. et al., 1987). They were dissociated into single cells. The retina cells were preplated in tissue culture petri dish at 37°C in a 5% CO_2 atmosphere for 45 to 60 min to remove rapidly attaching nonneuronal cells. At the end of the preplating step, the supernatants containing neuronal cells were harvested and the cell numbers were adjusted. About 2-5 x 10^5 cells/ml were plated in petri dishes containing coverslip-mounted modified ILM (see tissue culture section). The cells were cultured for different time periods in F12 nutrient mixture with insulin,

sodium selenite, conalbumin, and penicillin-streptomycin in 5% CO_2 atmosphere at 37°C.

Histology

Silver staining

Retina explants were fixed in 4% paraformaldehyde in CMF or Bodian's fixative overnight and stained by the Golgi method as modified by Rager et al., (1979) for embryonic tissue.

Toluidine blue staining

Retina explants or cultured retina cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 30 to 60 min at room temperature, washed 3 times with PBS and stained with toluidine blue (0.2% in 1% Na_2CO_3) as described (Lafont et al., 1992).

Neurite outgrowth assays

Neurite density assays: The explants were fixed and stained with toluidine blue and examined by phase contrast. Because the axons grew from the retina strips in parallel bundles, the density of axons was determined by counting the numbers of axonal bundles intersecting a 2 mm line parallel to the strip. Although there was a range in number of axons per bundle in any one explant, there is no

evidence that the number of axons per bundle was different under different experimental conditions.

Neurite growth rate assays: The outgrowing explants were photographed in culture every 2 hr. The lengths of the axon bundles were measured on the photographs.

Cell survival assay

Retina cells were dissociated and cultures as described in the cell culture section. The cell survival assay of Lafont et al. (1992) was conducted with some modifications. In brief, the number of live cells was determined from five independent fields of either control or enzyme treated ILM at time zero by the trypan blue exclusion method.

Cell adhesion assay

Retina cells were plated at 2-5 x 10^5 cells/ml per 35-mm petri dish which contained coverslip-mounted, enzyme-treated ILM and control ILM. After 1 hr at 35.5°C, non-adherent cells were gently washed away (Keilhauser et al., 1985). The cells were fixed with 4% paraformaldehyde for 15 min and counted under a microscope.

Results

Axonal behavior varies with age of the axon, not age of the ILM

In vertebrate retina the ganglionic cell axons are in direct contact with the ILM, on which they send out their axons to form the optic fibers that innervate the optic tectum. At about 2 to 3 days of incubation ganglion cells of chicken embryonic neural retina start to send out their axons, which migrate towards optic fissure. At about 5 to 7 days of incubation internal ends of the Müller cells (retinal glia) starts to produce the ILM (Romanoff, 1960). In the retina of the 8-day chicken embryo, most cells closest to the ILM have already differentiated into ganglionic cells except those around the lens. The first wave of the optic fibers reaches optic tectum of the brain at about 8 days of incubation. Most fibers have already reached and formed connections with the optic tectum in 10-day old embryos. At 12 to 13 days of incubation all ganglionic cells have differentiated, and there are no new optic fibers are extending to the optic tectum. We have investigate whether the age-dependent difference in axonal behavior is a function of changes in the ILM. Six-, eight-, ten-, and thirteen- or fourteen-day old retina explants were grown on seven- or eight-day old ILM (Fig. IV-2). Retina ganglion cells usually migrated out of the six- and seven-day old chick retina explants and sent out their axons while those of older retina explants remained within their explants (Fig. IV-2). Axons from six- and seven-day old retina explants fasciculated, making the density of individual fibers lower than that of the less

fasciculated eight-day explants. The axons from ten-day old retina explants were not fasciculated and also had a lower density. The 13-day old explants were also not fasciculated and had the lowest density.

Density of the axons from explants was influenced by age of the explants. The explants of neural retina from 6-day old donors formed relatively by dense axons (Fig. IV-2A). Eight-day old explants showed very dense axons (Fig. IV-2B). Ten-day old retina explants showed very low density axons (Fig. IV-2C). Thirteen day old explants had only few fibers from retina explants (Fig. IV-2D). When ILMs from donors of different ages were used as substrata, the axonal density of explants from the same age did not show any difference (Fig. IV-3A) . Thus, the density of retinal axons varied with the age of the tissue explants not with the age of the substrata, ILM.

The growth rate of axons was also affected by the age of the explants. The six-day old retina explants showed the highest growth rate (about 1 mm within 24 hr). The eight-day old retina explants showed a moderate growth rate (about 0.7-0.8 mm within 24 hr). The ten-day old retina explants showed a very slow growth rate (about 0.4-0.5 mm within 24 hr). Thirteen- and fourteen-day old retina explants showed the lowest growth rates on the ILM. When explants from the same ages of retinas grew on different age of ILMs, they did not appear to be different in their axonal growth rate (Fig. IV-3B).

Heparitinase-treated ILM does not support axonal outgrowth in 6-day explants

Figures IV-4 and IV-5 show that explants of 6-day and 13-day old chick retina grew on enzymatically modified 8-day old ILM. When 6-day old explants grew on the ILM that had been treated with heparitinase, both axonal density and growth rate were greatly reduced (Fig. IV-4 and IV-5). Neither axonal density nor growth rate of 13-day old retina explants were affected on the ILM treated with heparitinase. Chondroitinase did not have any effect on either axonal density or growth rate of either 6- and 13-day old retina explants (Fig. IV-6).

Removal of heparan sulfate from ILM by HSase does not affect cell survival

To find out if removal of the HSPGs in the ILM with heparitinase could cause cell death, we examined survival of cells from 6-day retinas on the substrate in the culture after 6, 16, and 24 hr by using trypan blue exclusion method. Cell survival on the ILM was not affected by pre-treating the substrate with heparitinase (Table IV-1).

HSPGs of Müller cell endfeet do not affect axonal growth

In an earlier study (Chapter II and III) we have shown by both autoradiography and immunohistochemistry that HSPGs are mainly located in the ILM but not on the Müller cell endfeet. In order to confirm that the influence of HSPGs is of HSPGs in the ILM not on Müller cell endfeet, we investigated the behavior of 6-day cells on both Müller cell endfeet and ILM HSPGs. By treating

with both detergent and enzyme we found that the reduction of neurite density and growth rate was affected only by heparitinase-treatment after removal of Müller cell endfeet (Table IV-2).

The action of HSase is on the ILM not cell membrane HSPGs

It is possible that the effect of the HSase in Fig. IV-4 and IV-5 was due not to the removal of HS but to residual heparitinase acting on the cell surface proteoglycans. To test for residual activity we incubated radioactive-labeled retina proteoglycans in cultures which contained either heparitinase-treated or non-treated ILM for 20 hours. After 20 hours incubation no degradation of the proteoglycans could be detected by Sephadex G-50 chromatography (Fig. IV-7).

HSPGs in the ILM act neurite-promoting molecules rather than adhesion molecules

In order to investigate whether the action of HSPGs in the ILM on axonal growth rate or density was due to an influence on cell adhesion, we cultured dissociated, single, 6-day retinal neuronal cells on heparitinase-treated, chondroitinase-treated, or untreated ILM. After 1 hour at 37°C, we gently washed the cell cultures to remove nonadhering-cells and loosely attached cells and calculated the number of cells on the substrate. The numbers of cells adhering to heparitinase-treated or chondroitinase-treated ILMs were not affected when compared to those on the untreated ILM (Fig. IV-8). This result suggests that HSPGs in the ILM did not influence axonal growth by affecting adhesion to the

ILM. On the other hand, as was the case with the influence of HSase-treated ILM on explants, neurite outgrowth was reduced in the single cells. The number of the cells bearing neurites greater than two cell body diameters were greatly reduced on the heparitinase-treated ILM but not chondroitinase-treated or untreated ILM (Fig. IV-9). Cells on the control ILM (Fig. IV-10A) or chondroitinase-treated ILM (Fig. IV-10B) sent out long neurites (usually greater than two to five cell body diameters), and cells on the periphery of aggregates, when cultured on an ILM, also sent out long neurites (Fig. IV-11A). By contrast, cells on heparitinase-treated ILM usually sent out short neurites (smaller than two cell body diameter) (Fig. IV-10C) and peripheral cells of aggregates sent out short neurites (Fig. IV-11B).

Discussion

In this study, we have used the ILM as a natural substrate to investigate possible roles of proteoglycans on the outgrowth of chicken embryonic retina ganglion cell axons. The ILM was first used as a substratum for neuron culture by Henke-Fahle et al., (1984) and then adopted by Halfter et al., (1987) to study behavior of neuronal cells on the substratum. The ILM has proven to be an excellent natural substrate on which transplanted neuronal cells can grow and migrate (Halfter et al., 1987; Nakayama et al., 1989) without any cellular or axonal contamination (see Chapter III, Chai and Morris, 1990).

Most functional studies on ECM molecules still rely on the technique with which one or two ECM molecules are coated on plastic petri dishes or applied to an in vitro culture system. The limitation of such techniques is that the architecture of applied molecule or molecules usually is unnatural for cells and, therefore, it is unreasonable to expect the cells to function normally. In nature, however, cells are usually exposed to a far more complicated environment in which many different molecules are interacting together. In this report we used ILM as a substrate, modified it with selective enzymes, and studied neuronal cell behavior on it. This technique allow us to investigate influences of proteoglycans on neuronal cell migration in a more natural environment.

Axonal outgrowth depends on the age of explant not ILM

In our first set of experiments, we demonstrated that the age of ILM did not affect the axonal outgrowth, but outgrowth varied with the age of the retinal explants. With increase in age of explants, both density and growth rate of axons from the explants were decreased. These results suggest that the ability of axonal outgrowth solely depends upon the age of the tissue explants but not age of the ILM. These indicate that the ILM is not a determinant, but rather, a permissive factor for maximum axonal outgrowth. The optimal axonal outgrowth of explant age was between day 6 and day 8, as shown by their maximum axonal density and growth rate (Fig. IV-2, 3) on the ILMs of all ages we tested. We also found, in our second set of experiments, that heparitinase-treated ILM did not affect axonal

outgrowth of 13-day old chick retina explants when compared with control ILM, even though the enzyme did reduce axonal outgrowth of 6-day old explants. The decrease of axonal outgrowth with increase of the age of the explant on the ILM, or loss of responsiveness of older neural explants on the heparitinase-treated ILM, may possibly be due to three reasons. First, one or more molecules, for instance HSPGs, laminin or other neurite promoting factors, to which the retinal ganglion cells respond, have been lost from the ILM. However, the losses of responsiveness of retina ganglion cells to the HSPGs in the ILM can not account for the loss of these molecules from the substratum, since the HSPGs persistently exist in all ages (4 day old to 14 day old retinas) when we used immunohistochemical techniques with a basal lamina HSPG monoclonal antibody (Chapter II). Also laminin is found in older chick retina (Halfter et al., 1987). Second, the ability of older neurons to regenerate their neurites has been lost. However, our results have shown that older chick retina axons are able to regrow after they were removed from ganglionic cell bodies (Appendix A). Other studies have also demonstrated that rat retinal ganglionic cells are able to regrow neurites when cultured on some substrates (Leifer et al., 1984; McCaffery et al., 1984). Furthermore, adult rat retinal ganglionic cells are also able to regrow into sciatic nerve grafts when optic nerves are transacted (So and Aquayo, 1985). Third, the substrate requirements of the neuronal cells have changed. It has been found that day 11 neuronal retinal cells are able to extend neurites when cultured on monolayers of astrocytes, even though they are not able to do so on laminin

substrata. The lack of neurite extension on matrix substrates does not reflect a loss of the ability to extend neurites (Cohen et al., 1986). The older retina neuronal cells in single cell culture have also been found to lose the ability to extend their axons on either collagen gels or laminin substratum (Hall et al., 1987). Developmental changes in the responsiveness of ciliary ganglion neurons to substrates coated with heart cell-conditioned medium with age have also been shown (Collins and Lee, 1982).

Influences of glycosaminoglycans on axonal outgrowth of chicken embryonic retina

In our second set of experiments, we used split-coverglass substrata to determine the possible effects of proteoglycans in the ILM on the behavior of the chick retinal ganglion cells. This technique permitted us to directly compare two different treatments of the substrata on which retina ganglion cell explants or neuronal cells grew under the same conditions. Our results showed that heparitinase-treated ILM without pre-removal of Müller cell endfeet did not affect either growth rate or axonal density. However, if we removed the Müller cell endfeet prior to selective enzymatic treatment, the heparitinase greatly reduced the axonal growth rate and density. These results suggest that it is the heparan sulfate in the ILM that was directly involved in outgrowth behavior of ganglionic cell axons. Reduction of axonal density and growth rate on the heparitinase pre-treated ILM might possibly be due to cell death caused by lack of heparan sulfate in the ILM. Alternatively, residual heparitinase left on the ILM acted on cell membrane-

bound heparan sulfate glycosaminoglycans and in turn affected axonal outgrowth on the ILM. However, our results did not support these possibilities.

If, however, the heparan sulfate glycosaminoglycans were strong cell adhesion molecules, then one would expect that removal of the heparan sulfate from the ILM would reduce the adhesion of the neuronal cells to the ILM. To test whether the neurite promoting behavior of the heparan sulfate in the ILM was due to an adhesion property of this molecule, we treated ILM with heparitinase and then cultured single cells on these substrata. In this study, we detected only a slight loss (less than 3%) of cell adhesiveness to the enzymatically treated ILMs when compared with non-treated ones. Therefore, there was no reason to speculate that heparan sulfate glycosaminoglycans in the ILM are merely adhesion molecules. With this slight loss of cell adhesion to the enzymatically treated ILM, reduction of both axonal density and growth rate can not only be accounted for by only the adhesion property of the HSPGs. Our experiments do not rule out the possibility that removal of heparan sulfate glycosaminoglycans resulted in a loss of undemonstrated adhesive interactions that could play a role in axonal extension. So far we are unable to eliminate this possibility. However, *in vitro* studies have shown that heparan sulfate glycosaminoglycans do not promote neuronal cells adhesion when they are coated on dish as a substrate even though heparan sulfate can block adhesion of neuronal cells to laminin (Werz & Schachner, 1988) or enhance neurite extension (Dow et al., 1988). It is unlikely that heparan sulfate glycosaminoglycans act solely as adhesion molecules in this case. On the other

hand, if we cultured the cells for a longer period of time (16 hr), we found that more than twice as many cells send out neurites greater than two cell body diameters on the control ILMs than those on the enzymatically treated ones. These results suggest that heparan sulfate glycosaminoglycans may act more directly as neurite promoting molecules, perhaps through their ability to act as receptors for growth factors (Cirillo et al., 1990; Fayein et al., 1990b; Soubrane et al., 1990).

We did not detect any effect of chondroitin sulfate in the ILM on either growth rate or axonal density in our system, even though it has been found to influence neurite growth in other *in vitro* systems (Snow et al., 1990, 1991; Snow & Letourneau, 1991). CSPGs may promote neuronal cell aggregation by steric exclusion (Morris, 1979, 1993). Recent findings have indicated that CSPGs *in vivo* and applied to surfaces *in vitro* can inhibit axonal growth and repel elongating chick retina ganglion axons (Snow & Letourneau, 1991; Snow et al., 1991). The most recent finding also shows that CSPGs are correlated with axon boundaries in the developing central nervous system and the gradual regression of these molecules may help control the onset of ganglion cell differentiation and initial direction of the their axons (Brittis et al., 1992). This suggests that these molecules affect neural pattern formation (Brittis et al., 1992). If CSPGs influence cell migration by steric exclusion (Morris, 1993), they would have to be highly concentrated in order to effectively repel elongating axons. In a previous study (Chai et al., 1990), we demonstrated ^{35}S-sulfate-labeled CSPGs in the ILM. CSPGs have also been localized in the basal lamina in several tissues (Yurchenco

& Schittny, 1990) and, at least in one case, in the ILM of rat retina by immunostaining technique (Aquino et al., 1984a), but nothing is known about their functions in these sites (reviewed by Yurchenco and Schittny, 1990).

In conclusion, the major findings in this report show that both density and growth rate of ganglionic cell axons from 6-day retinas can be reduced on the ILM when it is pretreated with heparitinase, but chondroitinase has neither a positive nor negative effect on this cell behavior. We have eliminated gross effects of heparan sulfate on cell adhesion as a mechanism for its influence on axons, and the results cannot be explained by direct action of residual heparitinase on the cells. We suggest that the influence of HSPG may be attributable to its ability to sequester and modulate, and act as reservoir for bFGF (Wagner & D'Amore, 1986; Walicke et al., 1986; Neufeld et al., 1987; Damon et al., 1988; Cirillo et al., 1990; Fayein et al., 1990b)

Fig IV-1. Flow chart of the experimental protocol.

(1) Glass coverslips were scratched in the middle and autoclaved. They were then incubated with polylysine overnight and washed twice with BSS. Chicken embryonic retinas were anchored on membrane filters with the inner limiting membrane (ILM) side up.

(2) Each retina was transferred to a polylysine-coated coverslip with the ILM contacting the polylysine-coated surface. After 20 min the membrane filter with attached retina was removed by a stream of medium and the ILM was left bound to coverslip. The ILM was treated with 2% Triton X-100 in water, extensively washed five times with BSS, and left in the BSS for an additional 2 to 2.5 hours.

(3) The coverslip was then broken into two pieces by applying pressure to the scratch. One half was treated with specific enzymes. The control half was treated in the same medium but without any chemical modifications.

(4) After treatment, the two halves of the coverslip were recombined in a 35 mm plastic petri-dish and immobilized on the dish by silicone vacuum grease.

(5) and (6) A 0.3 mm-wide strip of chicken retina was placed on the ILM across two halves of the coverslip in a minimum volume of medium. It was allowed to bind to the ILM for 2 hours. More medium was then carefully added, and the retina strip was cultured in 5% CO_2 environment for 16-24 hours. In some cases dissociated single cells were used instead of tissue explants. The axonal outgrowth on the two halves of the coverslip was compared.

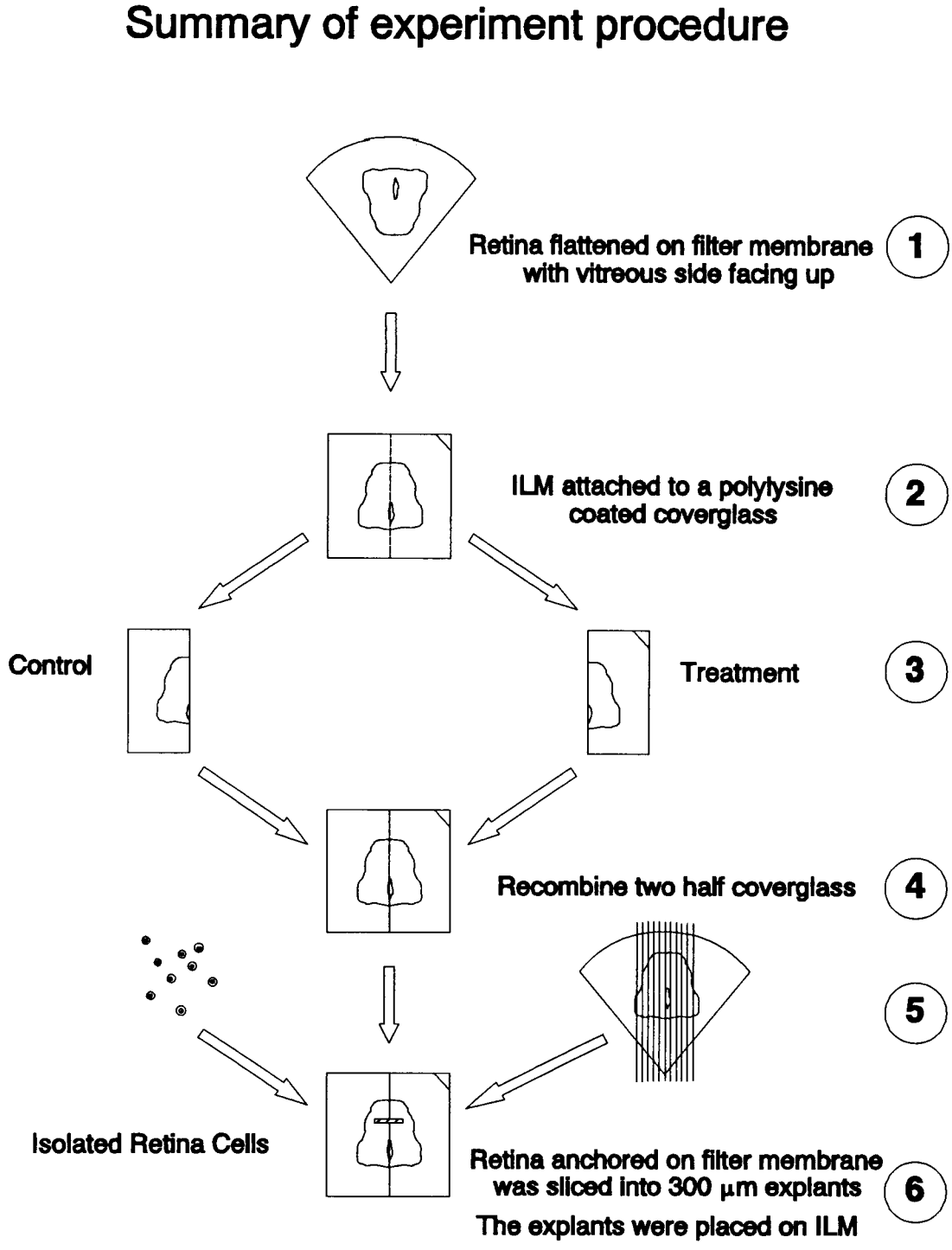

Summary of experiment procedure
Retina flattened on filter membrane with vitreous side facing up
1
ILM attached to a polylysine coated coverglass
2
Control
Treatment
3
Recombine two half coverglass
4
5
Isolated Retina Cells
Retina anchored on filter membrane was sliced into 300 μm explants
The explants were placed on ILM
6

Fig. IV-1 (Cont.)

Fig. IV-2. Micrographs of explants of different ages cultured on inner limiting membrane. (A) Six-day old retina explant; (B) Eight-day old retina explant; (C) Ten-day old retina explant; and (D) Thirteen-day old retina explant growing pattern on 8-day old inner limiting membrane after 24 hr in culture. (Explants showed a similar pattern on 7-, 10-, and 13-day old inner limiting membrane (Figures not shown)). Arrows indicate the ganglionic cell bodies. Bar = 100 μm

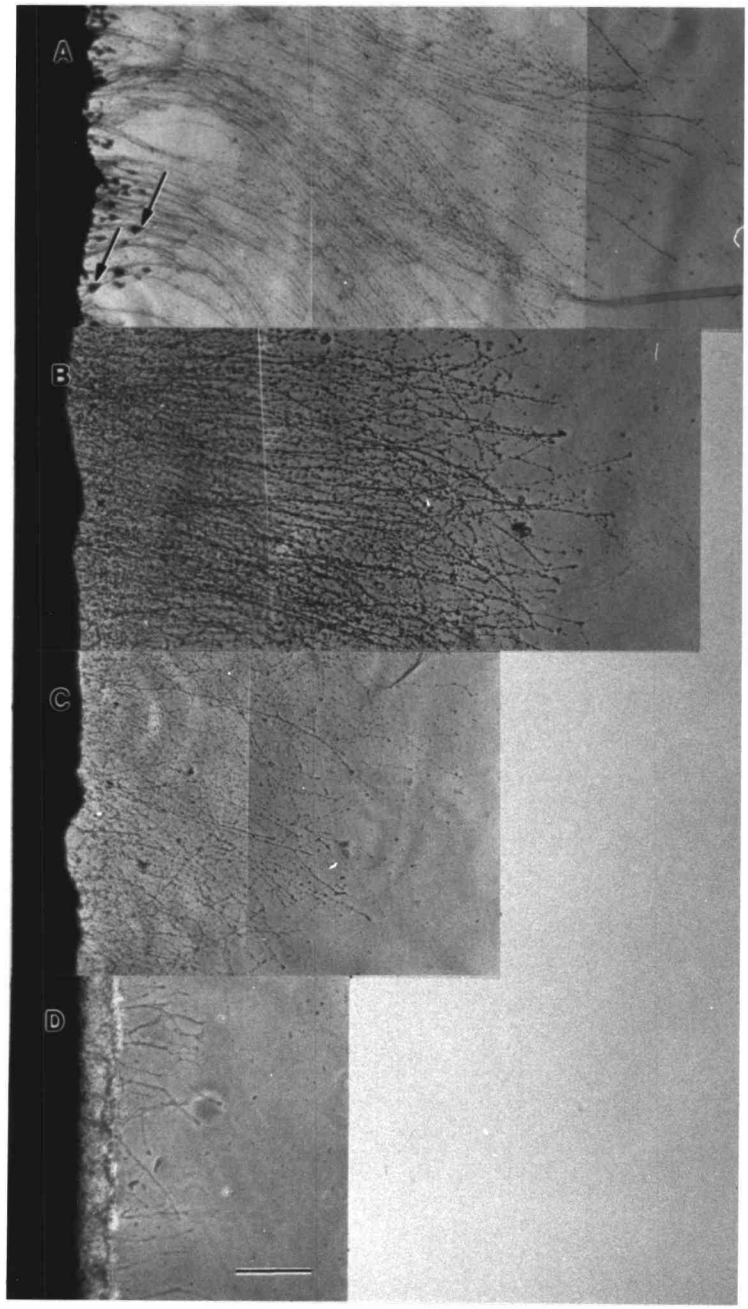


Fig. IV-2 (Cont.)

Fig. IV-3. Axonal behavior of different ages of explants on different ages of inner limiting membrane. Strips of retina from 6-, 8-, 10-, and 13-day old chicken embryos were transplanted on inner limiting membranes from the same or different age retinas. Both density (A) and growth rate (B) of ganglion cell axons decreased with age of the retina explants but not with age of the ILM. Bars show mean, $\pm$ standard deviation, $n = 5$

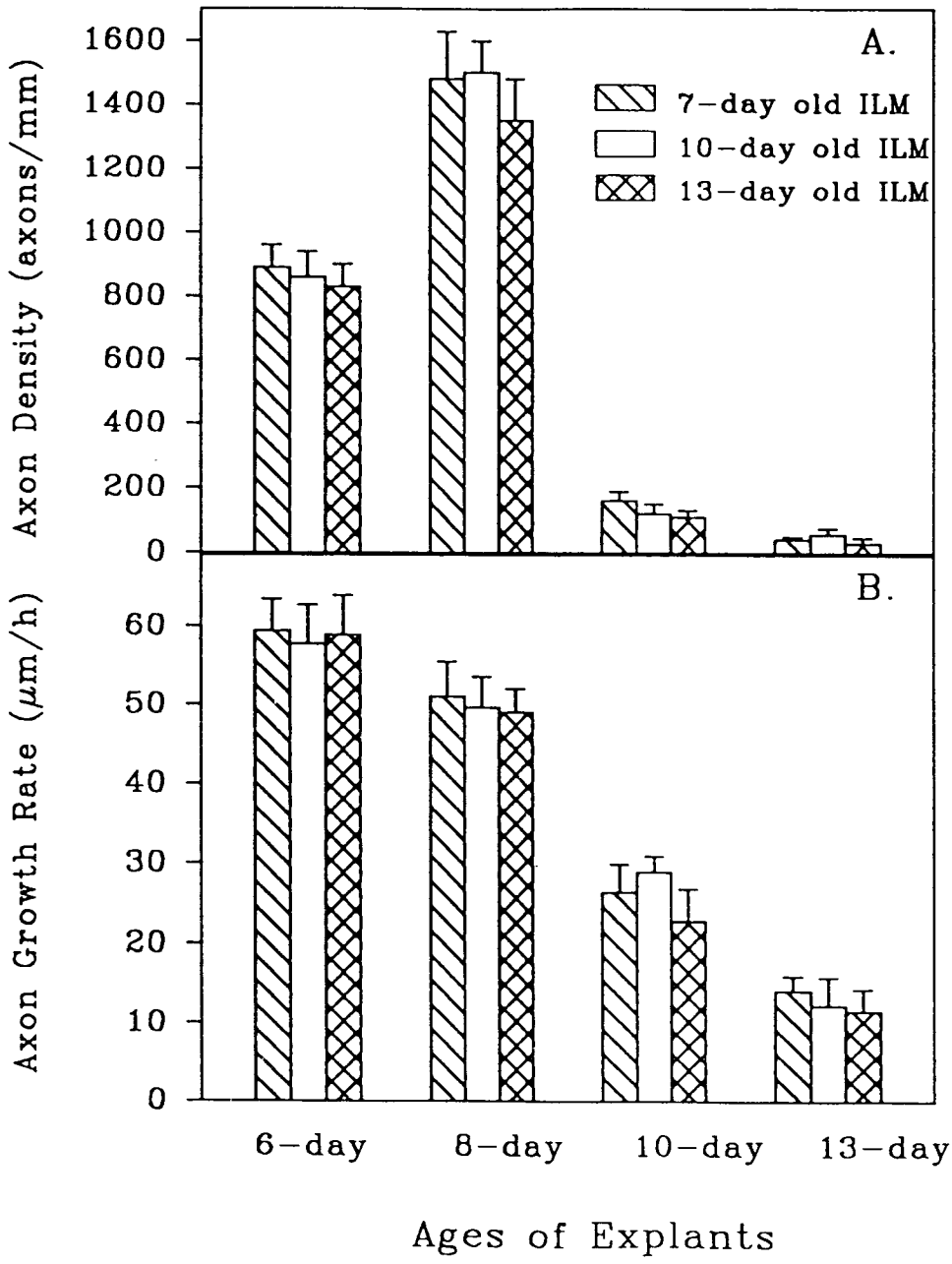

A.
7-day old ILM
10-day old ILM
13-day old ILM
Axon Density (axons/mm)
1600
1400
1200
1000
800
600
400
200
0
B.
Axon Growth Rate (μm/h)
60
50
40
30
20
10
0
6-day
8-day
10-day
13-day
Ages of Explants

Fig. IV-3 (Cont.)

Fig. IV-4. Micrographs of growth pattern of 6-day old embryonic chicken explant on the heparitinase-treated inner limiting membrane. (A) Axons from the explant on the control ILM (left) show normal outgrowth, whereas both axonal extension and density of the explant on the HSase-treated ILM are inhibited (right). Arrow points to split between two halves of the coverslip. (B) A higher magnification of axonal outgrowth on a control ILM and (C) on a HSase-treated ILM. Bar = 200 μm in A; Bar = 50 μm in B and C

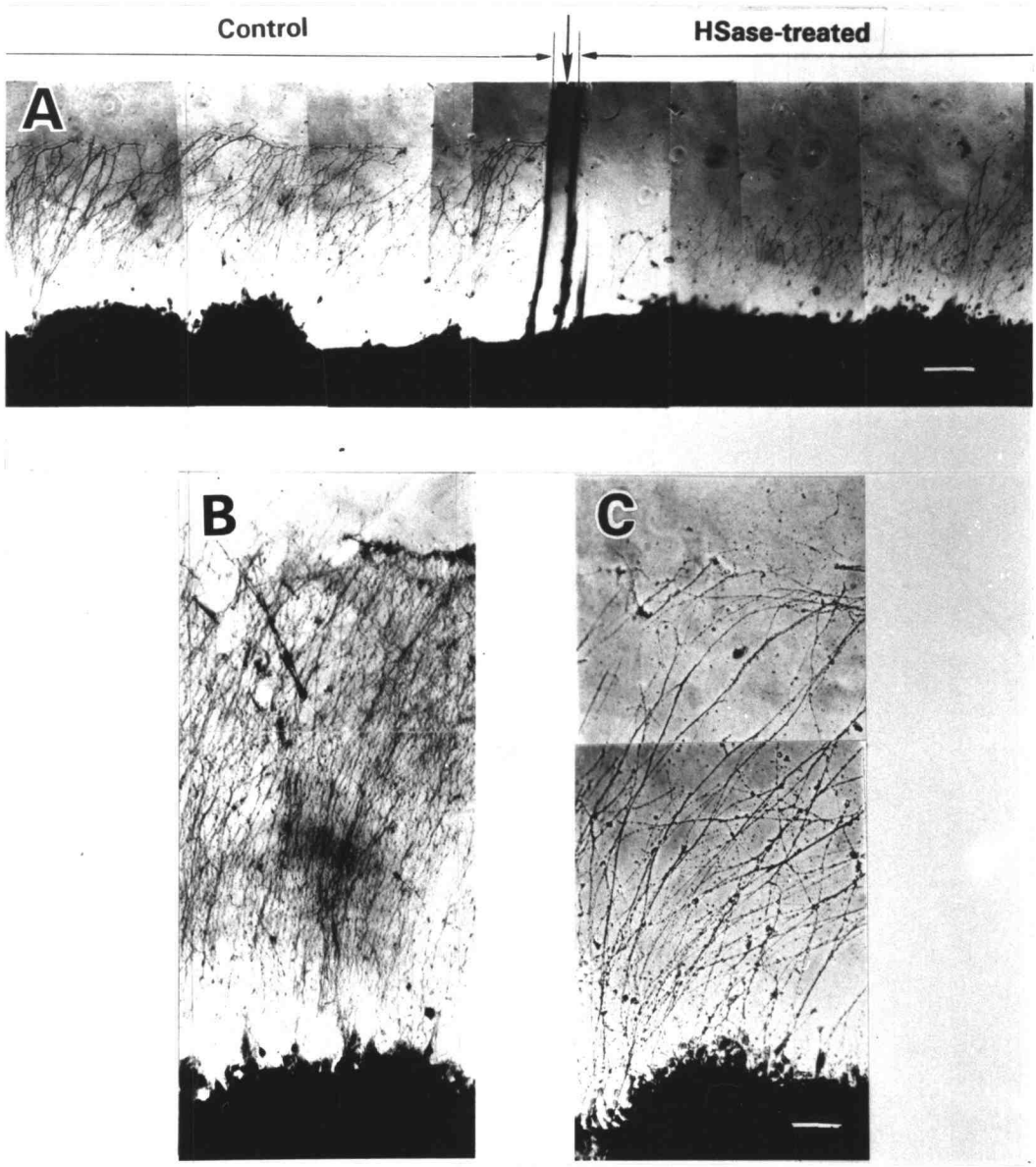


Fig. IV-4 (Cont.)

Fig. IV-5. Effects of heparitinase on axonal outgrowth of explants on inner limiting membrane. One half of the ILM was digested with HSase for 45 to 60 min and the other half was treated with medium as a control. The axonal density (A) and growth rate (B) of 6-day explants were significantly reduced by the enzyme treatment. However, neither axonal density (A) nor axonal growth rate (B) of 13 day-old explants was affected. Bars show mean, $\pm$ standard deviation, $n = 5$

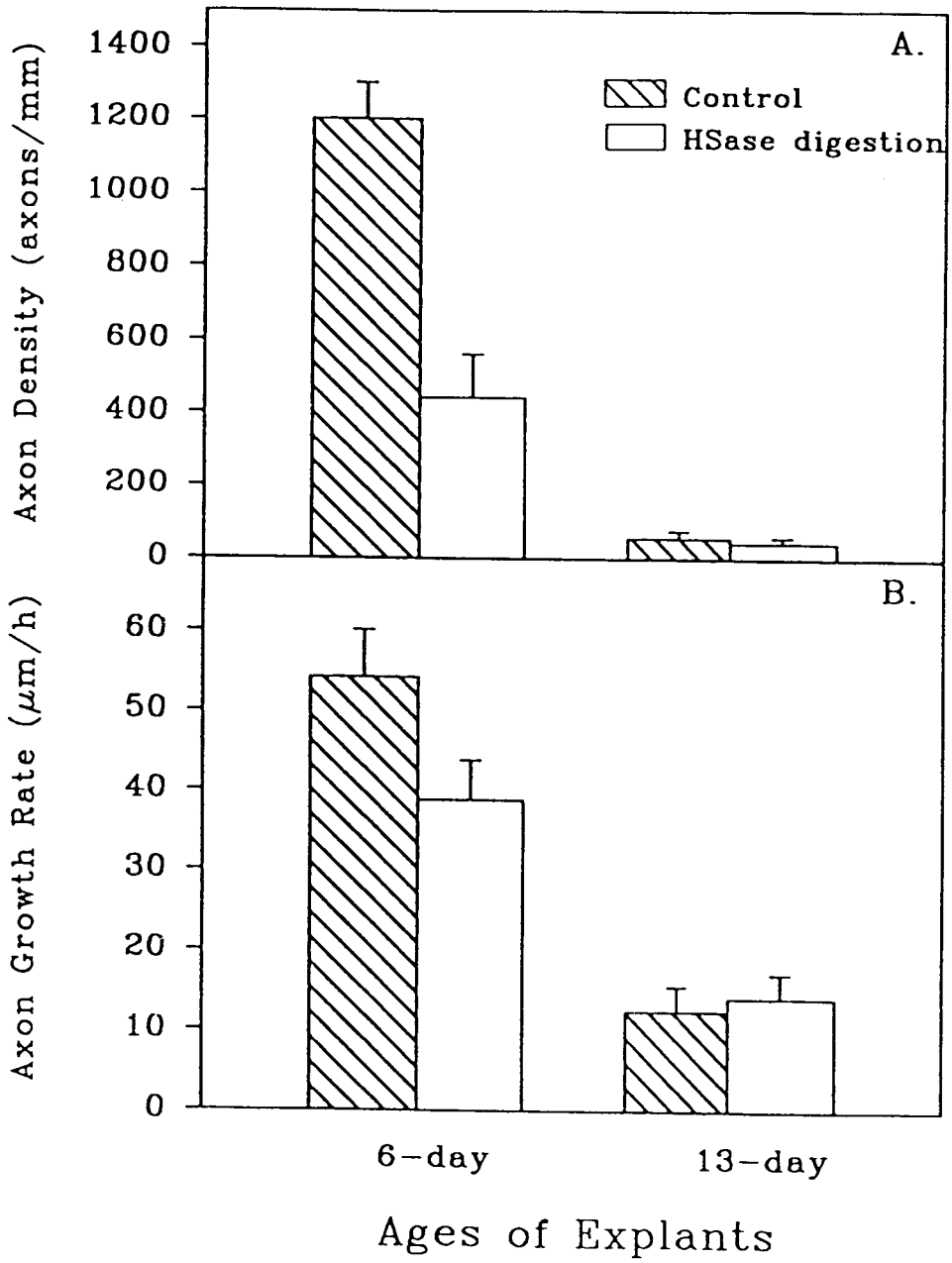

A.
Control
HSase digestion
Axon Density (axons/mm)
1400
1200
1000
800
600
400
200
0
B.
Axon Growth Rate (μm/h)
60
50
40
30
20
10
0
6-day
13-day
Ages of Explants

Fig. IV-5 (Cont.)

Fig. IV-6. Effects of chondroitinase ABC on axonal outgrowth of explants on inner limiting membrane. One half of the ILM was digested with CSase ABC for 45-60 min and the other half was treated with medium only as a control. Neither axonal density (A) nor axonal growth rate (B) of ether 6 day-old or 13 day-old retina explants on the enzyme-treated ILM were significantly inhibited by the enzyme treatment. Bars show mean, $\pm$ standard deviation, $n = 3$

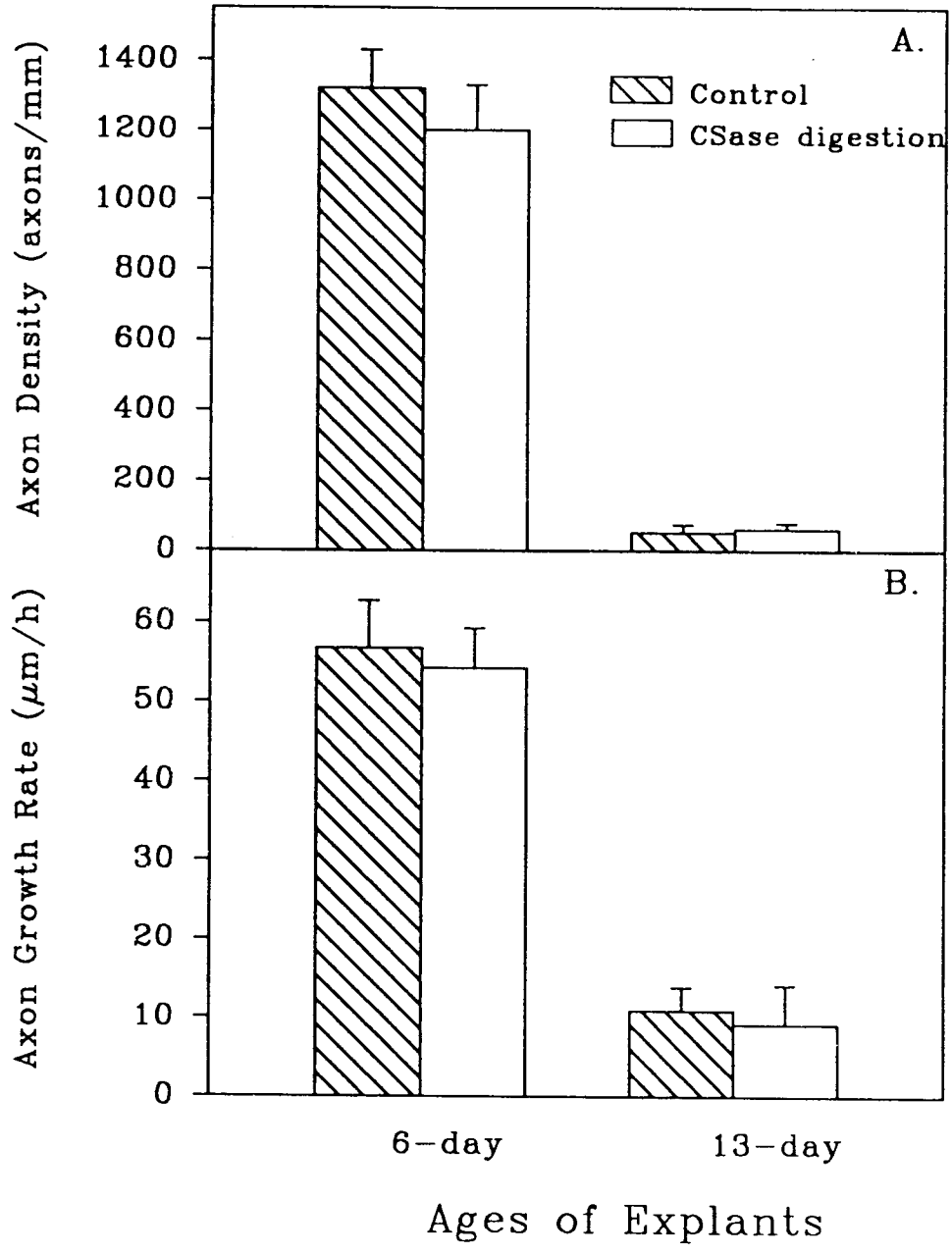

A.
Control
CSase digestion
Axon Density (axons/mm)
1400
1200
1000
800
600
400
200
0
B.
Axon Growth Rate (μm/h)
60
50
40
30
20
10
0
6-day
13-day
Ages of Explants

Fig. IV-6 (Cont.)

Fig. IV-7. Sephadex G-50 chromatography profiles of ^{35}S-sulfate labeled proteoglycans. To ensure that the inhibitory effect of HSase on axonal growth was not due to possible residual enzyme left on the inner limiting membrane (ILM), which might act on ganglion cell surface HSPGs, the same conditions as for cell culture were set up, but rather than retina explants or cells being placed on the ILM, ^{35}S-sulfate-labeled proteoglycans were incubated with the ILM. After 20 hr incubation at 37°C, the media from both HSase-treated and nonenzyme-treated incubations were applied to Sephadex G-50 columns. (A) The Sephadex G-50 chromatography profile of proteoglycans before incubation. (B) A similar profile between samples in heparitinase-treated (closed square) and control cultures (closed triangle) is shown. Degradation products, had they been present, should have appeared in fractions 7-9. This result indicated that no residual active HSase was left on the ILM.

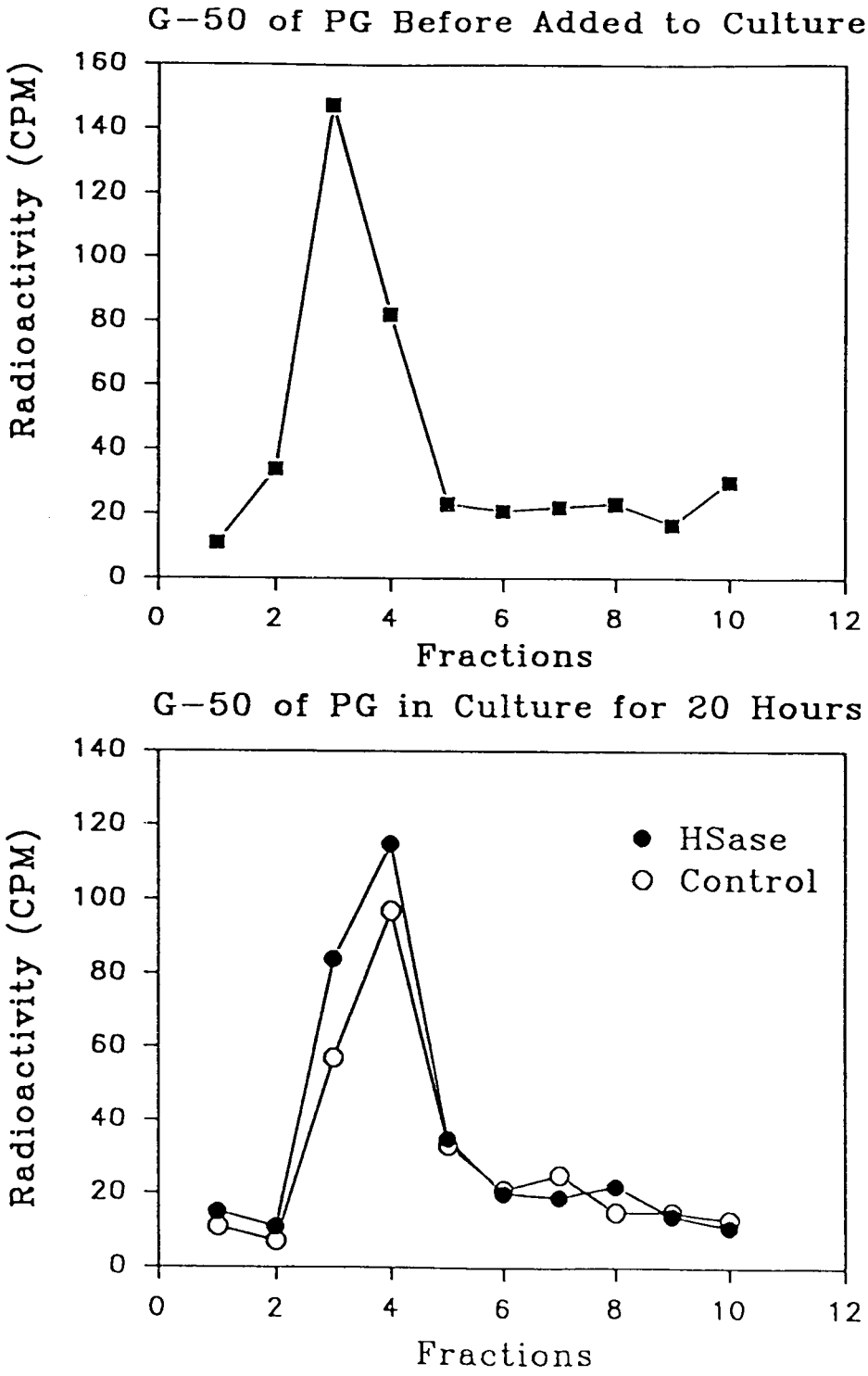

G−50 of PG Before Added to Culture
Radioactivity (CPM)
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Fractions
G−50 of PG in Culture for 20 Hours
Radioactivity (CPM)
140
120
100
80
60
40
20
0
HSase
Control
0
2
4
6
8
10
12
Fractions

Fig. IV-7 (Cont.)

Fig. IV-8. Effect of heparitinase or chondroitinase on neuronal cell adhesion to inner limiting membrane. Cells were placed on ILMs pretreated with HSase or CSase. The number of non-adhering cells was compared with the total number of cells placed in culture. The percentage of cells adhering to the ILM was not affected by either enzyme. Bars show mean, $\pm$ standard deviation, $n = 3$

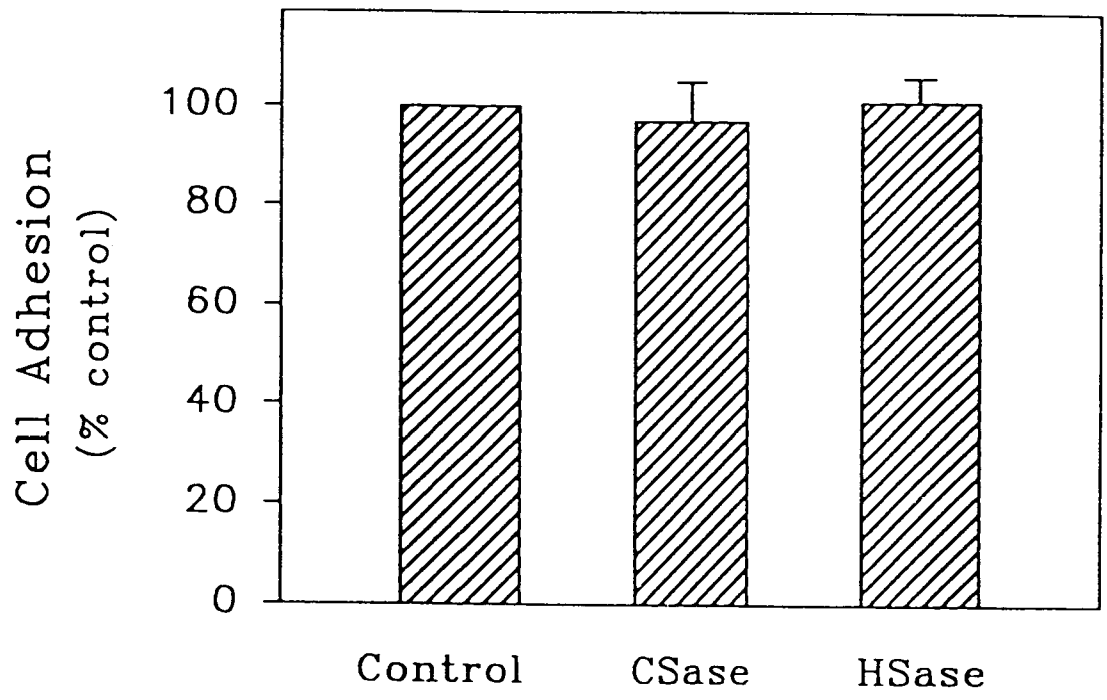


Fig. IV-8 (Cont.)

Fig. IV-9. Effect of heparitinase or chondroitinase on neurite outgrowth of isolated 6-day old retina cells on the inner limiting membrane. The number of neurite bearing cells was greatly reduced on the HSase-treated ILM but not on CSase-treated or control ILM after 16 hr in culture. Bars show mean, $\pm$ standard deviation, $n = 3$

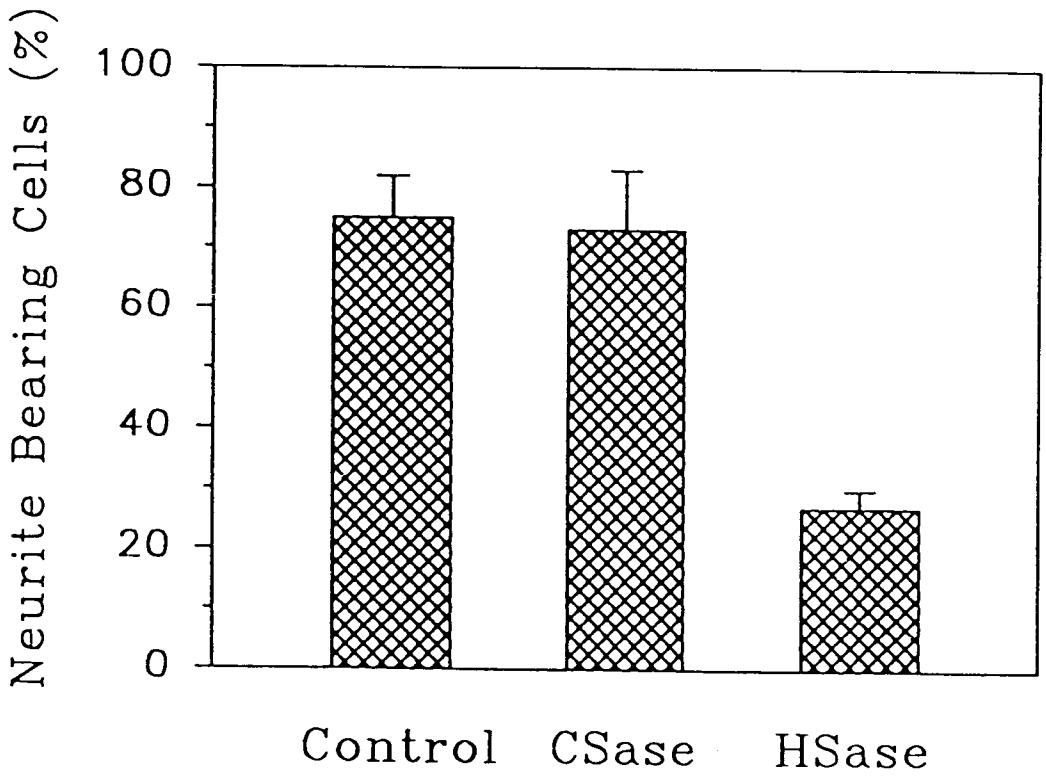


Fig. IV-9 (Cont.)

Fig. IV-10. Micrographs of isolated 6-day old embryonic chicken retina cells on the inner limiting membrane. When 6 day-old retina neuronal cells were cultured on non-treated (A) or CSase ABC-treated ILM (B), most sent out their axons after 16 hr in culture and the axons were usually greater in length than 2 cell body diameters. Most cells in the same culture dish but on the HSase-treated ILM had only short processes, and these processes usually were shorter than two cell-body diameters (C). Bar = 10 μm

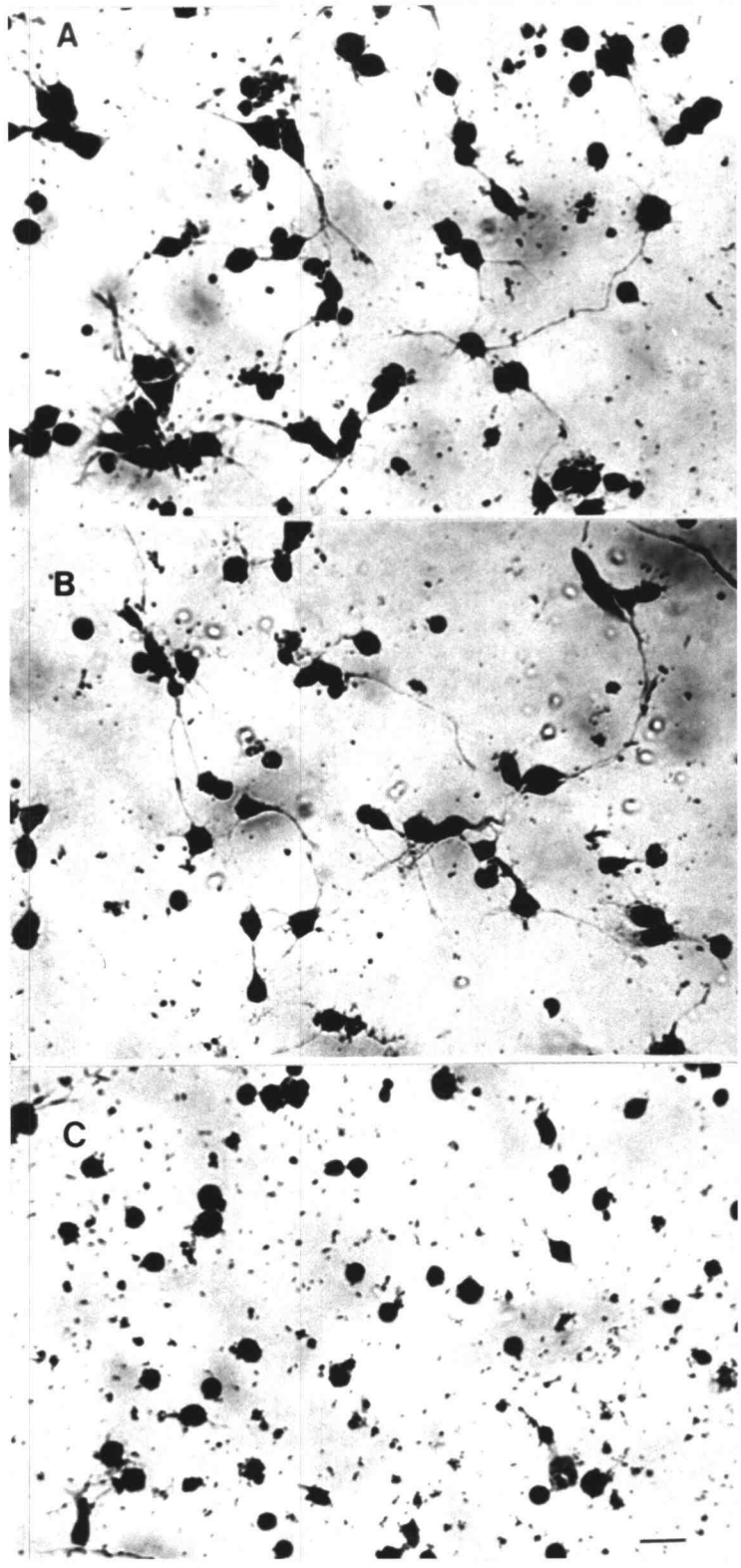


Fig. IV-10 (Cont.)

Fig. IV-11. Micrographs of 6-day old embryonic chicken retina cell aggregates on the inner limiting membrane. When the cells formed aggregates, they were loosely associated and those on the periphery had long processes, on both non-enzymatically treated ILM or CSase-treated ILM (A). On the other hand, aggregates on HSase-treated ILM, were tightly associated and very few cells from the aggregates send out their processes (B). Bar = 10 μm

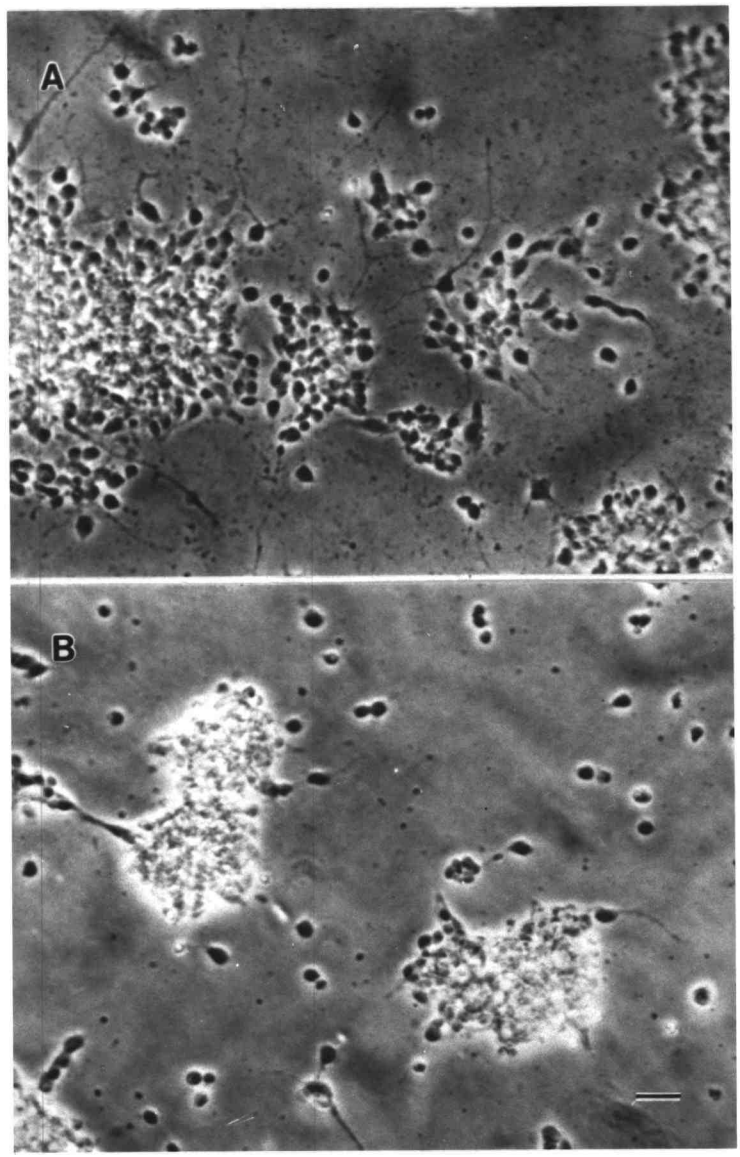


Fig. IV-11 (Cont.)

Table IV-1. Percentage of surviving neurons after 6, 16, or 24 hr on ILM in culture

	Time in culture (hours)		
	6	16	24
Conditions			
Control	87 ± 2	84 ± 4	86 ± 4
HSase	82 ± 6	84 ± 3	84 ± 4
CSase	83 ± 6	81 ± 5	82 ± 3

Data shown means ± SE, n = 3

The E6 or E7 chick retina neuronal cells were plated on the ILM in culture and the survival of retina cells was determined by trypan blue exclusion after 6, 16, or 24 hours. Five different microscope fields were counted for each determination.

Table IV-2. Comparison of axonal behavior of 6 day old retina on the ILM following treatment with a combination of enzymes and detergent*.

Conditions	Axon Density (% of Control)	Axon Growth Rate (μm/h)
Control	100 ± 0.0	57.7 ± 3.1
+ HSase, + TX 100	30 ± 5.2	36.4 ± 5.4
+ HSase, - TX 100	95 ± 3.7	54.0 ± 2.3
+ CSase, + TX 100	96 ± 6.1	57.2 ± 2.2
+ CSase, - TX 100	98 ± 4.3	56.9 ± 3.4
+ Collagenase, + TX 100	99 ± 1.2	60.2 ± 1.6
+ Collagenase, - TX 100	100 ± 1.5	58.7 ± 1.1
+ Trypsin, + TX 100	0	0.0
+ Trypsin, - TX 100	0	0.0

Data shown means ± SE

* The experimental procedure and calculations were as described in the methods. Briefly, the ILM was treated with TX-100 for 45 min, washed five times with BSS, and left in the BSS for an additional 2 to 2.5 hours. Then the ILM was digested with enzymes for 45 to 60 min and washed thoroughly before the retina explants were placed on it. Control experiments had neither enzyme nor detergent treatment.

CHAPTER V. SUMMARY AND CONCLUSION

The major observations in this thesis suggest *in situ* functions for proteoglycans in the inner limiting membrane (ILM) on behavior of neuronal cells. Heparan sulfate proteoglycans (HSPGs) were found to enhance both neurite density (neural sprouting) and growth rate (neurite elongation). Our results suggest that it is the glycosaminoglycans of these molecules rather than their protein cores that are responsible for the action. The enhancement of neurite outgrowth by these molecules is more readily explained by their direct growth promoting activity (e.g., as binding sites for growth factor accumulation) than by any ability to influence cell adhesion.

The significance of this work is that it provides an unique approach to examining and investigating functions of extracellular matrix molecules and behavior of neuronal cells on their natural substrate. The function of extracellular molecules has been studied mostly by applying one of these molecules alone or in combination on artificial culture substrata. In this type of assay, the molecules will form a microstructure and array of linkages determined by their simple environment. In nature, however, the environment surrounding cells is far more complex and cells usually encounter many different molecules. The specific organization of the native molecules has evolved developmentally specific interactions with cells to promote normal behavior. In order to study ECM

molecules in a more natural situation, we took advantage of the isolated ILM to investigate its influences on the behavior of retina neuronal cells.

Chapter I is a brief review of proteoglycans by nervous tissue. Chapter II describes the localization of proteoglycans in chicken embryonic neural retina. By using autoradiography and selective enzyme digestion, we found that HSPGs are mainly associated with three areas: the outer plexiform layer, the inner plexiform layer, and ILM in 10 day-old retinas. In 7 day-old retina, the only localization of HSPGs detected by this technique was the inner limiting membrane. The CSPGs in both cases (7 and 10 day embryos) were evenly distributed. Because of their localization beyond 7 days the results suggest that expression of HSPGs may be associated with synaptogenesis during the development of the retina. In an earlier study, biosynthesis of these two types of proteoglycans by chicken embryonic retinas was shown to undergo a developmental transition from predominantly HSPGs to predominate CSPGs (Morris 1978). By using immunohistochemical localization, we found that the core protein of HSPGs in the ILM may carry at least one epitope that is not present in the core protein of HSPGs in other regions of the retina. The epitope for basal lamina HSPGs was present in the ILM from early (4 day) to late (13 day) stages.

In chapter III, we studied the relative amount of heparan and CSPGs of retina using radioactively labeled retina that was mechanically separated from the inner limiting membrane. We studied these molecules quantitatively in ^{35}S-sulfate labeled retina by a combination of enzymatic and chemical digestion and

chromatographic separations. We found that the ILM contains about two-fold more metabolically active HSPGs than CSPGs. The remaining retina contains about equal amounts of metabolically active HSPGs and CSPGs. The relatively large amount of HSPGs in the ILM suggested that these molecules may play an important role in ganglion cell behavior. Our result also showed that ILM also contains fairly large amounts of CSPGs.

Chapters II and III describe topographic characterization of both HSPGs and CSPGs and give the basis for studying the functions of these molecules. Chapter IV provides insight into the functional aspect of these molecules. In this part of the study, we took advantage of the fact that the ILM can be isolated fully without contamination by intact cells or axons. By testing neuronal cell behavior on differently treated halves of ILM we found that both neurite density and growth rate on the membrane were reduced by treatment with heparitinase but not with chondroitinase. Heparitinase did not act by blocking the adhesion of neuronal cells to the ILM or by causing cell death. We found, however, that heparitinase treatment of the ILM did reduce the numbers of neurite-bearing cells. These results suggest that HSPGs act directly on neurites by promoting growth rather than by weakening adhesion. Furthermore, the fact that the ILM contains fairly large amounts of CSPGs (result from chapter III) whose removal from the substrata failed to block neurite growth indicates that, despite the ability of free chondroitin sulfate to influence cell adhesion and migration (see discussion in chapter I), when the role of these molecules in the construction of the substrate.

This thesis shows that HSPGs can play a significant role in stimulating neurite outgrowth of neuronal cells on the inner limiting membrane. Most ECM molecules (such as laminin, collagen, fibronectin etc.) also have been shown to promote neurite outgrowth (Halfter et al., 1987; Carri et al., 1988; Drazba and Lemmon, 1990). However, none of these molecules including HSPGs and CSPGs have been directly demonstrated to be guidance molecules for directing neurites of neuronal cells. How important are these molecules in directing axonal migration? Or, for that matter, how important is the ILM in guiding axonal migration? At least in the present study, we did not find that removal of heparan sulfate or chondroitin sulfate by the enzymes altered or influenced the direction of axonal migration from retina explants. The axons almost always extended out of the explant perpendicular to the explant as also reported by Halfter (1987). If the ILM does not influence the axonal direction, it may provide a supporting substrate and stimulating factors for neural axons. The guidance cues may come from neighboring cells, or the cells may be directed by some intrinsic mechanism. For example, as the axons continue to grow out, as also shown by Halfter (1987), they tend to curve in a clockwise direction. One study has shown that pioneer neurons of grasshopper embryo can migrate normally and find their target cells without basal lamina (Condic and Bentley, 1989). We also conducted a simple experiment to test how important the ILM is in direction of ganglion cell migration. We mechanically removed the ILM from 10 day old retina and then removed some axons from the retina. We cultured the retina for 6 hours in complete medium.

We examined this retina under the scanning electron microscope (see Appendix). One would expect the newly regenerated axons should migrate in all directions without the ILM if it is so important in guiding axons. However, we found that the regenerated axons all migrated in one direction parallel to exiting axons towards the optic fissure. This simple observation may indicate that the ganglion cell surface itself provide directional information for the migrating axons.

BIBLIOGRAPHY

Alberda, S. M., and Buck, C. A. 1990. Integrins and other cell adhesion molecules. The FASEB J. 4, 2868-2880.

Anderson, M. J., Champaneria, S., and Swenarchuk, L. E. 1991. Synaptic differentiation can be evoked by polymer microbeads that mimic localized pericellular proteolysis by removing proteins from adjacent surfaces. Dev. Biol. 147, 464-479.

Andres, J. L., Stanley, K., Cheifetz, S., and Massague, J. 1989. Membrane-anchored forms of betaglycan, a polymerphic proteoglycan that binds transform growth factor-ß . J. Cell Biol. 109, 3137-3145.

Aquino, D. A., Margolis, R. U., and Margolis, R. K. 1984a. Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. I. Adult brain, retina and peripheral nerve. J. Cell Biol. 99, 1117-1129.

Aquino, D. A., Margolis, R. U., and Margolis, R. K. 1984b. Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. II. Studies in developing brain. J. Cell Biol. 99, 1130-1139.

Arnott, S., and Mitra, A. K. 1984. X-ray diffraction analyses of glycosaminoglycans. Molecular Biophysics of the Extracellular Matrix. Arnott, S., Rees, D. A., and Morris, E. R. ed. Humana Press, Nj. 41-67.

Balazs, E. A., editor. 1970. Chemistry and molecular biology of the intercellular matrix. Vol. 1. Academic Press, London and New York.

Bayne, E. K., Anderson, M. J., and Fambrough, D. M. 1984. Extracellular matrix organization in developing muscle: correlation with acetylcholine receptor aggregates. J. Cell Biol. 99, 1486-1501.

Beck, K., Hunter, I., and Engel, J. 1990. Structure and function of laminin: anatomy of a multidomain glycoprotein. Faseb J. 4, 148-160.

Bernfield, M., Banerjee, S. D., Koda, J. E., and Rapraeger, A. C. 1984. Remodelling of the basement membrane: morphogenesis and maturation. Ciba Found. Symp. 108, 179-196.

Brandan, E., and Inestrosa, N. C. 1986. The synaptic form of acetylcholinesterase binds to cell-surface heparan sulfate proteoglycans. J. Neurosci. Res. 15, 185-196.

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.

Brower, D. L., Piovant, M., Salatino, R., Brailey, J., and Hendrix, M. J. C. 1987. Identification of a specialized extracellular matrix component in Drosophila imaginal discs. Dev. Biol. 119, 373-381.

Burg, M., and Cole, G. J. 1990. Characterization of cell associated proteoglycans synthesized by embryonic neural retina cells. Arch. Biochem. Biophs. 276, 396-404.

Burgess, W. H., and Maciag, T. 1989. The heparin-binding (fibroblast) growth factor family of proteins. Annu. Rev. Biochem. 58, 575-606.

Call, T. W., and Hollyfield, J. G. 1990. Sulfated proteoglycans in Bruch's membrane of the human eye: localization and characterization using cupromeronic blue. Exp. Eye Res. 51, 451-462.

Campbell, A. G., Fessler, L. I., Salo, T., and Fessler, J. H. 1987. Papilin: a Drosophila proteoglycan-like sulfated glycoprotein from basement membranes. J. Biol. Chem. 262, 17605-17612.

Carri, N. G., Perris, R., Johansson, S., and Ebendal, T. 1988. Differential outgrowth of retinal neurites on purified extracellular matrix molecules. J. Neurosci. Res. 19, 428-439.

Chai, L., Morris, J. E., and Potter, S. W. 1990. Isolation of proteoglycans from the inner limiting membrane of embryonic chick neural retina. J. Cell Biol. 111, 150a.

Cheifetz, S., and Massague, J. 1989. Transforming growth factors-β (TGF-β) receptor proteoglycan; cell surface expression and ligand binding in the absence of glycosaminoglycan chains. J. Biol. Chem. 264, 12025-12028.

Chernoff, E. A. 1988. The role of endogenous heparan sulfate proteoglycan in adhesion and neurite outgrowth from dorsal root ganglia. Tissue. Cell. 20, 165-178.

Chiquet, M., and Fambrough, D. M. 1984. Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunits. J. Cell Biol. 98, 1937-1946.

Chiquet-Ehrismann, R., Mackie, E. J., Pearson, C. A., and Sakakura, T. 1986. Tenascin: An extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. Cell. 47, 131-139.

Chiu, A. Y., Matthew, W. D., and Patterson, P. H. 1986. A monoclonal antibody that blocks the activity of a neurite regeneration-promoting factor: Studies on the binding site and its localization in vivo. J. Cell Biol. 103, 1383-1398.

Cirillo, A., Arruti, C., Courtois, Y., and Jeanny, J.-C. 1990. Localization of basic fibroblast growth factor binding sites in the chick embryonic neural retina. Diff. 45, 161-167.

Cohn, R. H., Banerjee, S. D., and Bernfield, M. R. 1977. Basal lamina of salivary epithelia. Nature of glycosaminoglycans and organization of extracellular matrix. J. Cell Biol. 73, 464-478.

Cole, G. J., and Akeson, R. 1989. Identification of a heparin binding domain of the neural cell adhesion molecule N-CAM using synthetic peptides. Neuron. 2, 1157-1165.

Cole, G. J., and Burg, M. 1989. Characterization of a heparan sulfate proteoglycan that copurifies with the neural cell adhesion molecule. Exp. Cell Res. 182, 44-60.

Cole, G. J., and Glaser, L. 1986. Heparin-binding domain from N-CAM is involved in neural cell-substratum adhesion. J. Cell Biol. 102, 403-412.

Cole, G. J., and McCabe, C. F. 1991. Identification of a developmentally regulated keratan sulfate proteoglycan that inhibits cell adhesion and neurite outgrowth. Neuron. 7, 1007-1018.

Cole, G. J., Schubert, D., and Glaser, L. 1985. Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. J. Cell Biol. 100, 1192-1199.

Cole, G. J., Loewy, A., and Glaser, L. 1986. Neuronal cell-cell adhesion depends on interactions of N-CAM with heparin-like molecules. Nature 320, 445-447.

Couchman, J. R., Caterson, B., Christner, J. E., and Baker, J. R. 1984. Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. Nature 307, 650-652.

Coulombre, A. J. 1955. Correlations of structural and biochemical changes in the developing retina of the chick. Am. J. Anat. 96, 135-189.

Damon, D. H., D'Amore, P. A., and Wagner, J. A. 1988. Sulfated glycosaminoglycans modify growth factor-induced neurite outgrowth in PC12 cells. J. Cell. Physiol. 135, 293-300.

David, G., Schueren, B., van der Marynen, P., Cassiman, J., and van den Berghe, H. 1992. Molecular cloining of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. J. Cell Biol. 118, 961-969.

Dow, K. E., Mirski, S. E. L., Roder, J. C., and Riopelle, R. J. 1988. Neuronal proteoglycans: biosynthesis and functional interaction with neurons in vitro. J. Neurosci. 8, 3278-3289.

Drazba, J., and Lemmon, V. 1990. The role of cell adhesion molecules in neurite outgrowth on Müller cells. Dev. Biol. 138, 92-93.

Edelman, G. M., and Crossin, K. L. 1991. Cell adhesion molecules: implications for a molecular histology. in Annu. Rev. Biochem. Annual Reviews Inc. 60, 155-190.

Edgar, D., Timpl, R., and Thoenen, H. 1984. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J. 3, 1463-1468.

Edgar, D., Timpl, R., and Thoenen, H. 1988. Structural requirements for the stimulation of neurite outgrowth by two variants of laminin and their inhibition by antibodies. J. Cell Biol. 106, 1299-1306.

Faassen, A. E., Schrager, A., Klein, D. J., Oegema, T. R., Couchman, J. R., and McCarthy, J. B. 1992. A cell surface chondroitin sulfate proteoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. J. Cell Biol. 116, 521-531.

Fayein, N. A., Courtois, Y., and Jeanny, J. C. 1990a. Ontogeny of basic fibroblast growth factor binding sites in mouse ocular tissues. Exp. Cell Res. 188, 75- 88.

Fayein, N. A., Courtois, Y., and Jeanny, J. C. 1990b. Ontogeny of basic fibroblast growth factor binding sites in mouse ocular tissues. Exp. Cell Res. 188, 75-88.

Fine, B. S., and Zimmerman, L. E. 1963. Observations on the rod and cone layer of the human retina. Invest. Ophthalmol. Vis. Sci. 2, 446-459.

Fujiwara, S., Wiedemann, H., Timpl, R., Lustig, A., and Engel, J. 1984. Structure and interactions of heparan sulfate proteoglycans from a mouse tumor basement membrane. Eur. J. Biochem. 143, 145-157.

Gordon, P. B., Choi, H. U., Conn, G., Ahmed, A., Ehrmann, B., Rosenberg, L., and Hatcher, V. B. 1989. Extracellular matrix heparan sulfate proteoglycans modulate the mitogenic capacity of acidic fibroblast growth factor. J. Cell. Physi. 140, 584-592.

Grant, D. S., and Leblond, D. P. 1988. Immunogold quantitation of laminin, type IV collagen, and heparan sulfate proteoglycan in a variety of basement membrane. J. Histo. Cytochem. 36, 271-283.

Grumet, M., Hoffman, S., Crossin, K. L., and Edelman, G. M. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissue that mediates glia-neuron interaction. Proc. Natl. Acad. Sci. USA. 82, 8075-8079.

Hageman, G. S., and Johnson, L. V. 1987. Chondroitin 6-sulfate glycosaminoglycan is a major constituent of primate cone photoreceptor matrix sheaths. Current Eye Res. 6, 639-646.

Hageman, G. S., Kirchoff-Rempe, M. A., Lewis, G. P., Fisher, S. K., and Anderson, D. H. 1991. Sequestration of basic fibroblast growth factor in the primate retinal interphotoreceptor matrix. Proc. Natl. Acad. Sci. USA. 88, 6706-6710.

Halfter, W., Reckhaus, W., and Kröger, S. 1987. Nondirected axonal growth on basal lamina from avian embryonic neural retina. J. Neurosci. 7, 3712-3722.

Hall, D. E., Neugebauer, K. M., and Reichardt, L. F. 1987. Embryonic neural retinal cell response to extracellular matrix proteins: developmental changes and effects of the cell substratum attachment antibody (CSAT). J. Cell Biol. 104, 623-634.

Hall, M. O., Ocumpauph, D. E., and Young, R. W. 1965. The utilization of 35S-sulfate in the synthesis of mucopolysaccharides by the retina. Invest. Ophthalmol. 4, 322-329.

Hantaz-Ambroise, D., Vigny, M., and Koenig, J. 1987. Heparan sulfate proteoglycan and laminin mediate two different types of neurite outgrowth. J. Neurosci. 7, 2293-2304.

Hardingham, T. 1981. Proteoglycans: their structure, interactions and molecular organization in cartilage. Biochem. Soc. Trans. 9, 489-497.

Hardingham, T. E., and Fosang, A. J. 1992. Proteoglycans: many forms and many functions. Faseb J. 6, 861-870.

Harrelson, A. L. 1992. Molecular mechanisms of axon guidance in developing insect nervous system. J. Exp. Zool. 261, 310-321.

Hascall, V. C., editor. 1981. Biology of Carbohydrates. Vol. 1. Wiley, New York.

Henke-Fahle, S., Reckhaus, W., and Babiel, R., editors. 1984. Influence of various glycoprotein antibodies on axonal outgrowth from chick retina. In Developmental Neuroscience: Physiological, Pharmacological and Clinical Aspects. Elsevier, Amsterdam.

Hoffman, S., and Edelman, G. M. 1987. A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. Proc. Natl. Acad. Sci. USA. 84, 2523-2527.

Hook, M. L., Kjellen, L., Johanson, S., and Robinson, J. 1984. Cell surface glycosaminoglycans. Annu. Rev. Biochem. 53, 847-869.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. Cell. 48, 549-554.

Iida, J., Skubits, A. P. N., Furcht, L. T., Wayner, E. A., and McCarthy, J. B. 1992. Coordinate role for cell surface chondroitin sulfate proteoglycan and $\alpha 4\beta 1$ integrin in mediating melanoma cell adhesion to fibronectin. J. Cell Biol. 118, 431-444.

Iijima, N., Oohira, A., Mori, T., Kitabatake, K., and Kohsaka, S. 1991. Core protein of chondroitin sulfate proteoglycan promotes neurite outgrowth from cultured neocortical neurons. J. of Neurochem. 56, 706-708.

Iwata, M., and Carlson, S. S. 1991. A large chondroitin sulfate basement membrane-associated proteoglycan exists as a disulfide-stabilized complex of several proteins. J. Biol. Chem. 266, 323-333.

Jalkanen, M., Rapraeger, A., and Bernfield, M. 1988. Mouse mammary epithelial cells produce basement membrane and cell surface heparan sulfate proteoglycans containing distinct core proteins. J. Cell Biol. 106, 953-962.

Kanwar, Y., Yoshinaga, Y., Liu, Z., Wallner, E., and Carone, F. 1992. Biosynthetic regulation of proteoglycans by aldohexoses and ATP. Proc. Natl. Acad. Sci. U. S. A. 89, 8621-8625.

Kashihara, W., Watanabe, Y., Makino, H., Wallner, E. I., and Kanwar, Y. S. 1989. Selective decreased de novo synthesis of glomerular proteoglycans under the influence of reactive oxygen species. Proc. Natl. Acad. Sci. USA. 89, 6309-6313.

Keilhauser, G., Faissner, A., and Schachner, M. 1985. Differential inhibition of neuron-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies. Nature(Lond.) 316, 728-730.

Keynes, R., and Cook, G. 1990. Cell-cell repulsion: clues from the growth cone? Cell. 62, 609-610.

Keynes, R. J., and Cook, G. M. 1992. Repellent cues in axon guidance. Curr. Opin. Neurobiol., 55-59.

Kiang, W.-L., Margolis, R. U., and Margolis, R. K. 1981. Fractionation and properties of a chondroitin sulfate proteoglycan and the soluble glycoproteins of brain. The J. of Biol. Chem. 256, 10529-10537.

Kleinman, H. K., McGarvey, M. L., Hassell, J. R., Star, V. L., Cannon, F. B., Laurie, G. W., and Martin, G. R. 1986. Basement membrane complexes with biological activity. Biochem. 25, 312-318.

Kroger, S., and Niehorster, L. 1990. Isolation, characterization, and substrate properties of the external limiting membrane from the avian embryonic optic tectum. J. Neurosci. Res. 27, 169-183.

Lafont, F., Rouget, M., Triller, A., Prochiantz, A., and Rousselet, A. 1992. In vitro control of neuronal polarity by glycosaminoglycans. Dev. 114, 17-29.

Lander, A. D., Fujii, D. K., Gospodarowicz, D., and Reichardt, L. F. 1982. Characterization of a factor that promotes neurite outgrowth: Evidence linking activity to a heparan sulfate proteoglycan. J. Cell Biol. 94, 574-585.

Lander, A. D., Fujii, D. K., and Reichardt, L. F. 1985. Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. J. Cell Biol. 101, 898-913.

Lander, D. A., Fujii, D. K., and Reichardt, L. F. 1985. Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media. Proc. Natl. Acad. Sci. USA. 82, 2183-2187.

Laterra, J., Silbert, J. E., and Culp, L. A. 1983. Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. J. Cell Biol. 96, 112-123.

Laurie, G. W., Leblond, C. P., and Martin, G. R. 1982. Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. J. Cell Biol. 95, 340-344.

Laurie, G. W., Leblond, C. P., and Martin, G. R. 1983. Light microscopic immunolocalization of type IV collagen, laminin, Heparan sulfate proteoglycan, and fibronectin in the basement membranes of a variety of rat organs. Amer. J. Anat. 167, 71-82.

Laurie, G. W., Bing, J. T., Kleinman, H. K., Hassell, J. R., Aumailley, M., Martin, G. W., and Feldmann, R. J. 1986. Localization of binding sites for laminin, heparan sulfate proteoglycan and fibronectin on basement membrane type IV collagen. J. Mol. Biol. 189, 205-216.

Laurie, G. W., Inoue, S., Bing, J. T., and Hassell, J. R. 1988. Visualization of the large heparan sulfate proteoglycan from basement membrane. Amer. J. Anat. 181, 320-326.

Lerner, L., and Torchia, D. A. 1986. A multinuclear study of the interactions of cations with proteoglycans, heparin and ficoll. J. Biol. Chem. 261, 12706-12714.

Li, M., Aggeler, J., Farson, D. A., Hatier, C., Hassell, J., and Bissell, M. J. 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA. 84, 136-140.

Liesi, P. 1990. Extracellular matrix and neuronal movement. Experientia. 46, 900-907.

Lin, W. 1990. Immunogold localization of basal laminar heparan sulfate proteoglycan in rat brain and retinal capillaries. Brain Res. Bull. 24, 533-536.

Lin, W. L., Essner, E., McCarthy, K. J., and Couchman, J. R. 1992. Ultrastructural immunocytochemical localization of chondroitin sulfate proteoglycan in Bruch's membrane of the rat. Invest. Ophthalmol. Vis. Sci. 33, 2072-2075.

Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F. M., Davis, G. E., and Varon, S. 1983. Laminin promotes neuritic regeneration from cultured peripheral and central neurons. J. Cell Biol. 97, 1882-1890.

Margolis, R. K., and Margolis, R. U. 1989. Structure and localization of glycoproteins and proteoglycans. Plenum Publishing Crop., New York.

Margolis, R. K., Goossen, B., Tekotte, H., Hilgenberg, L., and Margolis, R. U. 1991. Effects of β-xylosides on proteoglycan biosynthesis and morphology of PC12 pheochromocytoma cells and primary cultures of rat cerebellum. J. Cell. Sci. 99, 237-246.

Margolis, R. U., and Margolis, R. K. 1989. Nervous tissue proteoglycans. Dev. Neurosci. 11, 276-288.

Margolis, R. U., Margolis, R. K., Chang, L. B., and Preti, C. 1975. Glycosaminoglycans of brain during development. Biochemistry. 14, 85-88.

Martin, G. R., Kleinman, H. K., Terranova, V. P., Ledbetter, S., and Hessell, J. 1984. The regulation of basement membrane formation and cell matrix interactions by defined supramolecular complexes. Ciba Found. Symp. 108, 197-212.

Matthew, W. D., Greenspan, R. L., Lander, A. D., and Reichardt, L. F. 1985. Immunopurification and characterization of a neuronal heparan sulfate proteoglycan. J. Neurosci. 5, 1842-1850.

McCarthy, K. J., and Couchman, J. R. 1990. Basement membrane chondroitin sulfate proteoglycans: localization in adult rat tissues. J. Histochem. Cytochem. 38, 1479-1486.

McCarthy, K. J., Accavitti, M. A., and Couchman, J. R. 1989. Immunological characterization of a basement membrane-specific chondroitin sulfate proteoglycan. J. Cell Biol. 109, 3187-3198.

McCarthy, K. J., Horiguchi, Y., Couchman, J. R., and Fine, J. D. 1990. Ultrastructural localization of the core protein of a basement membrane-specific chondroitin sulfate proteoglycan in adult rat skin. Arch. Dermatol. Res. 282, 397-401.

Mecham, R. P. 1991. Laminin receptors. in Annu. Rev. Cell Biol. Annual Review Inc. 7, 71-92.

Meyer, M. R., Brunner, P., and Edwards, J. S. 1988. Developmental modulation of a glial cell-associated glycoprotein, 5B12, in an insect, Acheta domesticus. Dev. Biol. 130, 374-391.

Morris, J. E. 1976. Cell aggregation rate versus aggregate size. Dev. Biol. 54, 288- 296.

Morris, J. E. 1979. Steric exclusion of cells. A mechanism of glycosaminoglycan-induced cell aggregation. Exp. Cell Res. 120, 141-153.

Morris, J. E. 1984. Isolation of the major chondroitin sulfate/dermatan sulfate and heparan sulfate proteoglycans from embryonic chicken retina. Arch. Biochem. Biophys. 235, 127-140.

Morris, J. E. 1993. Proteoglycans and the modulation of cell adhesion by steric exclusion. submitted., .

Morris, J. E., Hopwood, J. J., and Dorfman, A. 1977. Biosynthesis of glycosaminoglycans in the developing retina. Dev. Biol. 58, 313-327.

Morris, J. E., Ting, Y.-P., and Birkholz-Lambrecht, A. 1984. Low buoyant density proteoglycans from saline and dissociative extracts of embryonic chicken retinas. J. Neurochem. 42, 798-809.

Morris, J. E., Yanagishita, M., and Hascall, V. C. 1987. Proteoglycans synthesized by embryonic chicken retina in culture: composition and compartmentalization. Arch. Biochem. Biophys. 258, 206-218.

Morriss-Kay, G., and Tuchett, F. 1989. Immunohistochemical localization of chondroitin sulfate proteoglycans and the effects of chondroitinase ABC in 9-to 11-day rat embryos. Dev. 106, 787-798.

Muir, H. 1983. Proteoglycans as organizers of the intercellular matrix. Biochem. Soc. Trans. 11, 613-622.

Nakayama, T., Sugiyama, H., and Furuya, S. 1989. Basal lamina enhances hippocampal neurite outgrowth in vitro. Dev. Brain Res. 49, 145-149.

Needham, L. K., Adler, R., and Hewitt, A. T. 1988. Proteoglycan synthesis in flat cell-free cultures of chick embryo retinal neurons and photoreceptors. Dev. Biol. 126, 304-314.

Neufeld, G., Gospodarowicz, D., Dodge, L., and Fujii, D. K. 1987. Heparin modulation of the neurotropic effects of acidic and basic fibroblast growth factors and nerve growth factor on PC12 cells. J. Cell Physio. 131, 131-140.

Ocumpaugh, D. E., and Young, R. W. 1966. Distribution and synthesis of sulfated mucopolysaccharides in the retina of the rat. Invest. Ophthalmol. 5, 196-203.

Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K., and Suzuki, S. 1980. Structural analysis of chick-embryo cartilage proteoglycan by selective degradation with chondroitin lyase (chondroitinases) and endo-β-D-galactosidase (keratanase). Biochem. J. 191, 193-207.

Olwin, B. B., and Rapraeger, A. 1992. Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. J. Cell Biol. 118, 631-639.

Oohira, A., Matsui, F., Matsuda, M., Takida, Y., and Kuboki, Y. 1988. Occurrence of three distinct molecular species of chondroitin sulfate proteoglycan in the developing rat brain. J. Biol. Chem. 263, 10240-10246.

Oohira, A., Matsui, F., and Katoh-Semba, R. 1991. Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC 12D cells. J. Neurosci. 11, 822-827.

Pacifici, M., Soltesz, R., Thal, G., Shanley, D. J., Boettinger, D., and Holtzer, H. 1983. Immunological characterization of the major chick cartilage proteoglycan and its intracellular localization in cultured chondroblasts. A comparison with type II procollagen. J. Cell Biol. 97, 1724-1736.

Paulsson, M., Fujivara, S., Dziadek, M., Timple, R., Pejler, G., Backstrom, G., Lindahl, U., and Engel, J. 1986. Structure and function of basement membrane proteoglycans. Ciba. Found. Symp. 124, 189-203.

Perris, R., and Johansson, S. 1987. Amphibian neural crest cell migration on purified extracellular matrix components: A chondroitin sulfate proteoglycan inhibits locomotion on fibronectin substrates. J. Cell Biol. 105, 2511-2521.

Perris, R., Krotoski, D., Lallier, T., Domingo, C., Sorrell, M., and Bronner-fraser, M. 1991. Spatial and temporal changes in the distribution of proteoglycans during avian neural crest development. Development. 111, 583-599.

Porrello, K., and LaVail, M. M. 1986. Histochemical demonstration of spatial heterogeneity in the interphotoreceptor matrix of the rat retina. Investigative Ophthalmology and Visual Science 27, 1577-1586.

Rada, J. A., and Carlson, E. C. 1991. Electron microscopic histochemical and immunochemical analyses of heparan sulfate proteoglycan distribution in renal glomerular basement membranes. Histol. Histopathol. 6, 149-160.

Rager, G., Lausmann, S., and Gallyas, F. 1979. An improved silver stain for developing nervous tissue. Stain Technol. 54, 193-200.

Ratner, N., Hong, D., Leiberman, M. A., Bunge, R. P., and Glaser, L. 1988. The neuronal cell-surface molecule mitogenic for Schwann cells is a heparin-binding protein. Proc. Natl. Acad. Sci. USA. 85, 6992-6996.

Reichardt, L. F., and Tomaselli, K. J. 1991. Extracellular matrix molecules and their receptors: functions in neural development. in Annu. Rev. Neurosci. 14, 531-570.

Robinson, J., Viti, M., and Hook, M. 1984. Structure and properties of an under-sulfate heparan sulfate proteoglycan synthesized by a rat hepatoma cell line. J. Biol. Chem. 250, 1877-1883.

Roden, L. 1980. Structure and metabolism of connective tissue proteoglycans. In The Biochemistry of Glycoproteins and Proteoglycans. Lennarz, W. J, ed. Plenum Publishing Corp. New York. 267-371.

Röhlich, P. 1970. The interphotoreceptor matrix: Electron microscopic and histochemical observations on the vertebrate retina. Exp. Eye Res. 10, 80-96.

Romanoff, A. L., editor. 1960. The avian embryo: structural and functional development. The Macmillan Company, New York.

Ruoslhti, E., and Yamaguchi, Y. 1991. Proteoglycans as modulators of growth factor activities. Cell. 64, 867-869.

Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M., and Sunshine, J. 1988. The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. Science 240, 53-57.

Sanes, J. R. 1989. Extracellular matrix molecules that influence neural development. in Annu. Rev. Neurosci. Annual reviews Inc. 12, 491-516.

Schlosshauer, B., Grauer, D., Dütting, D., and Vanselow, J. 1991. Expression of a novel Müller glia specific antigen during development and after optic nerve lesion. Dev. 111, 789-799.

Schubert, D., and LaCorbiere, M. 1985a. Isolation of a cell surface receptor for chick neural retina adherons. J. Cell Biol. 100, 56-63.

Schubert, D., and Lacorbiere, M. 1985b. Isolation of an adhesion-mediating protein from chick neural retina adherons. J. Cell Biol. 101, 1071-1077.

Schubert, D., Lacorbiere, M., Klier, G., and Birdwill, C. 1983. A role for adherons in neural retina cell adhesion. J. Cell Biol. 96, 990-998.

Shinomura, T., Jensen, K. L., Yamagata, M., Kimata, K., and Solursh, M. 1990. The distribution of mesenchyme proteoglycan (PG-M) during wing bud outgrowth. Anat. Embryol. Berl. 181, 227-233.

Shively, J. E., and Conrad, H. E. 1976. Formation of anhydrosugars in the chemical depolymerization of heparin. Biochem. 15, 3932-3942.

Snow, D. M., and Letourneau, P. C. 1991. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). J. Neurobiol. 23, 322-336.

Snow, D. M., Steindler, D. A., and Silver, J. 1990. Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. Dev. Biol. 138, 359-376.

Snow, D. M., Watanabe, M., Letourneau, P. C., and Silver, J. 1991. A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Dev. 113, 1473-1485.

Soubrane, G., Jerdan, J., Karpouzas, I., Fayein, N. A., Glaser, B., Coscas, G., Courtois, Y., and Jeanny, J. C. 1990. Binding of basic fibroblast growth factor to normal and neovascularized rabbit cornea. Invest. Ophthalmol. Vis. Sci. 31, 323-333.

Sperry, R. W. 1963. Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA. 50, 703-710.

Stow, J. L., Sawada, H., and Farquhar, M. G. 1985. Basement membrane heparan sulfate proteoglycans are concentrated in the laminae rarae and in podocytes of the rat renal glomerulus. Proc. Natl. Acad. Sci. USA. 82, 3296-3300.

Swenarchuk, L. E., Champaneria, S., and Anderson, M. J. 1990. Induction of a specialized muscle basal lamina at chimeric synapses in culture. Development. 110, 51-61.

Tessier-Lavigne, M. 1992. Axon guidance by molecular gradients. Curr. Opin. Neurobiol., 60-65.

Tessier-Lavigne, M., and Placzek, M. 1991. Target attraction: are developing axons guided by chemotropism? Trands Neurosci. 14, 303-310.

Threlkeld, A., Adler, R., and Hewitt, A. T. 1989. Proteoglycans biosynthesis by chick embryo retina glial-like cells. Dev. Biol. 132, 559-568.

Tomaselli, K. J., and Reichardt, L. R. 1989. Integrins, cadherines, and cell adhesion molecules of the immunoglobulin superfamily: neuronal receptors that regulate axon growth and guidance. in The assembly of the nervous system. pp81-108, Alan R. Liss,Inc.

Tosney, K., and Landermesser, L. 1985. Development of the major pathways for neurite outgrowth in chick hindlimb. Dev. Biol. 109, 193-214.

Vainio, S., Jalkanen, M., and Thesleff, I. 1989. Syndecan and tenacin expression is induced by epithelial-mesenchymal interaction in embryonic tooth mesenchyme. J. Cell Biol. 108, 1945-1953.

Vila-Porcile, E., Picart, R., Vigny, M., Tixier-Vidal, A., and Tougard, C. 1992. Immunolocalization of laminin, heparan-sulfate proteoglycan, entactin, and

type IV collagen in the rat anterior pituitary. I. An in vivo study. Anat. Rec. 232, 482-492.

Wagner, J. A., and D'Amore, P. A. 1986. Neurite outgrowth induced by an endothelial cell mitogen isolated from retina. J. Cell Biol. 103, 1363-1367.

Walicke, P., Cowan, W. M., Ueno, N., Baird, A., and Guillemin, R. 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc. Natl. Acad. Sci. USA. 83, 3012-3016.

Wang, L., and Denburg, J. L. 1992. A role for proteoglycans in the guidance of a subset of pioneer axons in cultured embryos of the cockroach. Neurons 8, 701-714.

Werz, W., and Schachner, M. 1988. Adhesion of neural cells to extracellular matrix constituents. Involvement of glycosaminoglycans and cell adhesion molecules. Dev. Brain Res. 43, 225-234.

Weysse, A. W., and Burgess, W. S. 1906. Histogenesis of the retina. Am. Nat. 40, 611-638.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell. 64, 841-848.

Yurchenco, P. D., and Schittny, J. C. 1990. Molecular architecture of basement membranes. Faseb J. 4, 1577-1590.

Zaremba, S., Guimaraes, A., Kalb, R. G., and Hockfield, S. 1989. Characterization of an activity-dependent, neuronal surface proteoglycan identified with monoclonal antibody Cat-301. Neuron. 2, 1207-1219.

Zimmerman, L. E., and Eastham, A. B. 1959. Acid mucopolysaccharide in the retinal pigment epithelium and visual cell layer of the developing mouse eye. Amer. J. Ophthalmol. 47, 488-499.

APPENDICES

APPENDIX I. EXAMINATION OF CHICKEN EMBRYONIC RETINA AXONAL ORGANIZATION AFTER MECHANICAL REMOVAL OF INNER LIMITING MEMBRANE

Introduction

The correct connection of neurons to their targets during embryonic development is critical for normal function of an animal nervous system. During early development the neurons send out their axons along their pathways to their correct target cells or tissues. Retinal axons accomplish with precision the task of finding their correct target tissue, the optic tectum. A number of studies have indicated that this precise target-finding process may involve interaction between the ganglion cells and their surrounding environment, the extracellular matrix molecules, and/or their neighboring cells, for instance, Müller cells.

In order to understand the neuronal behavior of embryonic ganglionic cell axons it is necessary to study their organization developmentally and the property of their regeneration. Studies of ganglionic axon behavior and organization in the retina has been deduced from sectioned tissue and staining with silver Golgi staining or DiI or transmission electron microscopy of thin sections. To obtain a more complete understanding of developmental changes in neurite patterns we have removed the inner limiting membrane from the surface of the ganglion cells layer (and ganglionic cell axons in some cases) of a chicken embryonic sensory retina

and examined the exposed surface by scanning electron microscopy. We found that by 5 days of development, axons have already started to form fascicles. By 6 or 7 days of development, fascicles have become more organized. By 11 days of development the fascicles have increased their diameter and by 14 days interconnections between fascicles have become established, blurring the distinction between individual fascicles. The existence of fascicles previously has only been suggested by studies of tissue sections and no attempt has been made to determine their changes during development. In this study we also report the pattern of axonal regeneration with inner limiting membrane removed. After 6 hours of incubation damaged axons have regenerated but not fasciculated. The inner limiting membrane was not reformed. The newly-regenerated axonal fibers were parallel to pre-existing axonal fascicles and correctly directed toward the optic fissure. These results suggest that the direction of axonal migration and orientation are independent from existing inner limiting membrane but may depend on the orientation of pre-existing axonal or glial fibers.

Materials and Methods

Removal of retina inner limiting membrane

Retinas from different ages (5-, 6-, 7-, 10-, and 14-days embryos) were dissected free from connective tissues but without removal of the vitreous body. The retinas were then mounted on DEAE paper with vitreous side facing up in

balanced saline solution. The retinas were flattened on the paper by rolling the vitreous body over their surface. The inner limiting membrane (in some cases, ganglionic cell axons) was carefully separated from the retinas mechanically, using glass microelectrodes as dissecting needles. The region of the retinas examined was typically within 0-2 mm from the optic fissure.

Tissue cultures

Retina tissues were cultured in MEM supplemented with 10% fetal bovine serum, 50% chicken embryonic extract and antibiotics (see chapter III).

Scanning electron microscopy

The inner limiting membrane was partially or fully peeled from freshly dissected retinas or cultured retina tissues. The retinas were then fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 1 hour, washed twice with phosphate buffer and postfixed with 1% OsO_4 in 0.1 M phosphate buffer. They were dehydrated and critical point dried for observation with an Amray AM-1000 scanning electron microscope.

Results and Discussion

Developmental organization of ganglionic axons

In a first set of experiments we investigated the developmental organization of ganglionic cell axons of embryonic chicken retinas. Fig.A-I-1 showed that the inner limiting membranes partially lifted from underlying ganglionic axons, exposing the ganglionic axons in 7-, and 11-day old retinas. At 5 days of development, the chick retina ganglionic cells send out fewer axons (Fig. A-I-2A) than older retinas (Fig. A-I-2B, A-I-2C, and A-I-2D). At this stage, ganglionic axons have already begun fasciculation, but the fascicles are small and poorly organized. Fascicles are usually more separated than those of older ganglionic cells. Unfasciculated axons can be seen and ganglionic cell bodies sometimes are not covered by the axons (or fascicles). At 6 and 7 days of development, the chick retina develops bigger and more organized fascicles and ganglionic cells are completely covered under the fascicles (Fig. A-I-2A and A-I-2B). At 11 day of development the axons formed even bigger fascicles (Fig. A-I-2C). At 14 day of development, the ganglionic cell axons are established and interwoven between the fascicles (Fig. A-I-2D). Due to interconnections between neighboring fascicles the separated fascicles cannot be easily identified (Fig. A-I-2E).

In this study we have shown that the organization of the ganglionic axons are developmentally regulated. Fewer fascicles and single migrating axon fibers at

5 days of development indicate that ganglionic cells at this stage are still undergoing differentiation.

Regeneration of ganglionic axons

In a second set of experiments we investigated regeneration of 11 day old retina ganglionic axons. To study axonal regeneration we removed axonal fascicles after removal of inner limiting membrane. After removal of fascicles no "stub" of remaining axons can be seen (Fig. A-I-3). After 6 hr of incubation, ganglionic cells began to regenerate their axons. The orientation of the axons were parallel to pre-existing axons (Fig. A-I-4).

In this study we demonstrated that the orientation of regenerating axons are parallel to the pre-existing axons. This result suggests that inner limiting membrane may not be necessary to provide guiding cues for the axon migration and orientation.

Fig. A-I-1. Scanning electron microscopic micrographs of lifted inner limiting membrane from 7 day old (A), and 11 day old (B) chick retinas, showing their relationship to the ganglionic cell axon bundles (fascicles). ILM = inner limiting membrane; F = fascicle. Scale = 20 μm.

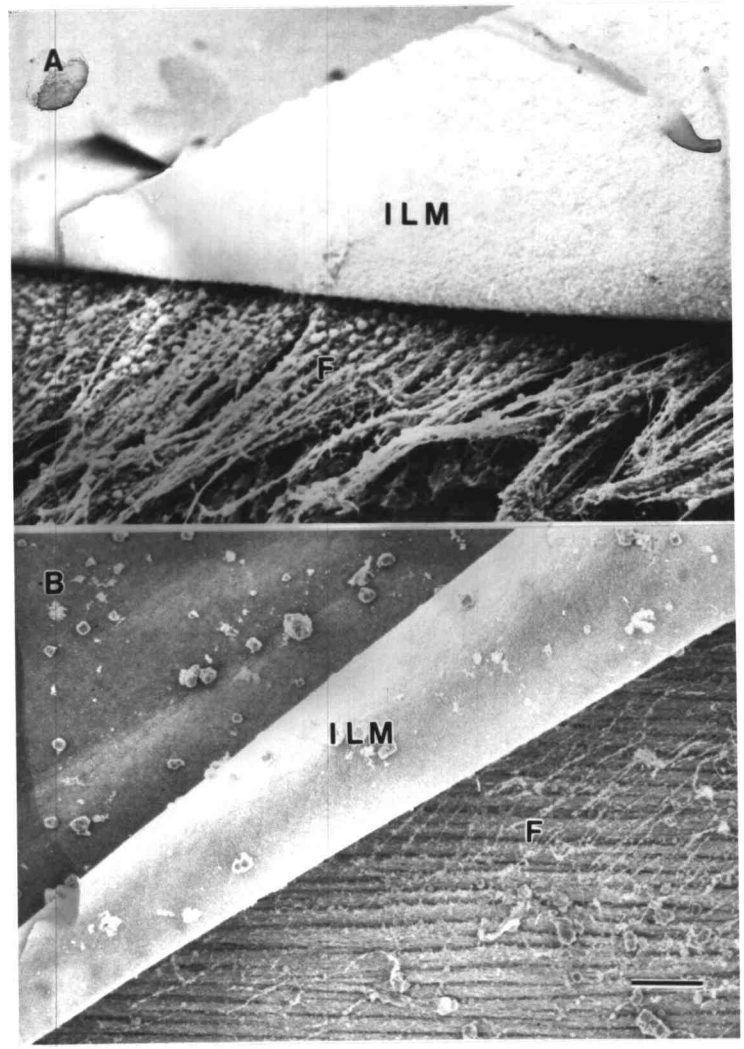


Fig. A-I-1 (Cont.)

Fig.A-I-2. Scanning electron microscopic micrographs of organization of the developing embryonic chick retina ganglionic cell axons. A. At 5 days of development, the axons have already begun fasciculation, but the fascicles are small and poorly organized. B and C. Unfasciculated single axons can be seen and some ganglionic cell bodies are still exposed. At 6 and 7 days of development (B and C, respectively), the chick retina develops bigger and more organized fascicles, and the ganglionic cells are completely covered by the fascicles. D. At 11 days of development, the fascicles increase their diameter. The endfeet of retracted Müller cell are seen as buttons and knobs. E and F. At 14 days of development, interconnection between fascicles have established and obvious boundaries between individual fascicles are disappearing due to interweaving between them. ILM = inner limiting membrane; F = fascicle; GCA = ganglionic cell axons; SA = single axon; GC = ganglionic cell. Scale = 10 μm in A, B, C, D, and E; 50 μm in F.

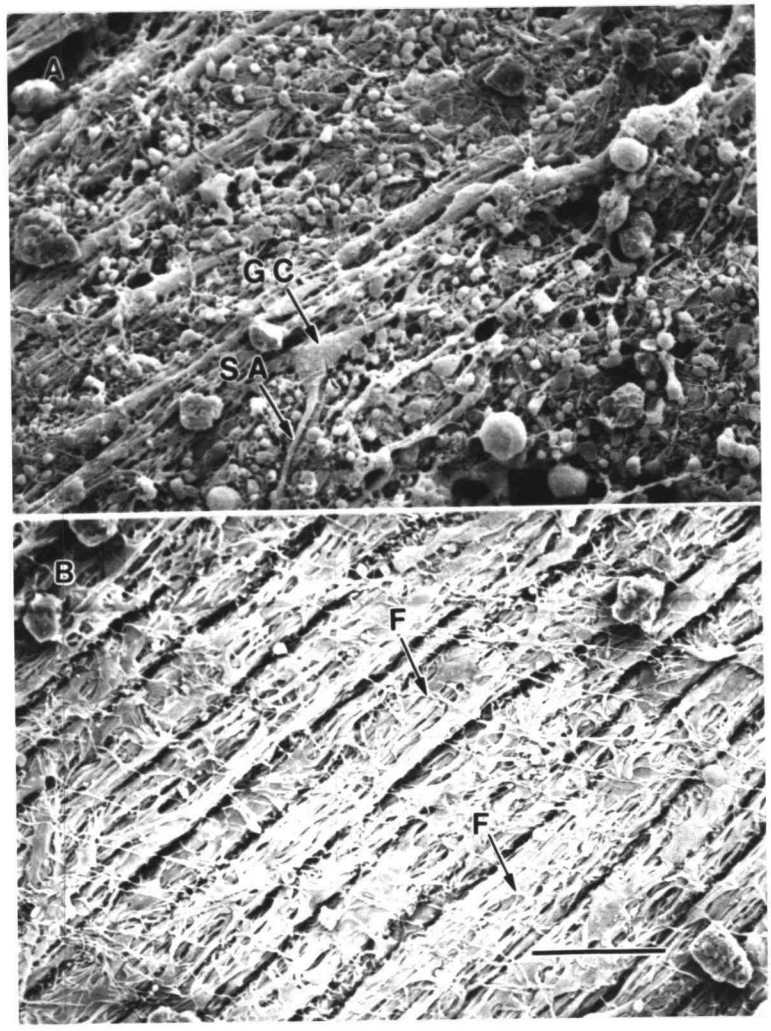
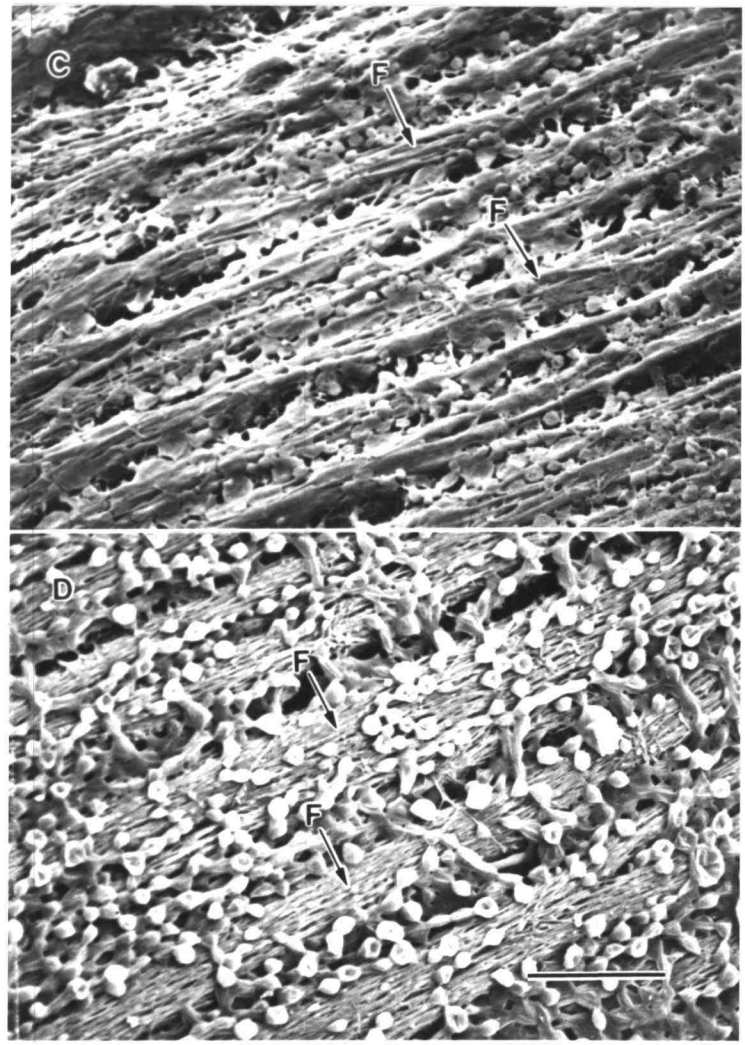


Fig. A-I-2 (Cont.)



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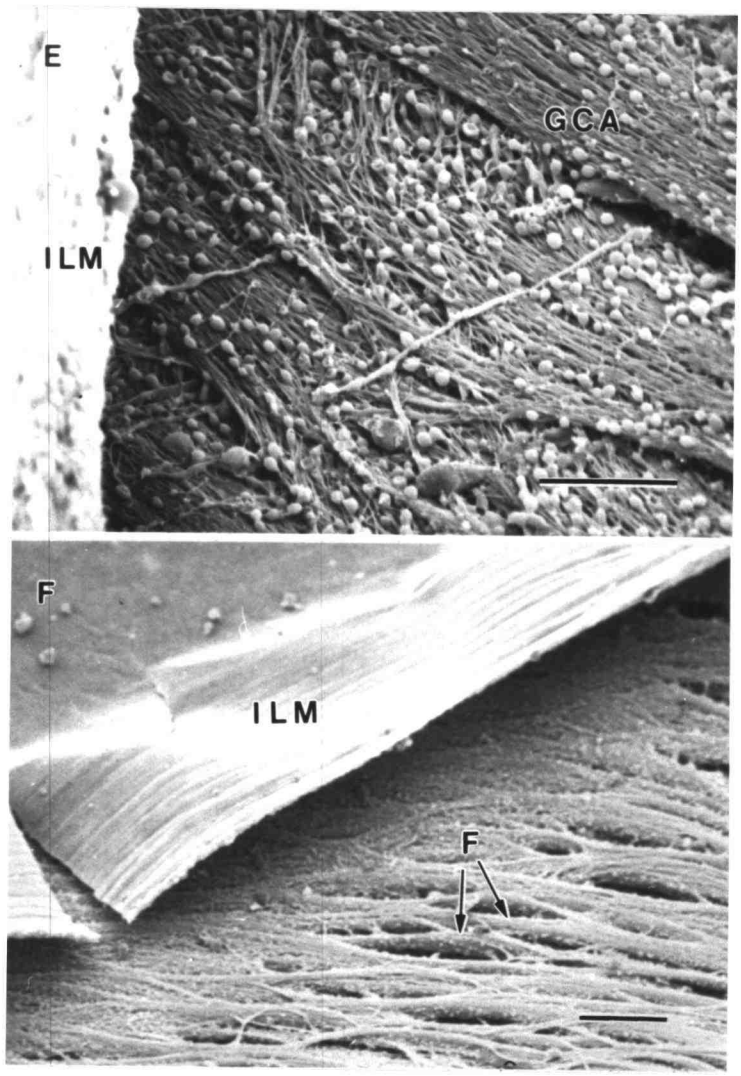

Fig. A-I-2 (Cont.)

Fig.A-I-3. Scanning electron microscopic micrographs of retina tissues with inner limiting membrane and ganglionic cell axons partially or completely removed. A. The ganglionic cell axon layer (GCA) is exposed by removal of inner limiting membrane. B. The ganglionic cell layer (GCL) is exposed by removal of both inner limiting membrane and ganglionic cell axons. C. Both inner limiting membrane and ganglionic cell axon layer are partially rolled back. D. At a greater magnification of the area enclosed by the box in C, picture D shows small bundles of axons (arrowhead) joining a larger fascicle (F) and no 'stub' of remaining axons after removal of fascicles. ILM = inner limiting membrane; A = Axons; LA = lifted axons; F = fascicle; GCA = ganglionic cell axons; GCL = ganglionic cell layer. Scales = 20 μm in A and B, = 100 μm in C, = 10 μm in D.

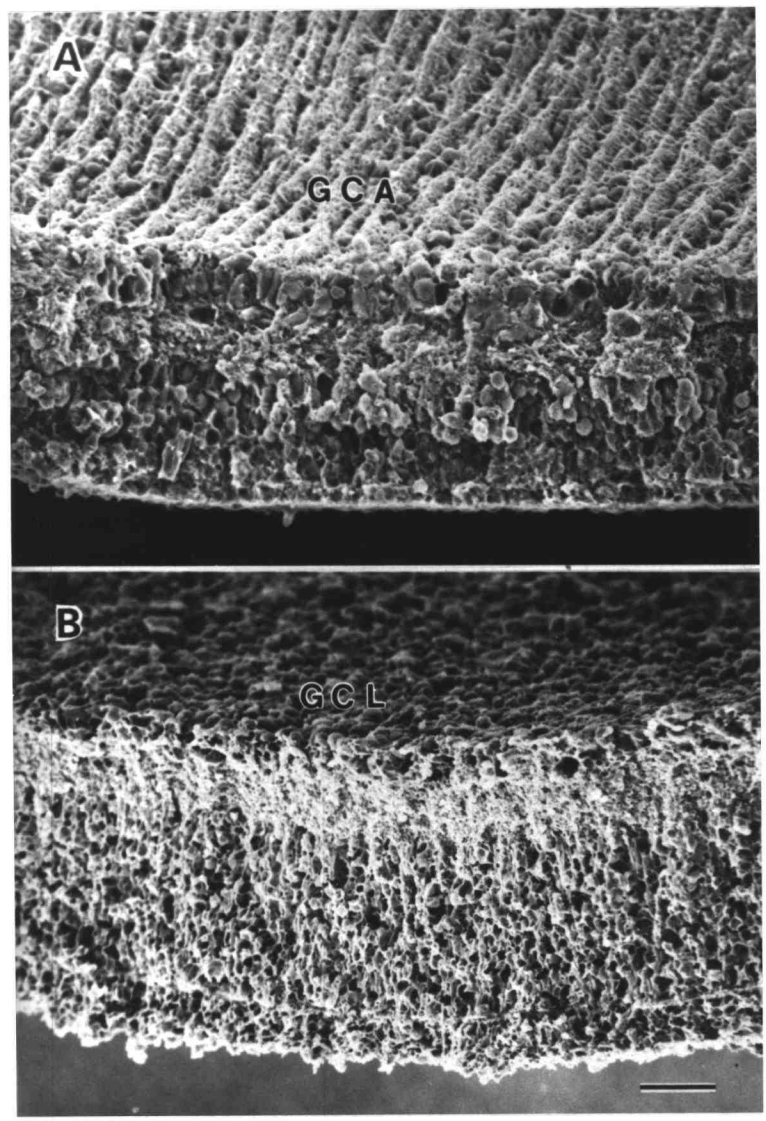

Fig. A-I-3 (Cont.)

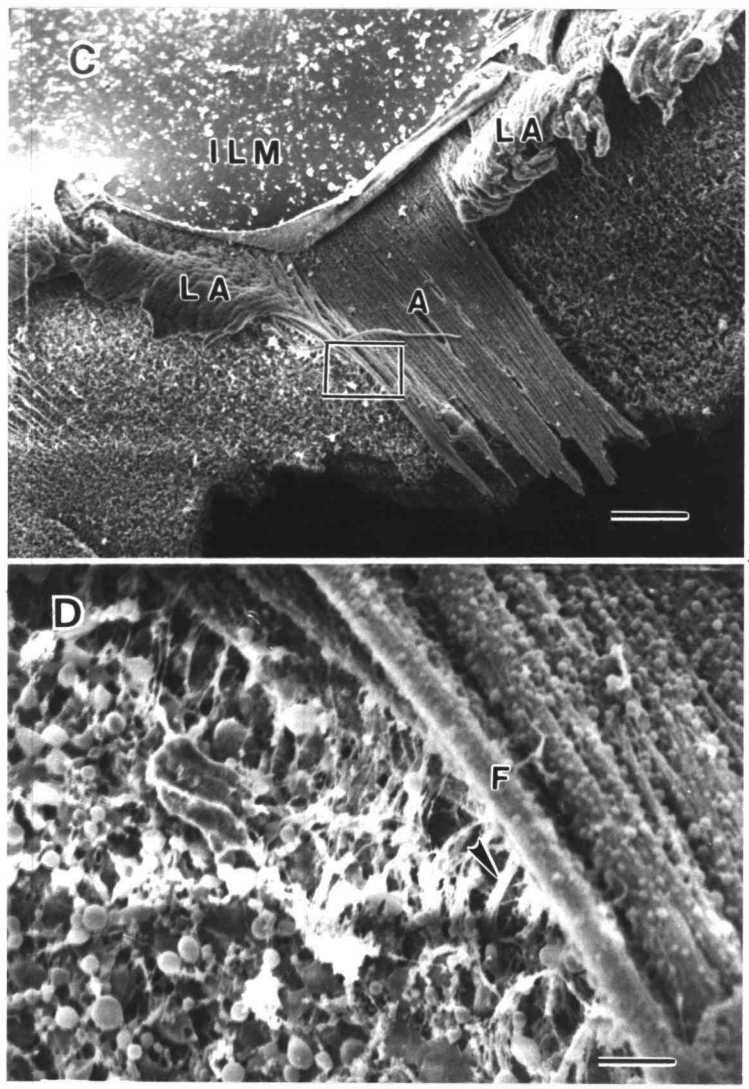

Fig. A-I-3 (Cont.)

Fig.A-I-4. Scanning electron microscopic micrographs of retina tissue cultured for 6 hrs. A. Newly-formed axon fibers are parallel to the pre-existing axons (EA). B. At a greater magnification of similar tissue region of a cultured retina tissues. Arrows indicate the direction of the optic fissure (not shown). SA = single axon fiber; EA = pre-existing axons. Scales = 100 μm in A, = 10 μm in B.

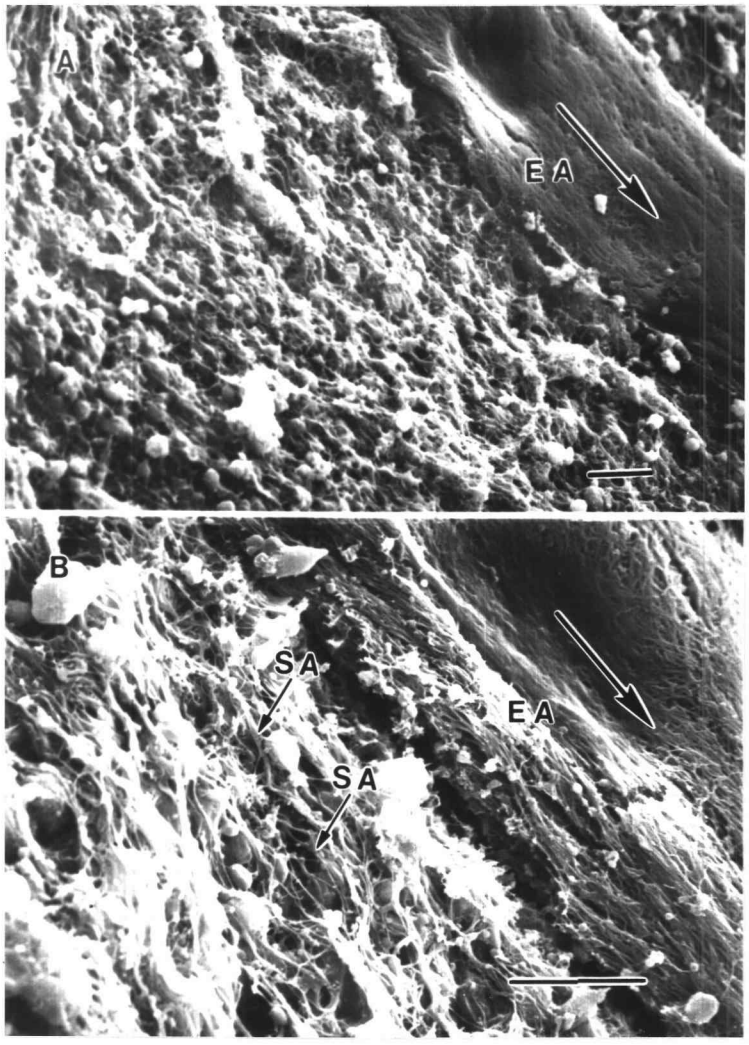

Fig. A-I-4 (Cont.)

APPENDIX II. DEVELOPMENT OF POLYCLONAL ANTISERA TO CELL SURFACE-ASSOCIATED HEPARAN SULFATE PROTEOGLYCANS OF EMBRYONIC CHICKEN CENTRAL NERVOUS SYSTEM

Objectives

In order to confirm our autoradiographic observations (chapter II) that heparan sulfate proteoglycans (HSPGs) were associated with inner and outer plexiform layers, and investigate if the core proteins of HSPGs of inner limiting membrane (ILM) contain an epitope(s) distinct from those of inner and outer plexiform layers, in this study we developed two different polyclonal antisera, designed as saHSPG-1 and saHSPG-2.

Materials and Methods

Radioactive labeling

Two 10 day old embryonic chicken brains (for saHSPG-1) or retinas (for saHSPG-2) were dissected free from connective tissues and chopped into small pieces (for saHSPG-1) and washed with balanced salt solution (BSS). They were then cultured in sulfate-free BSS for 30 min and transferred into 10 ml of fresh D-MEM supplemented with 200 μCi/ml ^{35}S-sulfate. After 12 hr incubation the tissues were washed with BSS 3 times with mild centrifugation. The tissues were

extracted with 8M urea overnight, and the extract was chromatographed on DEAE ion exchange columns (for extraction procedure see below). The isolated radioactively-labeled proteoglycans were used as tracers to aid in the preparation of larger quantities of proteoglycan antigen.

Isolation of membrane bound heparan sulfate proteoglycans

Ten- to twelve-day old embryonic chicken brains (for saHSPG-1) or retinas (for saHSPG-2) were dissected free from surrounding connective tissues and homogenized in balanced salt solution supplemented with DNAse and protease inhibitors. The homogenate was left in BSS at room temperature for an additional 30 min for further DNAse digestion. The homogenate was then extracted in 8 M urea + 0.15 M NaCl + 2% Triton X-100 + protease inhibitors overnight at 4°C. The homogenate was centrifuged at 17,000 g for 5 hr at 4°C. The supernatant was collected and the pellet was reextracted in the same buffer for 1 h. The reextracted sample was centrifuged at 17,000 g for 2 h. The pellet was discarded and all supernatants were pooled. The supernatant were combined with radioactive labeled isolated proteoglycans and applied on 20 ml of DEAE column. The column was washed with same buffer with 3 bed volumes of 0.5% Triton X-100. The sample was eluted with 0.6 M NaCl in 8 M urea after 0.15 M NaCl and 0.35 M NaCl in 8 M urea washes. The eluate was then concentrated on Centriprep™ 30 and applied onto G-50 chromatography to transfer into 0.1 M Tris/acetate buffer. The sample then was digested with chondroitinase ABC for 45 min.

Digested chondroitin sulfate and its protein core were washed through a 1-ml DEAE chromatography column with 0.35 M NaCl. The HSPGs were eluted with 0.6 M NaCl and applied to 3 ml of an affinity matrix consisting of monoclonal antibody (Hybridoma Development Bank) against basal laminin HSPG attached to Sepharose DL-4B beads (for saHSPG-1 only). The basal lamina HSPGs were bound to the column and the wash-through, which consisted of cell surface-associated HSPGs, was used for saHSPG-1 antigen.

For saHSPG-2 the HSPGs were applied to a DEAE column and washed with 3 column volumes each of 0.15 M and 0.3 M NaCl. The proteoglycans were eluted with 0.6 M NaCl plus 0.5% CHAPS (defined in chapter III), diluted 20 times with same buffer, and applied to 3 ml of an Octylsepharose column. The HSPGs from retina cell membranes, with hydrophobic domains, were bound to the column. The column was eluted with a 0-1% of Triton X-100 gradient. The fractions containing proteoglycans (Fig. A-II-5) were pooled and concentrated on a 1-ml DEAE column. The eluate was used as saHSPG-2 antigen.

Development of polyclonal antiserum to cell membrane bound heparan sulfate proteoglycans (saHSPG)

Two female New Zealand white rabbits were injected separately with either saHSPG-1 or saHSPG-2 emulsified with complete Fraud's adjuvant subcutaneously. They were injected biweekly twice intradermally and boosted

once intramuscularly respectively. The rabbit was bleed 4 days before the injection (to prepare pre-immune serum) and 10 days after final boost.

Isolation of polyclonal antiserum

The newly-collected rabbit blood was left at room temperature for 2 hr and then stored at 4°C overnight. The serum was separated from red blood cells by centrifugation at 1500xg for 10 min and was mixed with saturated ammonium sulfate (SAS) over 2 hr on ice. The precipitate was then centrifuged at 1500xg for 10 min, washed with 33% SAS, and then dissolved in phosphate buffer pH 7.4 as antiserum stock solution. The antiserum stock solution was diluted 100x before use.

Immunohistochemistry

Embryonic chick tissues were dissected, mounted on a nitrocellulose membrane filter (for retina only), embedded in O.C.T. compound, frozen in liquid nitrogen, and sectioned with a cryostat microtome. The fresh-frozen sections were incubated in phosphate buffer containing 10% horse serum for 10-20 min and then with antiserum (100 to 500 dilutions) against embryonic chicken saHSPG antiserum or preimmune serum for 30-60 min. After several 5-min washes the sections were stained with the fluorescein conjugated to goat anti-rabbit IgG (1:100 dilutions) (Molecular Probes, Eugene, Oregon) in the same buffer for 30-60 min. After

several rinses the sections were observed under a Zeiss fluorescence microscope equipped with epi-illumination.

Results

A. saHSPG-1

Antiserum staining of the cross-sections and sagittal-sections of whole embryos

The antiserum stained on all basal lamina of the 3.5-day embryos, as seen on the cross sections (Fig. A-II-1), and 4-day embryos, as seen on sagittal sections (Fig. A-II-2).

Antiserum staining of the embryonic retina and brain

The antiserum stained the inner limiting membrane of the retina (Fig. A-II-3A) and outer limiting membrane of the brain (Fig. A-II-3B). It showed a strong reaction with remaining retina especially on both inner and outer plexiform layers as well as the ganglionic cell axon layer. The connective tissue such as sclera surrounding the retina and the blood brain barrier were not stained (not shown).

Our results indicated that the antiserum saHSPG-1 recognized cell surface HSPG of both retina and brain as well as inner limiting membrane of the retina and outer limiting membrane of the brain. This antiserum also recognized other basal lamina HSPG of non-neuronal tissues.

B. saHSPG-2

Isolation of cell-surface-associated HSPGs

In order to isolate membrane bound HSPG antigen on the basis of its physical properties rather than its lack of basal laminal HSPG properties, we used two purification steps. (1) We isolated total proteoglycans by ion exchange and digested them with chondroitinase ABC to remove chondroitin sulfate proteoglycans; (2) we separated the remaining PGs with hydrophobic domains from ones without hydrophobic domains by Octylsepharose chromatography. We first isolated radioactively-labeled PGs by ion exchange chromatography by pooling fractions at 0.47 to 0.54 M NaCl fractions (Fig. A-II-4A). These fractions were used as a 'hot' PG spike for preparing the antigen, which was obtained as a single peak at 0.47 to 0.54 M NaCl peak (Fig. A-II-4B). This peak mainly contained HSPGs (Morris et al., 1987). When we separated hydrophobic PGs from hydrophilic PGs by Octylsepharose chromatography, we obtained one single sharp peak at about 0.33% triton X-100 (Fig. A-II-5). About two thirds of extract failed to bind to the column. The PGs bound to the column were considered to be membrane bound HSPGs.

Immunohistochemistry and immunocytochemistry

The antiserum mainly reacted with photoreceptor cells, inner and outer plexiform layers, and ganglionic cell axons as well as ganglionic cell surfaces of the retinas (Fig. A-II-6A). Quite unlike the antiserum to saHSPG-1, it did not

react with inner limiting membrane of the retina (Fig. A-II-6A) or external limiting membrane of the brain (Fig. A-II-6B).

Discussion

In present study, we have developed two polyclonal antisera against HSPGs, saHSPG-1 and saHSPG-2. The saHSPG-1 antigen was isolated by a combination of ion-exchange chromatography, selective enzyme digestion, and affinity-absorb chromatography. The antiserum saHSPG-1 not only reacted with cell surface and neuronal axons but also strongly reacted with basal lamina. The nondistinguished reaction of the antiserum (saHSPG-1) with both cell surface and basal lamina may possibly due to (1) The monoclonal antibody affinity column was insufficient to remove basal lamina HSPGs from the sample, since we found that only less that 1% of radioactivity was bound to the affinity column during preparation. Our previous results (Chapter III) showed that ILM contained about 5 to 10% of total radioactivity in the isolated PGs. (2) Both cell surface-associated HSPGs and basal laminal HSPGs may share some identical epitopes. At the present study we can not eliminate either of the possibilities.

For antiserum saHSPG-2 we isolated the antigen based on both its charge and hydrophobic properties. The generated antiserum reacted with both inner and outer plexiform layers, and also ganglionic axon layer but failed to react with ILM of retina and ELM of brain. The antiserum also reacted with cell surfaces of both

retina and brain. These results suggest that core proteins of basal lamina HSPGs probably bear different epitopes from those of cell membrane HSPGs.

In this study we found that both antisera strongly reacted with inner and outer plexiform layers as well as ganglionic axon layer, indicating that HSPGs mainly reside in axon-rich region.

In summary, we found (1) saHSPG-1 stained the basal lamina of all basal lamina-containing tissues at early stages (3.5 and 4 day old) in both cross- and sagittal-sections. It strongly reacted with nerve fibers, medial longitudinal fasciculus, in the brain. (2) saHSPG-1 strongly reacted with 13-day old retina with more reactions in inner and outer plexiform layers, optic fibers and ILM. It also strongly reacted with external limiting membrane (ELM) of mesencephalon of 13-day old brain. The ILM of the retina and ELM of the brain are histologically basal laminae. (3) saHSPG-2 reacted with inner and outer plexiform layers and optic fiber layers but failed react with either ILM of the retina or ELM of the brain. (4) saHSPG-2 also reacted with neuronal cell surfaces of chick retina.

Fig. A-II-1. Composite micrograph of a cross-section of a 3.5 day old embryonic chicken stained with a polyclonal anti-cell-surface-associated heparan sulfate proteoglycan antiserum (saHSPG-1). The section was indirectly stained with saHSPG-1. A. dark field; B. phase contrast. NT = neural tube; N = notochord; S = somite; DA = descending aorta; WB = wing bud; MK = mesonephric kidney; FG = foregut. Bar = 1mm

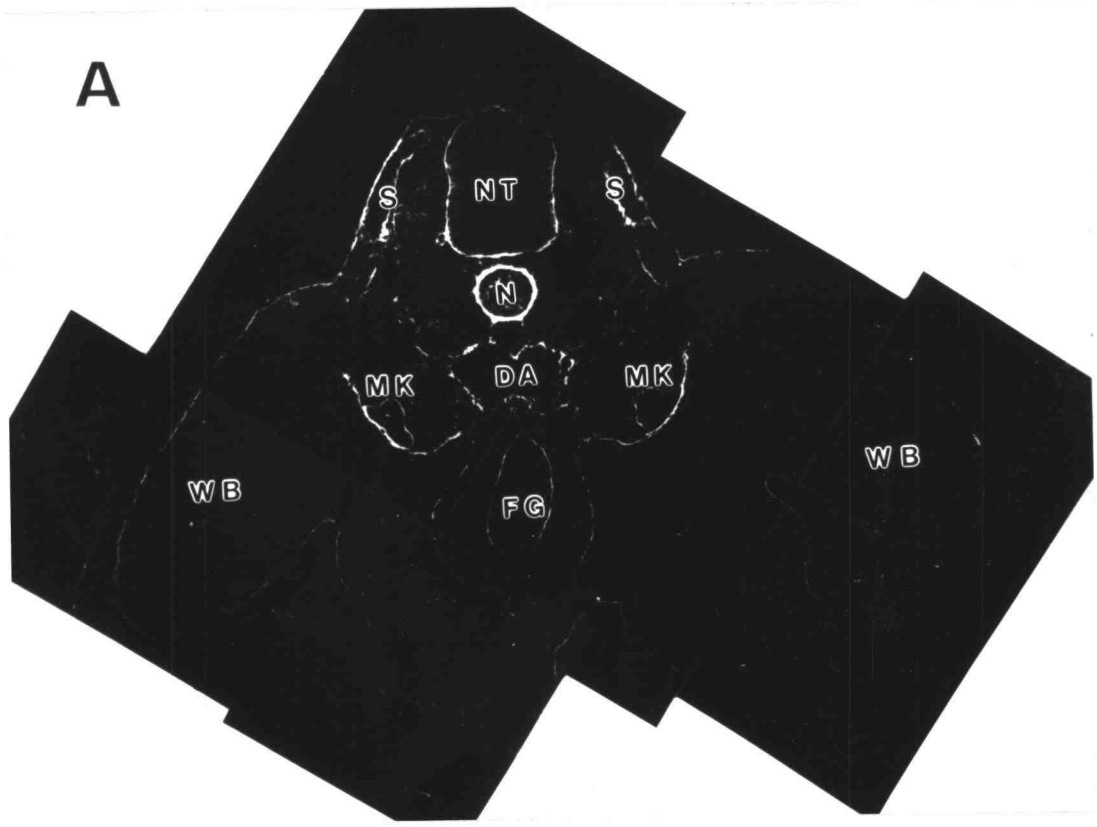

Fig. A-II-1 (Cont.)

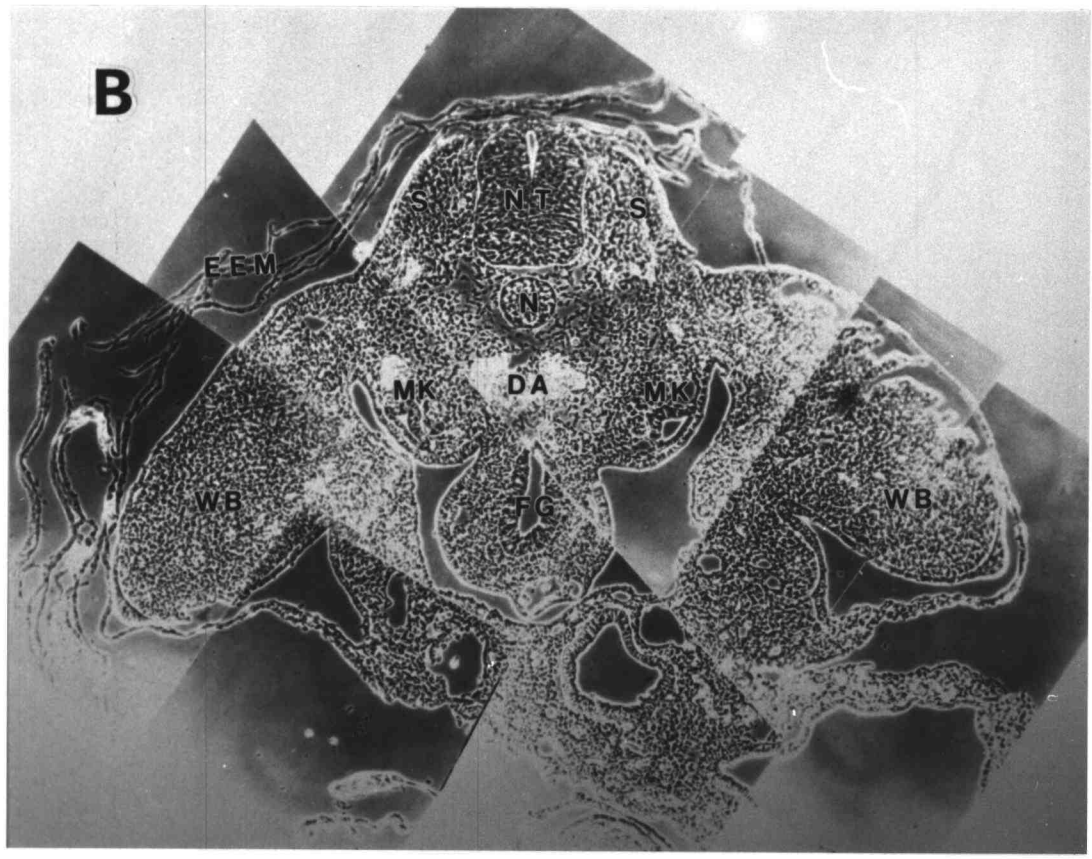

Fig. A-II-1 (Cont.)

Fig. A-II-2. Composite micrograph of a sagittal-section of a 4 day old embryonic chicken head stained with saHSPG-1. The section was indirectly stained with saHSPG-2. Insert indicates the portions of the brain. TELEN = telencephalon; DIEN = diencephalon; MESEN = mesencephalon; METEN = metencephalon; MYELEN = myelencephalon; ELM = external limiting membrane; BM = basement membrane of skin epithelium; LF = medial longitudinal fasciculus; OT = optic tectum; OC = optic chiasma. Bar = 1 mm.

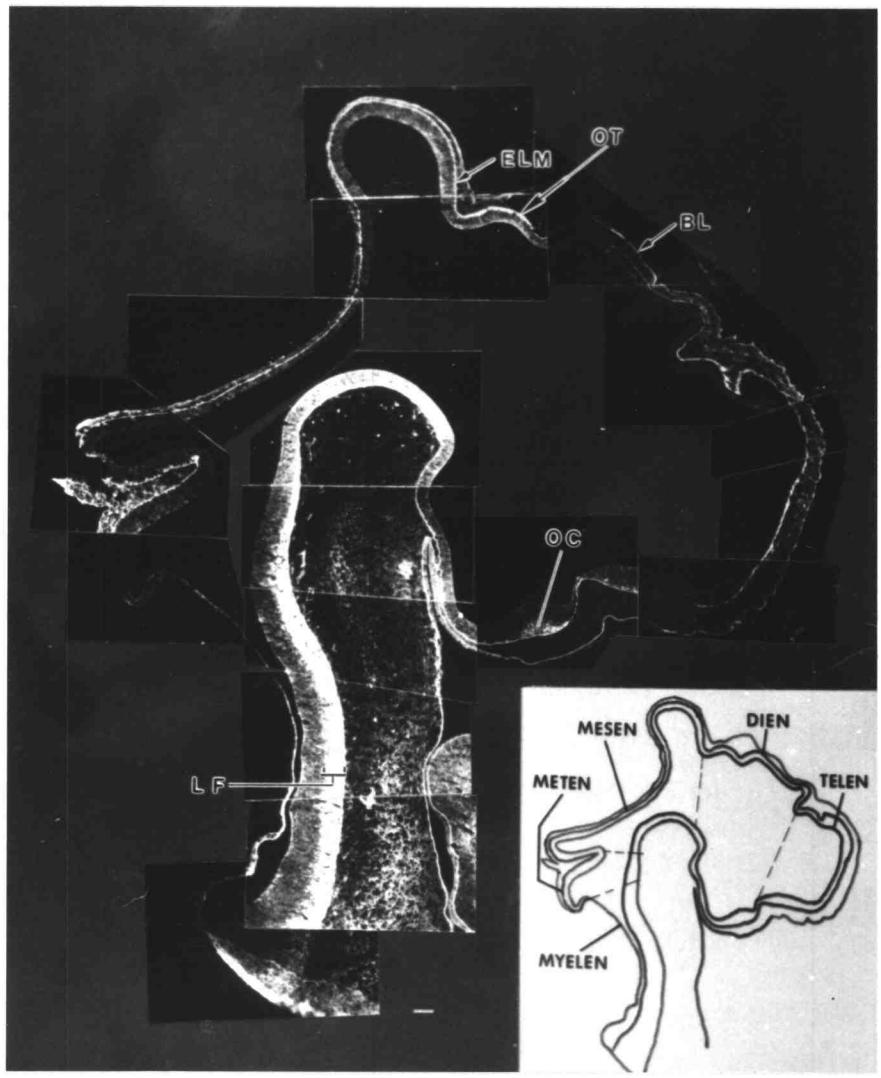

Fig. A-II-2 (Cont.)

Fig. A-II-3. Micrographs of cryosections of a 13-day old embryonic chicken retina and mesencephalon of the brain stained with saHSPG-1. The sections were indirectly stained with saHSPG-1. A. retina; B. brain. PL = photoreceptor layer; OPL = outer plexiform layer; IPL = inner plexiform layer; OF = optic fiber layer; ELM = external limiting membrane. Bar = 20μm in A and B.

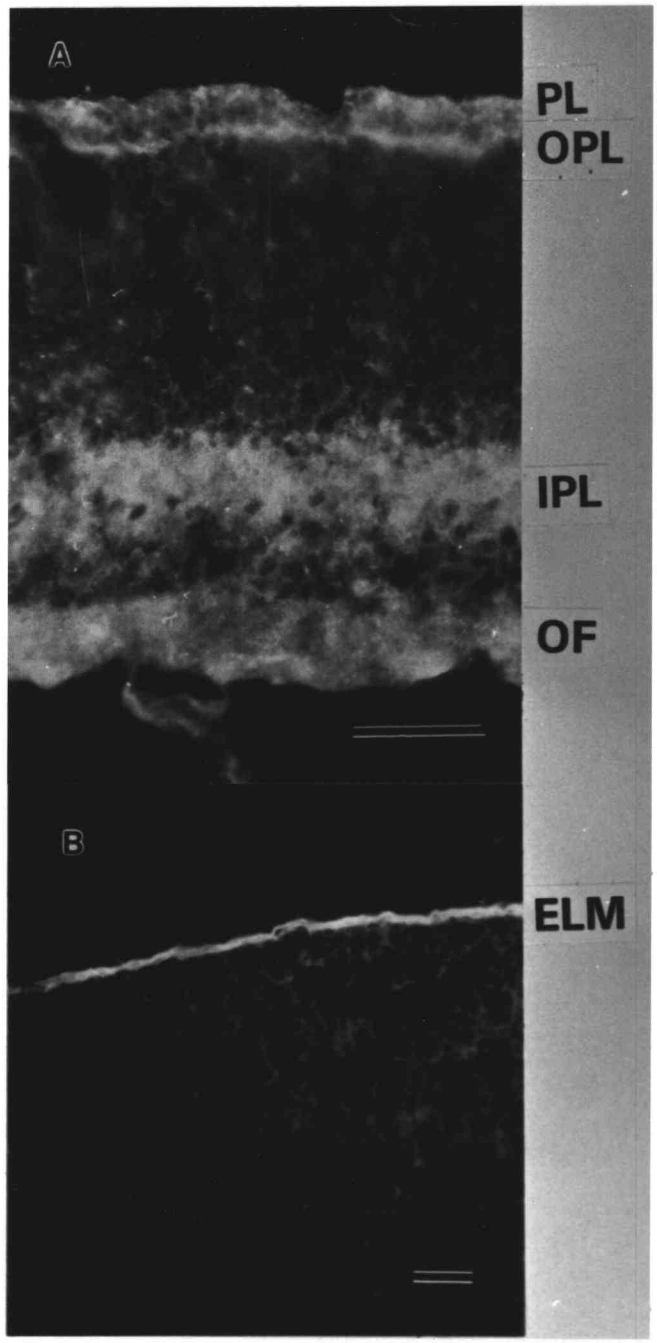

Fig. A-II-3 (Cont.)

Fig. A-II-4. Profile of DEAE gradient of proteoglycans isolated from 12 day old embryonic chick retina for antiserum saHSPG-2. A. primary DEAE profile for preparing the labeled PG tracer; B. secondary DEAE profile for the antigen (secondary DEAE profile was obtained from the pooled fractions from primary DEAE. Horizontal bars in A. and B. indicate the fractions pooled.

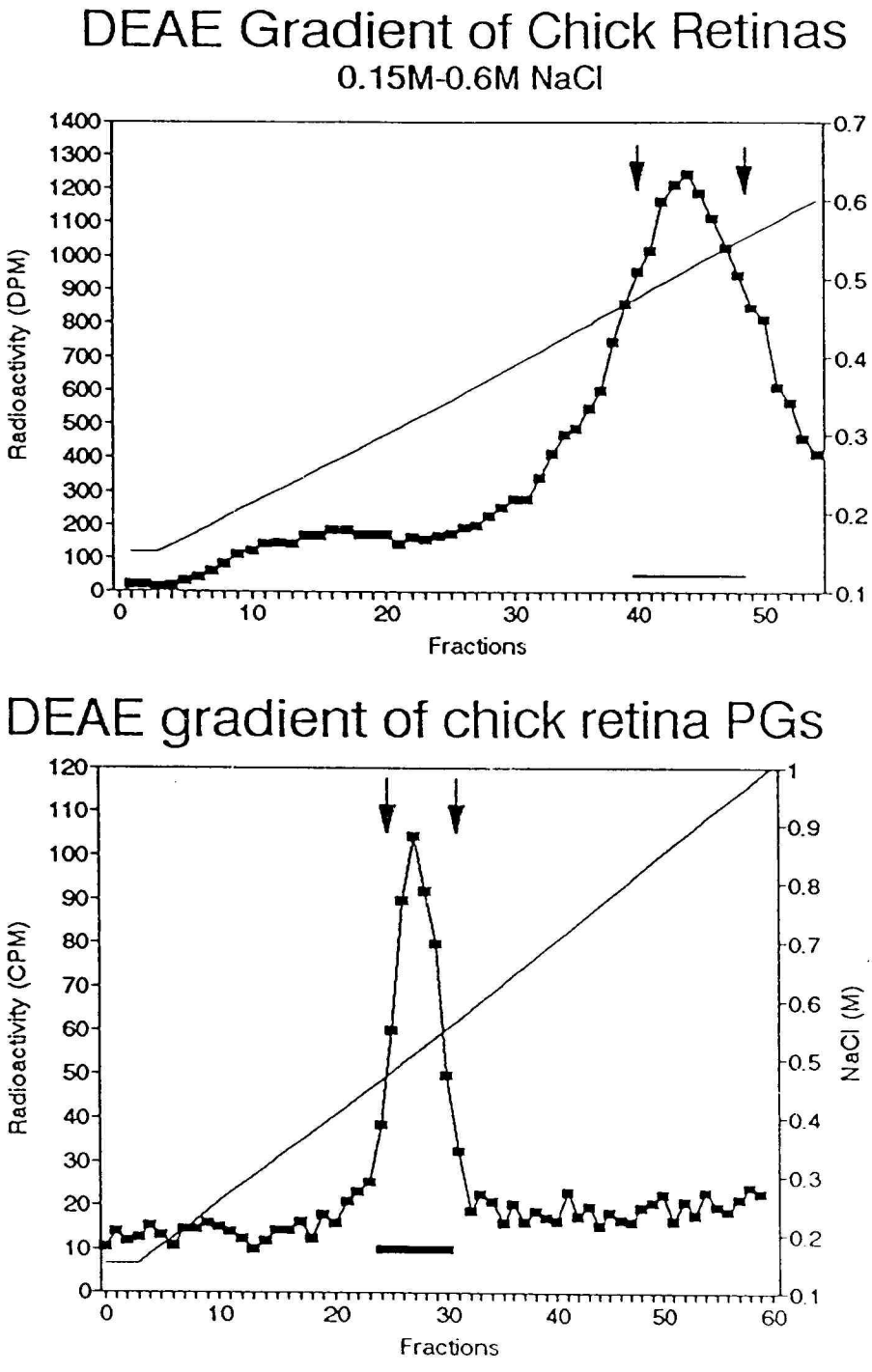

Fig. A-II-4 (Cont.)

Fig. A-II-5. Profile of the Octylsepharose gradient of proteoglycans from fractions of secondary DEAE chromatography (Fig. A-II-4). Horizontal bar indicates the fractions pooled used as antigen for antiserum saHSPG-2.

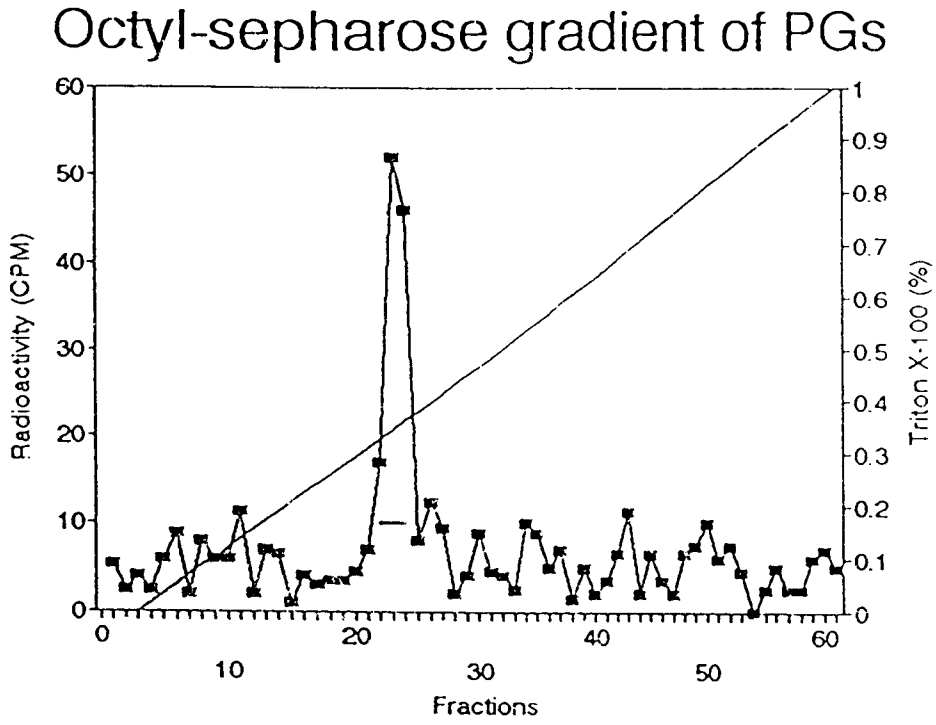

Fig. A-II-5 (Cont.)

Fig. A-II-6. Micrographs of cryosections of 10-day old embryonic chicken retina and mesencephalon of the brain stained with saHSPG-2. The sections were indirectly stained with saHSPG-2. A. retina; B. brain. PL = photoreceptor layer; OPL = outer plexiform layer; IPL = inner plexiform layer; OF = optic fiber layer; ELM = external limiting membrane. Bar = 20 μm in A; = 10 μm in B.

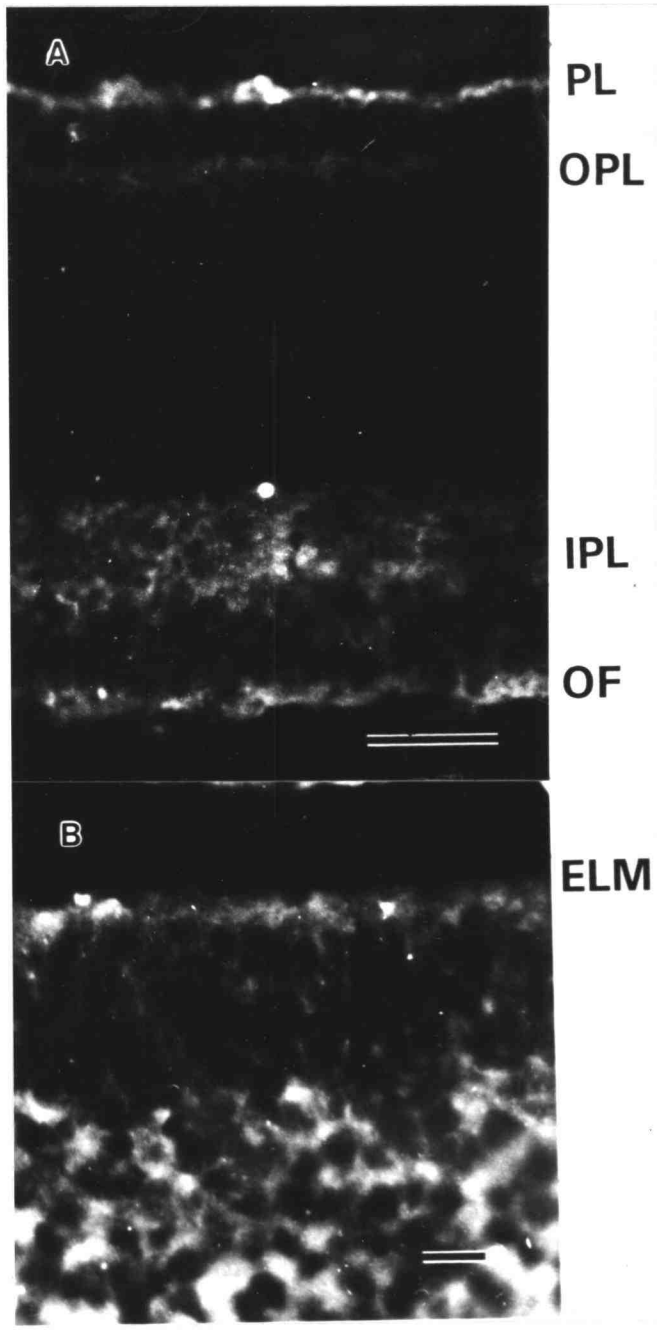

Fig. A-II-6 (Cont.)

Fig. A-II-7. Micrograph of an isolated 10-day old neuronal cell stained with saHSPG-2. The cells from embryonic chicken retina were maintained in culture for 6 to 12 hr. The cells were stained indirectly with saHSPG-2. Arrow indicates cell body of a neuron. Arrowheads indicate axon of the neuron. Bar = 10μm

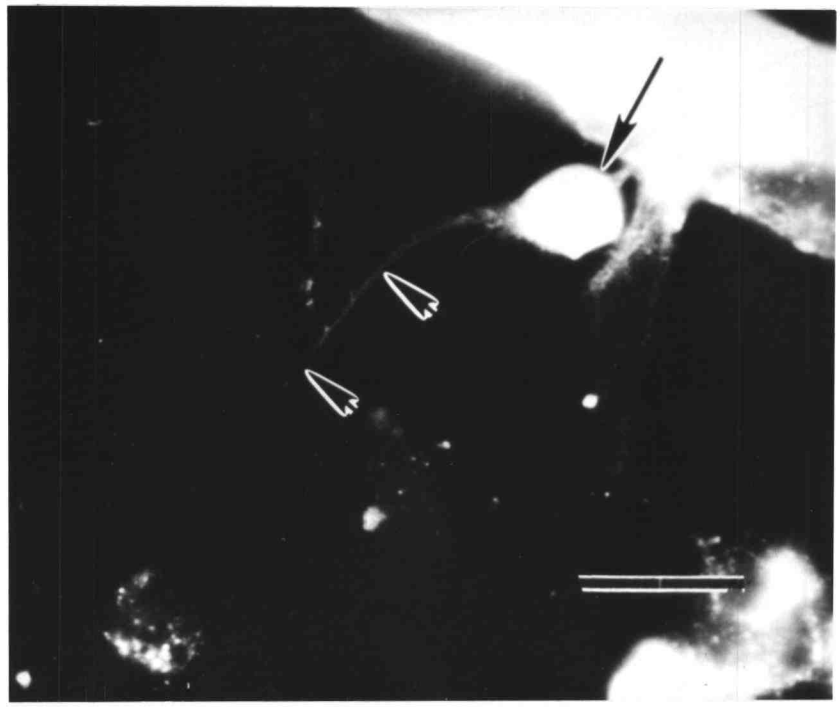

Fig. A-II-7 (Cont.)