

Spatiotemporal Expression Patterns of N-Syndecan, a Transmembrane Heparan Sulfate Proteoglycan, in Developing Retina

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PURPOSE. N-syndecan is a transmembrane heparan sulfate proteoglycan, that is highly expressed in neural tissues. In the current study, changes in N-syndecan expression during retinal development were examined.

METHODS. Localization of N-syndecan in developing rat retina was examined by immunohistochemistry and in situ hybridization. The amount of the core protein was evaluated by immunoblot analysis, using retinal homogenates at various developmental stages. In addition, mRNA expression was semi-quantified by reverse transcription-polymerase chain reaction (RT-PCR). To understand better the localization of N-syndecan in retinal neuronal cells, we performed immunocytochemistry using retinal ganglion cells in culture.

RESULTS. N-syndecan is highly expressed in nerve fiber-rich layers of the retina at early postnatal stages (between postnatal day [P]0 and P14). In contrast, immunoreactivity was faint during embryonic stages and late postnatal stages. In addition, in retinal flatmounted sections, N-syndecan immunoreactivity was observed on the axons of retinal ganglion cells. Intense signals were observed in the ganglion cell layer during in situ hybridization. Immunoblot analyses demonstrated that the amount of N-syndecan core protein reached a peak at approximately P14. The RT-PCR analyses using N-syndecan primers showed that an intense amplified band was observed in the cDNA derived from P14 retinas, whereas only faint bands were detected in the embryonic day (E)16 and P42 retinas. In retinal ganglion cells in culture, N-syndecan was located on the long, extended neurites.

CONCLUSIONS. The data show that N-syndecan is transiently expressed, primarily in retinal neural fibers, during retinal development, indicating that it may be involved in formation of the retinal neural network. (*Invest Ophthalmol Vis Sci.* 2002; 43:1616-1621)

The presence of heparan sulfate has been shown in retinal tissues by previous histochemical and biochemical studies. It has also been reported that heparan sulfate proteoglycans are expressed primarily in the basal laminae of retinal vessels, inner limiting membrane, and Bruch membrane.¹⁻⁵ More recently, the development of molecular biologic studies have enabled us to understand the molecular characterization of a number of proteoglycan core proteins. A few heparan sulfate proteoglycans, such as glypican⁶ and agrin,⁷ are known to be expressed in the optic nerve during retinal development, indicating that these proteoglycans may be involved in neuronal development of retinal tissues.

Syndecans are transmembrane proteins that carry predominantly heparan sulfate side chains.⁸ During embryonic and postnatal development, syndecans have specific and highly regulated expression patterns that are distinct from the expression in adult tissue, suggesting an active role in morphogenetic processes.⁹ Although all four mammalian syndecans are expressed in brain tissue,⁹ N-syndecan (syndecan-3), which was cloned initially from neonatal rat Schwann cells,¹⁰ is the principal syndecan expressed during early postnatal development in the central and peripheral nervous systems.¹¹ It has been reported that neurite-promoting factors, such as heparin-binding growth-associated molecule (HB-GAM),¹² basic fibroblast growth factor (bFGF),¹³ and midkine (MK)¹⁴ bind heparan sulfate side chains linked to the core protein of N-syndecan at the neuronal cell surface. In addition, it is thought that N-syndecan communicates with the cytoskeleton to enhance neurite outgrowth.¹⁵⁻¹⁸

Thus, it is possible that heparan sulfate proteoglycans play a pivotal role in formation of the neural network. However, knowledge regarding the expression of heparan sulfate proteoglycans during retinal development is limited. In this study, we report the temporal and spatial regulation of N-syndecan expression during retinal development.

MATERIALS AND METHODS

Immunohistochemistry

In the studies reported herein, Wistar rats at various developmental stages (embryonic day [E]16 to postnatal day [P]42) were used. All animals were killed by an intraperitoneal overdose injection of pentobarbital. All experimental animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines for animal experimentation of our institute. Retinal frozen sections (15 μ m) were obtained by fixation of rat eyes with 4% paraformaldehyde in phosphate buffered saline (PBS). Sections were incubated with blocking solution (2% bovine serum albumin [BSA], 2% normal horse serum and 2% normal goat serum in PBS) and then incubated for 1 hour with an anti-rat N-syndecan polyclonal antibody¹⁹ diluted 1:1000. After sections were washed with PBS, they were incubated for 30 minutes with fluorescein-conjugated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) diluted 1:200. The sections were then observed under a confocal microscope (LSM410; Carl Zeiss, Oberkochen, Germany).

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In immunohistochemical studies using flatmounted sections, after fixation in 4% paraformaldehyde, the cornea and lens were removed from the eyes. The immunohistochemical procedure just described was then performed, after which the retinas were removed from the sclera and mounted on slides.

Cell Culture

As described previously,^{20,21} retinal ganglion cells from P8 rat retina were purified by a two-step immunopanning procedure using an anti-rat macrophage monoclonal IgG (Chemicon International, Inc., Temecula, CA) and an anti-Thy-1.1 monoclonal IgG (Chemicon International, Inc.). The purified retinal ganglion cells were plated at a low density of approximately 500 cells/cm² on 12-mm glass coverslips coated with 50 µg/mL poly-L-lysine (PLL) and 10 µg/mL laminin. The cells were then cultured in medium (Neurobasal; Life Technologies, Rockville, MD) with 1 mM glutamine, 10 µg/mL gentamicin, B27 supplement (Life Technologies), 40 ng/mL human brain-derived neurotrophic factor (Dialclone Research, Besançon, France), 40 ng/mL rat ciliary neurotrophic factor (Dialclone Research), and 5 µM forskolin (Sigma, St. Louis, MO), as described previously.^{21,22} Cultures were maintained at 37°C in a 5% CO₂ incubator. Immunocytochemical studies using the anti-N-syndecan antibody were performed at 1 and 3 days after seeding of the cells. The immunohistochemical results were confirmed from five experiments in at least four different eyes at each developmental stage.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Retinal total RNA at several stages of development (E16, P14, and P42) was collected by the acid guanidium thiocyanate-phenol chloroform extraction method, as described previously.²³ cDNAs for subsequent RT-PCR experiments were synthesized from the retinal total RNA with the use of reverse transcriptase (First-Strand cDNA Synthesis Kit; Amersham Pharmacia Biotech, Uppsala, Sweden), as described previously.²⁴ After normalization of each cDNA concentration using primers to β -actin, AGCTGAGAGGGAAATCGTGC (sense) and ACCAGACAGCACTGTGTTGG (antisense),²⁵ PCR experiments using primers to N-syndecan, AGTACCCTACCACCCACTA (sense) and TAGATGAGCAACGTGACCAG (antisense),²⁶ were performed. The following conditions were used: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and polymerization at 72°C for 1 minute for 20 cycles (β -actin primers) or 30 cycles (N-syndecan primers). The PCR products were then separated by 2% agarose gel electrophoresis. To investigate relative levels of N-syndecan gene expression, semiquantitative analysis was performed by measurement of the optical densities of the PCR bands using NIH Image (NIH Image, ver. 1.59; provided in the public domain by the National Institutes of Health, Bethesda, MD, and available at <http://rsb.info.nih.gov/nih-image/>). A standard curve was generated from the optical densities of bands from serial dilutions of template cDNAs, and the linearity of the created standard curve among the selected concentrations was confirmed. The relative levels of mRNA expression were then calculated.

In Situ Hybridization

The PCR product (1000 bp), generated with primers to N-syndecan, CAATGAGAAGCTTCGAGAGGC (sense) and CCGTCTAGTATGCTCTCT (antisense),²⁶ was inserted into a vector (pCR4-TOPO; Invitrogen, Carlsbad, CA). Digoxigenin (DIG)-labeled RNA probes were raised with a DIG-RNA labeling kit (Roche, Basel, Switzerland). The subsequent procedures of hybridization, washing, and detection were performed according to the previous report.²⁷

Immunoblot Analysis

As described previously,²⁴ rat retinal tissues at various developmental stages (E16–P42) were homogenized in 50 µL ice-cold PBS containing 10 mM *N*-ethylmaleimide (NEM), 20 mM EDTA, and 2 mM phenylmeth-

ylsulfonyl fluoride (PMSF). The homogenates were then mixed with 200 µL of 20 mM Tris-HCl buffer (pH 7.5) containing 2% sodium dodecyl sulfate (SDS), 10 mM NEM, 20 mM EDTA, and 2 mM PMSF and boiled for 5 minutes. The protein concentration of the boiled solution was measured by a protein assay kit (DC; Bio-Rad Laboratories, Tokyo, Japan). Proteins (200 µg) were precipitated from the solution by adding 3 volumes of 95% ethanol-1.3% potassium acetate. The pellet was washed with 400 µL of 70% ethanol containing 1.0% potassium acetate and recentrifuged. The pellet was suspended in 104 µL of distilled water. To this suspension, 15 µL of 1 M Tris-HCl-0.3 M sodium acetate (pH 7.5), 15 µL of 50 mM calcium acetate, 4 µL inhibitor mixture-I (20 mM PMSF and 7.2 mM pepstatin) and 8 µL inhibitor mixture-II (0.1 M EDTA and 0.1 M NEM) were added. To digest the heparan sulfate side chains linked to core proteins, 4 µL heparitinase I (EC 4.2.2.8; Seikagaku, Tokyo, Japan) enzyme solution (1 mU/µL) was added to the sample.¹⁹ After the treatment with heparitinase I, the sample was electrophoresed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 3% stacking gel and a 6% separating gel and then transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was incubated in the blocking solution for 1 hour at room temperature and then incubated in the anti-N-syndecan antibody for 2 hours and subsequently incubated in biotinylated anti-rabbit IgG for 30 minutes at room temperature. After the incubation with an avidin-biotin complex (ABC) kit (Vectastain elite; Vector Laboratories), immunoreactive materials on the membrane were detected using 4-chloro-1-naphthol.

RESULTS

Immunohistochemistry for N-Syndecan during Retinal Development

At E16, N-syndecan immunoreactivity in rat retinas was faint (Fig. 1). Faint immunoreactivity was observed in the ganglion cell layer (GCL), retinal pigment epithelium (RPE), and choroidal tissue (CT). Around birth (P0), the inner layers, such as the nerve fiber layer (NFL), GCL, and inner plexiform layer (IPL), were clearly formed, and intense immunoreactivity was present in the NFL and IPL. Moreover, the optic nerve (ON) was intensely immunopositive (Fig. 2A). In other ocular tissues, the RPE, CT, and iris were immunopositive. In transverse sections of the retinas through the optic disc, intense immunoreactivity in the NFL and IPL was observed from the central regions near the ON to the peripheral regions of the retinas. Between P7 and P14, as the outer plexiform layer (OPL) developed, it also became immunopositive (Fig. 1). The immunoreactivities in the NFL were not uniform along the layer in postnatal rat retinal sections. We performed further immunohistochemical analysis using flatmounted sections to identify the detailed immunolocalization in the NFL. In flatmounted sections, N-syndecan immunoreactivity was observed on the axons of retinal ganglion cells (Fig. 2E). In addition, in the transverse retinal sections after P7, stratified immunostaining patterns were observed in the IPL. As differentiation of the retinal layers proceeded (from P21 to P42), the immunoreactivity gradually decreased (Fig. 1). In adult rat retinas (P42), N-syndecan immunoreactivity was faint (Figs. 1, 2D). The immunoreactivity in the ON also gradually decreased between P7 and P14 and had significantly decreased by P42 (Figs. 2C, 2D).

Immunocytochemistry for N-Syndecan in Retinal Ganglion Cells In Vitro

On the day after the purified retinal ganglion cells were seeded, the cells extended only short neurites on coverslips coated with PLL and laminin, as described previously,^{21,22} and N-syndecan immunoreactivity was observed on the surface of the soma and short neurites (Fig. 3A). At 3 days after seeding,

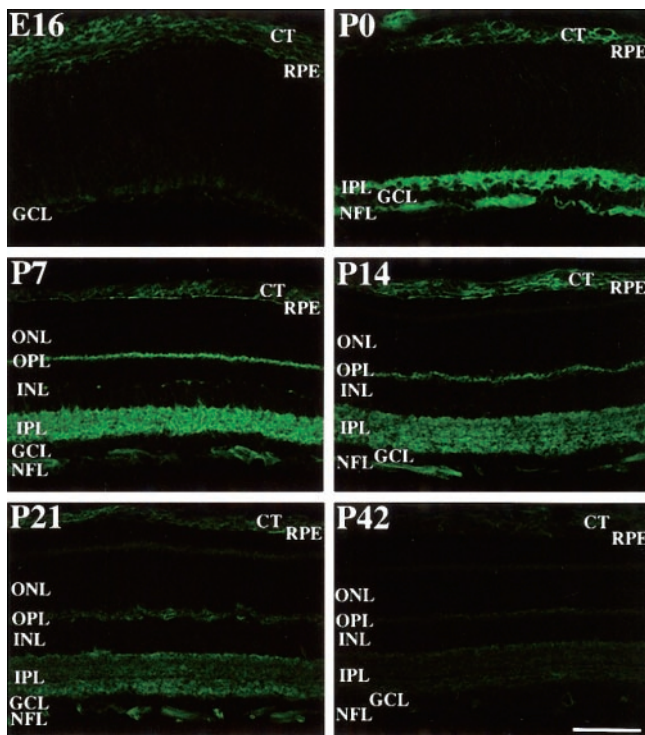


FIGURE 1. Immunohistochemistry for N-syndecan during retinal development. At E16, faint immunoreactivity was observed in the GCL, RPE, and CT. At P0, intense immunoreactivity was present in the NFL and IPL. The RPE and CT were faintly immunopositive. Between P7 and P14, the NFL, IPL, and OPL were intensely immunopositive. In addition, stratified immunostaining patterns were observed in the IPL. Between P21 and P42, immunoreactivity in the retina became gradually more faint. The immunoreactivity in the NFL was not uniform between P0 and P42. Scale bar, 100 μ m.

N-syndecan immunoreactivity was intense on the long neurites ($>50 \mu$ m) that extended from the cultured retinal ganglion cells (Fig. 3B).

Expression of N-Syndecan mRNA during Retinal Development

To examine the relative levels of mRNA expression of N-syndecan during retinal development, we performed semi-quantitative RT-PCR experiments after normalization to β -actin (Figs. 4A-C). An intensely amplified PCR band using N-syndecan primers (540 bp) was obtained in the cDNA derived from P14 rat retinas. In contrast, the cDNA derived from E16 and P42 retinas yielded only faint bands. The mean \pm SE expression at E16 and P42 was only $13.2\% \pm 7.6\%$ and $9.3\% \pm 4.5\%$, respectively, of that at P14 (defined as 100%).

To identify the localization of the N-syndecan gene expression, *in situ* hybridization was performed. Strong signals were detected in the cell bodies within the GCL (Fig. 4D). The cells in the inner part and outer part within the INL were also positive. In addition, diffuse signals were observed in the ONL. There were no obvious signals in the retinal section examined by the sense probe (Fig. 4E).

Glycosaminoglycan Side Chains of N-Syndecan in the Retina

On immunoblot analysis using brain homogenates before treatment with heparitinase I, a broad band that corresponded to a molecular mass of approximately 200 kDa was detected, as described previously (Fig. 5).^{13,19} After the homogenate was

digested with heparitinase I, the broad band became more distinct, with a molecular mass of 140 kDa, which indicates that the antibody reacts with N-syndecan core protein, as reported previously.¹⁹ When we performed the same procedure using homogenates from P14 rat retinal tissues without heparitinase I digestion, an immunopositive band of approximately 200 kDa was also detected, and after treatment with heparitinase I, an intense immunopositive band was observed at 140 kDa. In addition, a weakly heparitinase-resistant immunopositive band was detected at 120 kDa in retinal homogenates. Thus, our immunoblot analysis of retinal homogenates showed that the N-syndecan immunoreactivity in retinal sections was truly derived from N-syndecan core protein bearing heparan sulfate side chains.

Developmental Change in the Amounts of N-Syndecan in the Retina

To examine temporal alterations in the expression of N-syndecan core protein in developing retina, retinal homogenates (each, 50 μ g of protein) at various developmental stages from E16 to P42 were treated with heparitinase I and then subjected to immunoblot analysis (Fig. 6A). During perinatal stages (between E18 and P7), the intensity of the N-syndecan-immunopositive band increased gradually, being very faint at E16 and reaching a peak at P14. After P14 the intensity decreased gradually. Intensities of the immunopositive bands were semi-quantified by means of a densitometric analysis, and relative levels were calculated as the percentage of the mean level at peak (P14; Fig. 6B). The mean level \pm SE at E16 was $9.8\% \pm 1.3\%$ of the peak intensity at P14 (defined as 100%) and then increased gradually. The level at P0 was $41.1\% \pm 11.6\%$ and was near the peak level ($84.6\% \pm 0.7\%$) at P7. After the peak at P14, the intensity gradually decreased to $23.4\% \pm 1.4\%$ at P42.

DISCUSSION

Four different syndecans, designated as syndecans 1, through 4, are expressed in vertebrate tissues.⁸ Although all these syndecans are observed in central nervous system tissues,⁹ N-syndecan (syndecan-3) is most highly expressed in developing neural tissues.¹⁰ It has been reported that N-syndecan on the neural surface binds to neurite-promoting factors, such as HB-GAM,¹² bFGF,¹³ and MK.¹⁴ Furthermore, N-syndecan has been implicated in the receptor mechanism of those factors and has been thought to play a role in transducing extracellular signals into cellular responses, such as organization of actin filaments.²⁸ Our immunohistochemical analysis demonstrated the intense immunoreactivity of N-syndecan in nerve fiber-rich layers, such as the NFL, IPL, and OPL, during early postnatal stages. The results of immunoblot analyses showed that large amounts of N-syndecan core protein were expressed in P14 rat retinas, which is supported by results of our RT-PCR experiments. Thus, mRNA and protein expressions of N-syndecan are abundant during the early postnatal period. In contrast, in adult rat retinas N-syndecan expression dramatically decreases. Our data indicate that N-syndecan may play a pivotal role in some developmental processes in the nerve fiber-rich layers during early postnatal stages.

It has been known that active dendrite branching and the formation of synapses are observed in the IPL and OPL between P7 and P14.²⁹⁻³¹ It has also been shown that, at P14, the electroretinogram becomes the same as that of the adult rat.²⁹ Walz et al.³² reported that, when heparan sulfate derived from perlecan, which preferentially binds bFGF, is added exogenously to the developing optic pathway, the heparan sulfate severely disrupts target recognition, causing axons from the retina to bypass the optic tectum. Moreover, the heparan

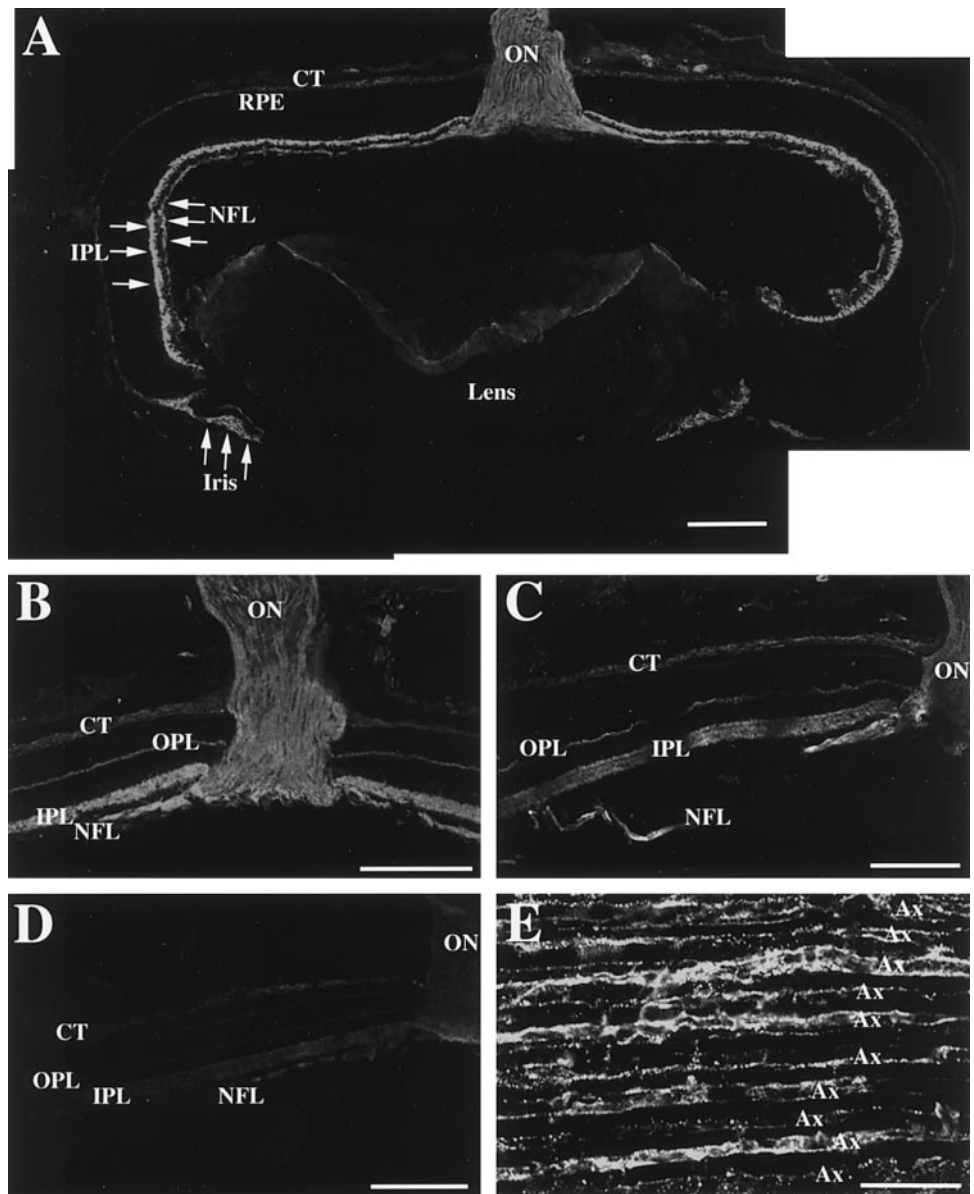


FIGURE 2. Immunolocalization of N-syndecan in rat eyes. (A) Immunoreactivity in neonatal rat eyes. Intense immunoreactivity was observed in the NFL (arrows), IPL (arrows), and ON. The intense immunoreactivity was observed from the central regions near the ON to the peripheral regions of the retina. The RPE, CT, and iris (arrows) were also immunopositive. Area surrounding the ON at (B) P7, (C) P14, and (D) P42. (E) Immunohistochemistry using a flat-mounted section at P7. N-syndecan was immunolocalized on the axons (Ax) from retinal ganglion cells. Scale bar: 250 μ m (A-D); 50 μ m (E).

sulfate promotes neurite outgrowth from retinal neuronal cells when the molecule binds to bFGF. Because heparan sulfate side chains linked to N-syndecan core protein also bind to bFGF,¹³ it is possible that N-syndecan may be involved in the target recognition of neurites and synaptogenesis in the nerve fiber-rich layers during early postnatal stages, binding to bFGF.

Also, some reports have shown that the high affinity of bFGF for the synaptic regions in those retinal layers is observed during retinal development.^{33,34} Agrin, another heparan sulfate proteoglycan highly expressed in the neural retina, is also present in the IPL during retinal development.³⁵ Heparan sulfate derived from those proteoglycans may be related to syn-

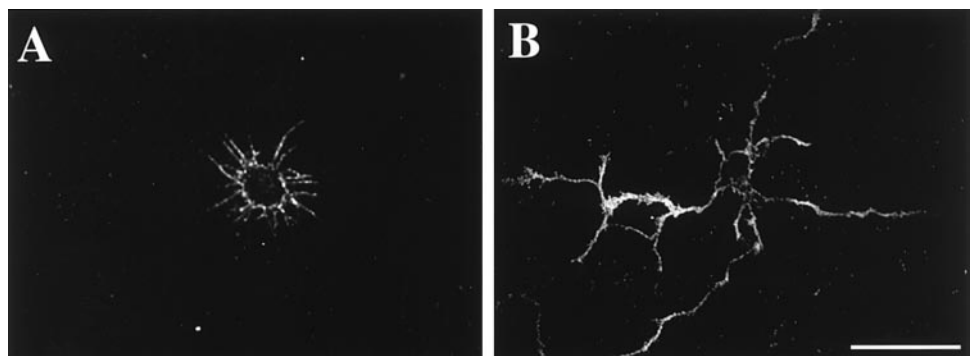


FIGURE 3. N-syndecan expression from retinal ganglion cells in vitro. (A) A retinal ganglion cell at 1 day after seeding. When the cells had short neurites only, surfaces of the cell bodies and the short neurites were immunopositive. (B) A cell at 3 days in vitro. N-syndecan immunoreactivity was intense on the long, extended neurites. Scale bar, 50 μ m.

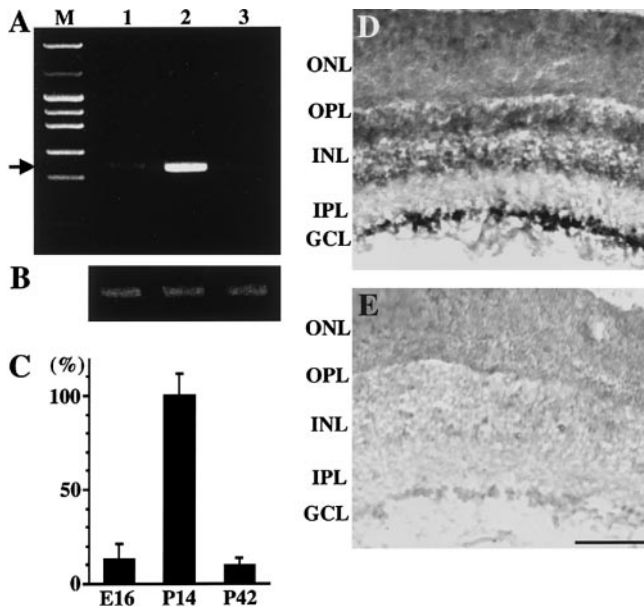


FIGURE 4. Representative PCR experiments to determine N-syndecan gene expression during retinal development. PCR was performed using (A) the N-syndecan primers after cDNA concentration was normalized to (B) β -actin gene expression. PCR products of the expected length (540 bp) were amplified (arrow). Lane 1: E16; lane 2: P14; and lane 3: P42. (C) Peak intensities of the PCR bands occurred at P14 ($n = 3$). Error bar; SE. (D) In situ hybridization using the antisense probe in the retinal section at P10. (E) The control experiment using the sense probe. Scale bar, 100 μ m.

apse formation in the IPL. Further studies are needed to identify the significance of N-syndecan expression in retinal neuronal cells.

It has been reported that N-syndecan is expressed in brain, spinal cord, and peripheral nerve tissue of rats at various ages¹¹ and that this expression increases greatly after birth and reaches a peak at about P7. After this, the expression declines gradually.^{11,19,26} This transient expression is similar to that of

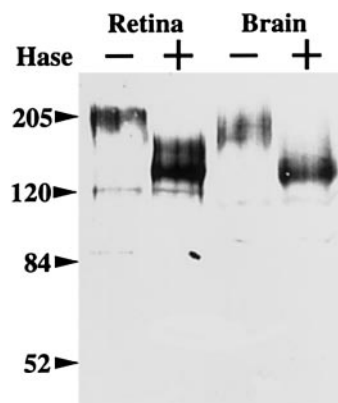


FIGURE 5. Immunoblotting for N-syndecan. In the retinal homogenate before treatment (-) with heparitinase I (Hase), a broad band that corresponded to a molecular mass of approximately 200 kDa was detected. After treatment (+), the broad band became more distinct with a molecular mass of 140 kDa. In addition, a heparitinase-resistant immunopositive band was weakly detected at 120 kDa. In the brain homogenate before treatment with heparitinase I, a broad band was detected at approximately 200 kDa, whereas, after treatment, a 140 kDa-band was observed. The positions of the molecular mass markers are indicated in kilodaltons.

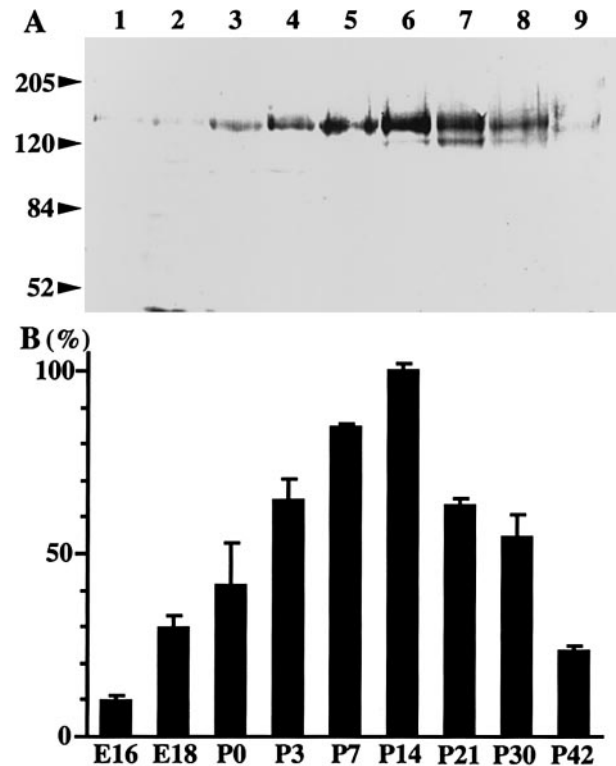


FIGURE 6. Immunoblot analysis for N-syndecan during retinal development. (A) Representative immunoblot analysis using retinal homogenates from E16 to P42 treated with heparitinase I. Intensity of the 140-kDa immunopositive band increased gradually as retinal development proceeded (between E16 and P14), and then decreased after P14. The positions of molecular mass markers are indicated in kilodaltons. (B) Densitometric analysis of intensities of immunopositive bands. The relative levels were calculated as the percentage of the mean levels at the peak (P14; $n = 3$). Error bar, SE.

change in retinal development. In the brain and peripheral nerve, N-syndecan is produced from oligodendrocytes¹¹ and Schwann cells,¹⁰ both of which play a role in myelination of nerve fibers during the development of neural tissue.¹¹ On the other hand, in embryonic rat brain neurons *in vitro*³⁶ and in cerebellar granule cells,³⁷ N-syndecan is expressed on the surfaces of the neurites. In retinal tissues, N-syndecan was highly expressed in the nerve fiber-rich layers during early postnatal stages. After P7, stratified immunoreactivity was observed in the IPL, indicating that the distribution of N-syndecan in the IPL may be associated with neural fibers from retinal neuronal cells, in that the neurites of ganglion cells,³⁸ bipolar cells,³⁹ and amacrine cells⁴⁰ form stratifications in the IPL. In addition, in our immunohistochemical analyses using transverse retinal sections, N-syndecan immunoreactivity in the NFL was not uniform. Because the immunohistochemical analysis using flatmounted sections clearly showed that its immunoreactivity is derived from the axons of retinal ganglion cells, the discontinuous staining of the NFL in the transverse sections suggests that N-syndecan is exclusively localized in the nerve fibers. Considering all evidence, it is thought that N-syndecan may be expressed from neuronal cells in retinas developing in the early postnatal period and distributed in their neurites. Moreover, *in situ* hybridization showed strong signals in the cell bodies within the GCL, probably retinal ganglion cells. The positive cells in the inner part and the outer part of the inner nuclear layer (INL) seem to correspond with amacrine cells and horizontal cells, respectively. Retinal ganglion cells in culture, which have lost their axons during the purifying pro-

cedure, extend their long neurites at 3 days after seeding. N-syndecan is immunolocalized on these extended neurites, supporting our hypothesis of N-syndecan expression from neuronal cells. Although we cannot exclude the possibility that retinal glial cells express N-syndecan, it is thought that neuronal cells are mainly responsible for production of N-syndecan in the retina.

In conclusion, we have shown spatiotemporal expression patterns of N-syndecan during retinal development. N-syndecan is mainly distributed in the neurites from retinal neuronal cells during early postnatal stages. Our data indicate that N-syndecan may play a role in the formation of the retinal neural network.

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