Expression and Localization of Neural Cell Adhesion Molecule and Polysialic Acid during Chick Corneal Development

Xiuli Mao, Tyler Schwend, and Gary W. Conrad

PURPOSE. To assay for expression and localization of neural cell adhesion molecule (NCAM) and polysialic acid (polySia) in the chick cornea during embryonic and postnatal development.

METHODS. Real time quantitative PCR and Western blot analyses were used to determine NCAM expression and polysialylation in embryonic, hatching, and adult chick corneas. Immunofluorescence staining for NCAM and polySia was conducted on cryosections of embryonic and adult corneas, whole embryonic corneas, and trigeminal neurons.

RESULTS. NCAM and ST8SiaII mRNA transcripts peaked by embryonic day (E)9, remained steady between E10 and E14 and slowly decreased thereafter during embryonic development. Both gene transcripts showed > 190-fold decline in the adult chick cornea compared with E9. In contrast, ST8SiaIV expression gradually decreased 26.5-fold from E6 to E19, increased thereafter, and rose to the early embryonic level in the adult cornea. Western blot analysis revealed NCAM was polysialylated and its expression developmentally changed. Other polysialylated proteins aside from NCAM were also detected by Western blot analysis. Five NCAM isoforms including NCAM-120, NCAM-180 and three soluble NCAM isoforms with low molecular weights (87–96 kDa) were present in chick corneas, with NCAM-120 being the predominate isoform. NCAM was localized to the epithelium, stroma, and stromal extracellular matrix (ECM) of the embryonic cornea. In stroma, NCAM expression shifted from anterior to posterior stroma during embryonic development and eventually became undetectable in 20-week-old adult cornea. Additionally, both NCAM and polySia were detected on embryonic corneal and pericorneal nerves.

CONCLUSIONS. NCAM and polySia are expressed and developmentally regulated in chick corneas. Both membrane-associated and soluble NCAM isoforms are expressed in chick corneas. The distributions of NCAM and polySia in cornea and on corneal nerves suggest their potential functions in corneal innervation. (Invest Ophthalmol Vis Sci. 2012;53:1234–1243) DOI:10.1167/iovs.11-8834

The cornea is the most densely innervated and sensitive tissue on the surface of the body. During early chick embryonic development, corneal nerves are derived from neural crest cells located in the ophthalmic lobe of the trigeminal ganglion.1–4 Trigeminal nerve axon fascicles reach the corneal periphery by embryonic day (E)5. The nerves are repelled from the cornea and form a perilimbal ring around the cornea until E9.5–7 Beginning at E9, nerves from the ring invade the anterior stroma, branch, and extend toward the center of the cornea. By E12, the nerves migrate from the stroma, penetrate the basement membrane, intermingle with the epithelium, and reach the center of the cornea by E15.1,5 By E18 the nerves complete the innervation of the cornea.6–9 Recently work has shown that secreted neuronal guidance proteins, such as Semaphorin 3A and Slit2, are involved in orchestrating this pattern of nerve development and distribution.10–19 However, in addition, carbohydrate moieties on proteins, such as polysialic acid (polySia) on neural cell adhesion molecule (NCAM), are functionally significant during axon outgrowth, guidance, plasticity, neural repair, and regeneration in the central nervous system (CNS) and peripheral nervous system (PNS).10–17 Expression of NCAM and associated posttranslational modifications have not been studied previously during embryonic development of corneal nerves.

NCAM is an immunoglobulin superfamily cell adhesion molecule. The three major isoforms of NCAM are NCAM-120, -140, and -180. NCAM-120 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein, whereas NCAM-140 and -180 are transmembrane proteins.18 Particularly high levels of NCAM are expressed in the nervous system,19 but also are expressed in nonneuronal tissues, such as lungs, muscles, kidneys, stomach, and heart.20 In addition to membrane-associated isoforms, soluble forms of NCAM have been found in rat brain, cerebrospinal fluid, and plasma21–23; human serum and amniotic fluid24; and culture media of chick retinal cells.25 Soluble NCAM exists in different isoforms, with various molecular weights ranging from 180 kDa to 100 kDa,26–31 which are produced via alternative splicing of the transcripts, enzymatic cleavage of the extracellular domain of membrane-associated NCAM, and detached NCAM-containing membrane fragments.32 All these NCAM isoforms can be modified posttranslationally with polySia.

PolySia is a unique and highly negatively charged homopolymer of sialic acid residues mostly with α2-8 linkage. The degree of polymerization of the polySia moieties on NCAM can be as high as 400 residues.33 The large, negatively charged, and highly hydrated structure of polySia on NCAM can increase the intermembrane space and disrupt the adhesive properties of NCAM,34–36 thus influencing cell-cell interaction and communication.37–39 During embryonic nervous system development, polySia on NCAM is regarded as a prominent regulator of neural cell migration and differentiation, nerve outgrowth, axon guidance, and targeting.12,34,40 It also contributes significantly to neurogenesis, synaptic plasticity, and repair in the postnatal nervous system.17,40–42

The function of polySia on soluble NCAM is not well understood. However, it has been demonstrated that soluble
NCAM can interfere with the homophilic interaction between membrane-associated NCAM molecules and reduce NCAM-mediated cell adhesion. Thus, soluble NCAM can modulate neurite outgrowth and branching.\textsuperscript{3,4} and also can promote Schwann cell migration.\textsuperscript{44}

In the CNS, the polySia on NCAM is downregulated during embryonic development and is persistently expressed at sites limited to ongoing neurogenesis or plasticity.\textsuperscript{1,11} In chick, NCAM is first detected in the gastrula (primitive streak) stage, continues to be expressed at high levels throughout the developing central and peripheral nervous system, and persists at low levels in the adult.\textsuperscript{15–48} Studies have shown that NCAM is expressed in chick retina and regulates retinal ganglion cell nerve outgrowth and guides ganglion cell axons.\textsuperscript{2,5,12} To date, NCAM expression, distribution, and the extent to which it is polysialylated in developing corneas have not yet been reported previously. In this work, the expression and localization of NCAM and polySia in chick corneas during embryonic and postnatal development were investigated, including polysialylation of NCAM and other proteins, and the distribution of NCAM and polySia in the chick cornea and on corneal and pericorneal nerves.

**METHODS**

**Chick Husbandry and Corneal Isolation**

Fertile White Leghorn chicken eggs, newly hatched chicks (1-day-old; 1 D) and adult chickens (20-week-old; 20 weeks) used in this work were all from a local hatchery. The fertile chicken eggs were incubated at 38°C and 45% humidity from E0. Hatched chickens were all handled under an approved Institutional Animal Care and Use Committee (IACUC) protocol. Corneas from chick embryos of the desired ages and chickens were dissected free of sclera and limbus tissue in sterile phosphate buffered saline (PBS), snap frozen in liquid nitrogen, and stored at −80°C until used.

**RNA Isolation and Real Time Q-PCR**

For each data point of real time quantitative (Q)-PCR, three separate RNA isolations, cDNA synthesis, and real time Q-PCR reactions were performed as previously described.\textsuperscript{53} Corneas of desired embryonic ages between E6 and E20, newly hatched chicks (1 D), and adult chickens (20 weeks) were all from a local hatchery. The fertile chicken eggs were incubated at 38°C and 45% humidity from E0. Hatched chickens were all handled under an approved Institutional Animal Care and Use Committee (IACUC) protocol. Corneas from chick embryos of the desired ages and chickens were dissected free of sclera and limbus tissue in sterile phosphate buffered saline (PBS), snap frozen in liquid nitrogen, and stored at −80°C until used.

**Total Protein Extraction**

The previously snap frozen corneas of chick embryos (E7, E9, E10, E12, E14, E16, E18, and E20), newly hatched chicks (1 D), adult chickens (20 weeks) and E12 chick brain were pulverized in liquid nitrogen and homogenized in an NP-40 extraction buffer containing protease inhibitors (Total Protein Extraction Kit; Millipore, Billerica, MA). The extraction buffer was composed of HEPES (pH 7.9), MgCl\textsubscript{2}, KCl, EDTA, sucrose, glycerol, sodium deoxycholate, NP-40, and sodium orthovanadate. Total protein was extracted according to the manufacturer’s protocol. The concentration of total protein was determined using an assay (BCA Protein Assay Kit; Pierce, Rockford, IL).

**Digestion with Peptide-N-Glycosidase F**

Each solution of 20 μg total protein extracted from corneas of the desired ages was diluted to 45 μL with deionized (DI) water followed by incubation with 2 μL peptide-N-glycosidase F (N-Glycanase; ProZyme, Hayward, CA) overnight at 37°C to deglycosylate proteins. The digestion was terminated by adding SDS-PAGE sample buffer and incubating at 70°C for 10 minutes.

**Immunoblotting Analysis**

Twenty micrograms of total protein, N-Glycanase-treated total protein from corneas of desired developmental stages or E12 chick brain were subjected to SDS-PAGE gel electrophoresis. Proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane, blocked with the blocking solution from a Western blotting kit (Invitrogen Western-Breeze Chromogenic Immunodetection Kit; Invitrogen, Carlsbad, CA), and then incubated with the antibody against the extracellular domain of NCAM (5e, 1:1000; Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), the cytoplasmic domain of NCAM (4d, 1:1000; Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), polySia-NCAM (2-2B, 1:500; Miltenyi Biotec Inc., Auburn, CA), or GAPDH (6C5, 1:1000; Thermo Scientific, Waltham, MA) in the blocking solution. This was followed by three washes in washing solution and incubation with an alkaline phosphatase-conjugated secondary antibody in the blocking solution. NCAM, GAPDH, or polySia was visualized with chromogenic solution (BCIP/NBT; Invitrogen). GAPDH was chosen as sample loading control.

**Table 1.** Real Time Q-PCR Primers for GAPDH, NCAM, ST8SialI, and ST8SialIV

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name/GI Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate-dehydrogenase/4105595</td>
<td>5′-GCTGAGAACGGGAAACTTGTGA</td>
<td>5′-GACATGATGTCCGAGCACTTT</td>
</tr>
<tr>
<td>NCAM</td>
<td>NCAM1 neural cell adhesion molecule-1/770790</td>
<td>5′-GTTGCATTCCAAGAAGAATAC</td>
<td>5′-AGGACACTGATTTGATTTGAT</td>
</tr>
<tr>
<td>ST8SialI</td>
<td>ST8 alpha-N-acetyl-neuraminide</td>
<td>5′-GGACCCGCTCATCGCTTT</td>
<td>5′-CTTACGGTCTGTCGATAGGG</td>
</tr>
<tr>
<td>ST8SialIV</td>
<td>ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase-2/414336</td>
<td>5′-AGTATTTCCGAGTTTGG</td>
<td>5′-GATTACATCTCCTGGCTT</td>
</tr>
</tbody>
</table>

GI, GenInfo Identifier.
The Western blot analysis results obtained with deglycosylated total protein using the anti-NCAM extracellular domain antibody were scanned and profiled. The intensity and relative amount of each NCAM isoform was determined (ImageQuant TL; GE Health Care Biosciences Corp., Piscataway, NJ).

### Immunostaining for NCAM on Corneal Sections

Localization of NCAM was performed using immunofluorescence staining on frozen sections of E7, E9, E14, E20, and 20 weeks corneas. Entire hemispheres, containing the cornea, from eyeballs of embryonic chicks, or one cornea of 20 weeks chickens, were embedded in OCT. Frozen sections (15 μm) were cut at −20°C using a cryostat (Bright Instrument Company Ltd., Huntingdon, England), mounted on slides (Fisher, Pittsburgh, PA) and stored at −80°C until used. The sections were then fixed in cold acetone at −20°C for 10 minutes, washed twice with PBS containing 0.025% Tween-20 for 5 minutes and then blocked with 10% bovine serum albumin (BSA) in PBS. NCAM was detected with anti-NCAM extracellular domain antibody (5e, 1:200), and a secondary antibody Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen). Negative controls were performed on each immunofluorescence staining with absence of primary antibody and showed negative staining. The sections were visualized and photographed using an epifluorescent microscope equipped with a digital camera (Leica, MZ16F microscope and DFC 320 camera; Leica Microsystems, Wetzlar, Germany). This epifluorescent microscope was used in the following experiments unless stated otherwise. The fluorescence intensity across each corneal section was profiled using ImageJ (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

### Whole-mount Double Immunostaining for NCAM or PolySia and Corneal Nerves

Whole-mount double immunofluorescence staining for neuronal-specific class III β-tubulin (β-tubulin) and NCAM or polySia was performed on E9 and E14 corneas. The corneas were dissected in PBS and fixed in 4% paraformaldehyde solution overnight at 4°C. After washing with PBS-T (PBS with 0.1% Triton X-100) three times for 5 minutes, the cornea was cut into two halves; one half was used for negative control and the other half was for double immunofluorescence staining. Each half cornea was slit through the pericornea to allow the immunofluorescence staining. The negative controls showed no staining. For PolySia staining, the cornea was stained using the anti-NCAM extracellular domain antibody (5e, 1:200), and a secondary antibody Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen). The sections were then fixed in formalin for 48 hours in media (Opti-MEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum, antibiotics (100 units penicillin, 0.1 mg/mL streptomycin; Sigma) and 25 ng/mL nerve growth factor (Sigma) to support neurogenesis of the trigeminal explants.3,55 After culture, neuronal cell bodies and their neurite extensions were washed briefly in Howard Ringer’s saline solution (7.2 g NaCl, 0.23 g CaCl₂, 0.37 g KCl in 1 liter DI water, pH 7.3), fixed at room temperature for 1 hour in 4% paraformaldehyde solution, followed by several washes in PBS-T. Neuronal tissue was incubated at room temperature for 1 hour in blocking solution (PBS, pH 7.2, containing 5% goat serum, 1% bovine serum albumin, 0.1% Triton X-100), followed by staining with anti-neuronal-specific class III β-tubulin (1:100), anti-NCAM extracellular domain (1:50), or anti-polySia-NCAM (1:50) in blocking solution overnight at 4°C with mild rocking. After several washes in PBS-T, tissue was incubated in appropriate secondary antibodies, Alexa Fluor 488 mouse IgG₂a antibody (neuronal-specific class III β-tubulin), Alexa Fluor 488 goat anti-mouse IgG₁ (NCAM) antibody or Alexa Fluor 488 goat anti-mouse IgM (polySia) antibody each used at a 1:100 dilution in blocking solution.

### Developmental Expression of NCAM, ST8SiaII, and ST8SiaIV mRNA in the Chick Cornea

Among the six known α 2, 8-sialyltransferases, only ST8SiaII and ST8SiaIV can add polySia to NCAM.56 Both ST8SiaII and ST8SiaIV can modify all the isoforms of NCAM57 and cooperatively control the expression of polySia.58,59 Here we investigated the mRNA expression of both NCAM and the polysialyltransferases to understand the expression and polysialylation of NCAM in the chick cornea. To determine the mRNA expression, real time Q-PCR was performed with the total RNA from embryonic (E6 to E20), hatchling (1 D), and 20 weeks adult corneas. Figure 1 presents the Ct numbers of the target genes (NCAM, ST8SiaII and ST8SiaIV) normalized to the housekeeping gene, GAPDH. The mRNA expression levels of NCAM and ST8SiaII show similar patterns of changes during embryonic and postnatal development. NCAM and ST8SiaII transcript levels first increased approximately 3.5-fold and 1.6-fold re-
Characterization of NCAM in the Chick Cornea

The presence of NCAM protein in the chick cornea was confirmed by Western blot analysis using antibodies against NCAM (Fig. 2). A smeared band with a molecular weight (MW) range of approximately 130 to 250 kDa was obtained using anti-NCAM extracellular domain antibody for each desired stage (Fig. 2A), whereas a narrower smeared band with a MW range of approximately 180 to 250 kDa was detected using the anti-NCAM cytoplasmic domain antibody for the selected stages (Fig. 2B). The presence of a smeared band instead of a single band in each lane in Figures 2A and 2B demonstrates that NCAM in the chick cornea is polysialylated. Consistent with the real time Q-PCR results, NCAM protein expression levels varied during development and after hatching. The amount of NCAM protein increased from E7 to E10, remained constant to E14 and then decreased dramatically thereafter. Eventually, NCAM became undetectable in 20 weeks adult cornea.

Western blot analysis using anti-polySia-NCAM antibody revealed several wide, smeared bands ranging from 120 kDa to 350 kDa for each embryonic developmental stage examined, with total protein from E12 chick brain as a positive control (Fig. 3). The expression of polySia showed similar developmental regulation as that of NCAM (Fig. 2). The intensity of the stained bands increased dramatically from E7 to E9 and then continued with high intensity to E16 and declined thereafter. Weak staining detected in 20 weeks corneas showed the persistent presence of polySia in adult corneas. Significantly, Western blot analysis, using antibodies against NCAM and polySia, showed very different band profiles. Firstly, the MW range of the staining using anti-polySia-NCAM antibody was much broader than that obtained using anti-NCAM extracellular domain antibody which can bind all NCAM isoforms (Fig. 2A). In addition, many bands that stained positive for polySia did not correspond in position to bands staining positive for NCAM. This suggests that there are other protein(s) in addition to NCAM that carry polySia residues.

NCAM Isoforms in the Chick Cornea

The anti-NCAM extracellular domain antibody binds all NCAM isoforms, whereas the anti-NCAM cytoplasmic domain antibody specifically recognizes the isoforms NCAM-140 and NCAM-180, which have intracellular domains. The Western blot analysis using anti-NCAM extracellular domain antibody detected bands of a wider MW range than using anti-NCAM cytoplasmic domain antibody (Figs. 2A, 2B). This demonstrates the existence of NCAM isoform(s) other than transmembrane isoform(s). To determine these NCAM isoforms in the chick cornea, we used peptide-N-glycosidase F (N-Glycanase) to remove the polySia chains which are attached to NCAM through N-glycans. Four NCAM isoforms with MWs of 87 kDa, 96 kDa, 110 kDa, and 160 kDa were detected in E7 cornea (Fig. 4A). When the embryo developed to E9, the isoforms with MWs of 87 kDa and 96 kDa disappeared and an isoform with MW of 92 kDa became present for the first time. This 92 kDa NCAM isoform, along with the two isoforms (110 kDa and 160 kDa), were consistently present from E9 to E20 and in hatching chick corneas. In contrast to Western blot analysis of the native NCAM forms (polysialylated forms) (Fig. 2A), NCAM isoforms
with Mws of 92 kDa and 110 kDa were detected in 20 weeks adult corneas. This occurs because the deglycosylation allowed each broadly spread native isoform with diverse polysialic acid chains to migrate instead as one single band in SDS-PAGE gel (Fig. 4A).

PolySias are attached to NCAM through the terminal α2-3(6) sialic acid of N-linked glycans. Previous studies have shown that after removing polySia with endoneuaminidase, the membrane-associated NCAM isoforms show the apparent Mws of 180 kDa, 140 kDa, and 120 kDa, commonly designated as NCAM-180, NCAM-140, and NCAM-120. Respectively, further removal of the N-glycans (using N-Glycanase) yields polypeptide chains with smaller apparent Mws of 160 kDa, 130 kDa, and 110 kDa. Therefore the bands with Mws of 160 kDa and 110 kDa in Figure 4A (produced by digestion of corneal proteins with N-Glycanase) were identified as NCAM-180 and NCAM-120. Other isoforms with lower Mws of 87 kDa, 92 kDa, and 96 kDa are defined respectively as NCAM-87, NCAM-92, and NCAM-96 in this work. A summary of NCAM isoforms in chick corneas and their Mws after desialylation and deglycosylation is listed in Table 2.

NCAM-120 is a GPI-anchored isoform and NCAM-180 is a transmembrane isoform. Besides the membrane-associated isoforms, NCAM can also exist in soluble form with various Mws ranging from 180 to 100 kDa. Thus, it can be predicted that NCAM-87, NCAM-92, and NCAM-96 are in soluble form. To confirm this, Western blot analysis were performed on the deglycosylated NCAM using the anti-NCAM cytoplasmic domain antibody (Fig. 4B). The relative amount of each isoform of NCAM at the selected stages was determined (ImageQuant TL; GE Health Care Biosciences Corp.) (D). The insets in (C) are zoom-in profiles of NCAM isoforms in E7 and 20 weeks adult corneas.

**TABLE 2. Summary of the Molecular Weight Change of NCAM Isoforms in Chick Cornea Treated with Neuraminidase and Peptide-N-Glycosidase F (N-Glycanase)**

<table>
<thead>
<tr>
<th>Native (kDa)</th>
<th>Desialylated (kDa) (Neuraminidase-treated)</th>
<th>Deglycosylated (kDa) (N-Glycanase-treated, Fig. 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCAM-87</td>
<td>—</td>
<td>87</td>
</tr>
<tr>
<td>NCAM-92</td>
<td>—</td>
<td>92</td>
</tr>
<tr>
<td>NCAM-96</td>
<td>—</td>
<td>96</td>
</tr>
<tr>
<td>NCAM-120</td>
<td>120</td>
<td>110</td>
</tr>
<tr>
<td>NCAM-180</td>
<td>180–250</td>
<td>180</td>
</tr>
</tbody>
</table>
main antibody. Only one isoform with an apparent MW of 160 kDa, which is NCAM-180, was detected (Fig. 4B). As expected, other isoforms showed no immunoreactivity to the antibody and therefore do not include an intracellular domain.

It has been reported that the anti-NCAM extracellular domain antibody shows the same binding ability to all NCAM isoforms. The lines in Figure 4A were scanned and the intensity of each band was profiled using software (ImageQuant TL; GE Health Care Biosciences Corp.) as shown in Figure 4C. The distribution of the NCAM isoforms on each lane was determined as shown in Figure 4D. All NCAM isoforms showed developmental changes resembling their polysialylated forms, as shown in Figure 2A. NCAM-120 is the most abundant isoform, constituting 90% of the total NCAM isoforms at E7, with amounts greater than 70% during later developmental and postnatal stages (Fig. 4D). The relative amount of NCAM-180 increased at early embryonic stages, decreased after E14, and lasted until undetectable in the 20 weeks adult cornea. The proportion of NCAM-92 increased slightly during embryonic stages and in postnatal corneas.

**Localization of NCAM by Immunostaining in Chick Corneas**

To localize NCAM in chick corneas, immunofluorescence staining was performed on frozen sections of E7, E9, E14, E20, and 20 weeks adult corneas. These developmental ages were selected due to their differential NCAM expression. NCAM was detected in the epithelium and stroma of embryonic corneas (Figs. 5K, 5M–O), and in the epithelium of embryonic lens (Figs. 5A–D). However, in 20 weeks adult cornea, NCAM was only found in the epithelium and endothelium (Fig. 5P). The fluorescence intensities across the anterior-posterior axis of corneas (along the arrows as shown in Figs. 5K, 5M–O) were profiled as in Figure 5Q. The continuous and high fluorescence intensity across the embryonic corneal stroma suggests that NCAM was expressed in the extracellular matrix (ECM) of the embryonic corneal stroma. This is consistent with the previous Western blot results indicating soluble NCAM isoforms in embryonic corneas. We also noticed a difference in the fluorescence intensities of the anterior versus the posterior stroma during embryonic development (Fig. 5Q). During early embryonic development stages from E7 to E14, NCAM is highly expressed in the anterior stroma with a decreased expression in epithelium from E7 to E9. By E20, higher amounts of NCAM were detected in posterior stroma cornea. No NCAM was found in 20 weeks adult stroma. Overall, NCAM is present in both embryonic and adult corneal epithelium. NCAM expression did not show obvious change in epithelial presence during embryonic and postnatal development. In the stroma, NCAM seems to shift from the anterior stroma to posterior stroma during embryonic development and disappear eventually in adult corneal stroma.

**Localization of NCAM and Polysial in Corneal Nerves**

To determine whether NCAM is expressed on corneal and pericorneal nerves, whole-mount immunofluorescence stainings for NCAM and polysial were performed on corneas and the surrounding pericorneal mesenchyme as well as isolated neurons from the ophthalmic lobe of the trigeminal ganglion (OTG) of E9 and E14 embryos. Corneas of E9 and E14 embryos were doubly-stained with antibodies against neuronal-specific class III β-tubulin and NCAM extracellular domain or polysial-NCAM. On E9, trigeminal sensory nerve bundles formed a ring around the cornea and started to invade the cornea (Fig. 6A). By E14, corneal nerves extended from the ring of nerve bundles around the cornea and migrated into the stroma along the whole circumference of the cornea (Fig. 6D). The colocalization of NCAM and β-tubulin in E9 (Figs. 6A–C) and E14 (Figs. 6G–I) corneas suggests NCAM is expressed on the surface of corneal and pericorneal nerves. The high fluorescent intensity in cornea obtained from immunofluorescence staining for NCAM (Fig. 6H) also reveals its existence in corneal epithelium and/or stroma (i.e., at sites other than corneal nerves), which is consistent with immunofluorescence staining results obtained on frozen sections (Fig.
indicating the presence of NCAM in epithelium and stroma. Similar results were obtained from the double immunostaining for β-tubulin and polySia. PolySia was detected on pericorneal nerve bundles and corneal nerves of both E9 (Figs. 7A–C) and E14 (Figs. 7D–I) embryos.

The majority of sensory nerves associated with the cornea are derived from the OTG. To confirm the expression of NCAM and polySia on nerves associated with the cornea and pericorneal nerves, we isolated explanted pieces of tissue from both E9 (Figs. 7A–C) and E14 (Figs. 7D–I) embryos.

The high-magnification image of immunofluorescence staining for NCAM in E14 cornea also showed high fluorescent intensity in cornea aside from corneal nerves indicating the expression of NCAM in epithelium and/or stroma, consistent with the results obtained using cryosections as shown in Figure 5. Arrows show NCAM staining on corneal nerves and pericorneal nerves.

Our work shows, for the first time, that NCAM is present in developing chick corneas and its expression and polysialylation are developmentally regulated in embryonic and postnatal chick corneas.

## DISCUSSION

In the present work, the expression and localization of NCAM and polySia in chick corneas and corneal nerves were investigated during embryonic and postnatal development.

![Figure 6](image_url) Double immunofluorescence staining for β-tubulin and NCAM in E9 (A–C) and E14 (D–I) corneas. Immunofluorescence staining for β-tubulin (A), NCAM (B), and the merged image (C) in E9 cornea suggests the localization of NCAM to trigeminal sensory nerve bundles shortly before their invasion into cornea. Immunofluorescence staining on E14 for β-tubulin (D, G), NCAM (E, H), and the merged images (F, I) revealed the expression of NCAM on both corneal and pericorneal nerves. The high-magnification image of immunofluorescence staining for NCAM in E14 cornea (H) also showed high fluorescent intensity in cornea aside from corneal nerves indicating the expression of NCAM in epithelium and/or stroma, consistent with the results obtained using cryosections as shown in Figure 5. Arrows show NCAM staining on corneal nerves and pericorneal nerves.

![Figure 7](image_url) Double immunofluorescence staining for β-tubulin and polySia in E9 (A–C) and E14 (D–I) corneas. The colocalization of polySia and β-tubulin indicates the expression of polySia on embryonic corneal and pericorneal nerves. Arrows indicate the colocalization of β-tubulin and polySia on corneal and pericorneal nerves.

Our work shows, for the first time, that NCAM is present in developing chick corneas and its expression and polysialylation are developmentally regulated in embryonic and postnatal chick corneas.

![Figure 8](image_url) NCAM and polySia expression on OTG neurons. (A–C) Cultures of explanted tissue from E9 OTG showing radial neurite outgrowth are positively labeled with antibodies for the neuronal marker β-tubulin (A) and also NCAM (B) and polySia (C). (D–F) Phase contrast images are shown directly below their accompanying fluorescent image for each antibody staining. (G–I) Staining for β-tubulin (G), NCAM (H), and polySia (I) on OTG explants harvested at a later stage, E14, remain positive for each antibody. (J–L) Phase contrast images are shown below their accompanying fluorescent image.
Both NCAM mRNA (Fig. 1) and protein (Fig. 2) showed increased expression levels from early developmental stage (E6/7) to E9 and maintained the high expression level from E9 to E14, coincident with the timing when trigeminal nerves start to invade the cornea at points around its entire circumference and migrate into the entire area of the cornea. The high amounts of NCAM in the cornea at these developmental stages might be related to corneal innervation. ST8SiaII and ST8SiaIV mRNA showed very different expression patterns in embryonic and adult chick corneas. ST8SiaII becomes downregulated from embryonic stages to adulthood. However, in the adult corneas, ST8SiaIVV showed a distinct regulation pattern and is the predominate polysialyltransferase. Hildebrandt et al. reported similar regulation for ST8SiaII and ST8SiaIV during rat brain development. ST8SiaII dominates during embryonic development and is undetectable in 6-month-old postnatal brains, whereas ST8SiaIV persists at relatively high levels in postnatal rat brains. They did not propose the mechanism of the upregulation of ST8SiaIV in mature rat brains. However, they localized the persistent ST8SiaIV expression in the subependymal layer, the glomerular layer of the olfactory bulb, the granule cell layer of the dentate gyrus, and in some widely dispersed cells of the isocortex where the polysialylated NCAM is expressed in postnatal brains. Here, in the chick cornea, the downregulation of NCAM and ST8SiaII mRNA expression and upregulation of ST8SiaIV mRNA expression in adult cornea imply that polySia is persistently expressed in adult corneas. The Western blot analysis using anti-polySia-NCAM antibody confirmed the presence of polySia in 20 weeks adult corneas (Fig. 3). The analysis also reveals that there are other polysialylated proteins besides NCAM in chick corneas. The persistent expression of polySia in adult corneas may be related to postnatal corneal plasticity.

Another contribution of this work is that we identified the NCAM isoforms in chick corneas. Membrane-associated isoforms, NCAM-120 and NCAM-180, and three soluble NCAMs were identified in the chick cornea, with NCAM-120 being the predominate isoform (Fig. 4). The membrane-associated NCAM isoforms arise from the alternative splicing of a single gene. However, the mechanism by which NCAM isoforms are generated is not clear. It has been reported that the expression of NCAM isoforms is developmentally regulated in the chick brain. NCAM-140 was expressed first followed by NCAM-180 which appeared after E3, and NCAM-120 was not detected until E14. Sunshine et al. also reported the differential expression of NCAM isoforms in the brains of frog and chick. However, they localized the persistent NCAM expression to the embryonic corneal stroma and corneal nerves. Prior reports revealed that NCAM was expressed in both the postnatal corneal epithelium and endothelium. In our work, NCAM was also detected in embryonic corneal stroma and its distribution in stroma is developmentally regulated from anterior stroma to posterior stroma (Fig. 5). Particular attention should be paid to the developmental stages between E9 and E14 during which NCAM is highly expressed in anterior stroma, coincident with the location of initiation of significant corneal innervation and stromal development.

NCAM can be expressed by Schwann cells, satellite cells of sensory and sympathetic ganglia of PNS. Inhibition of polySia in chicken sensory neuron cultures increases the thickness of neurite bundles and reduces neurite outgrowth. PolySia on NCAM can reduce the axon-axon interaction and increase nerve branching and promote neuronal regeneration. In the present work, both NCAM and polySia are highly expressed on corneal and pericorneal nerves of embryos (Figs. 6, 7, 8) suggesting their potential functions in corneal nerve development and regeneration.

In summary, NCAM and polySia are expressed and developmentally regulated in the chick cornea. Our results also showed there are other proteins that carry polySia in the chick cornea. NCAM-120, NCAM-180, and three soluble NCAMs were found in chick corneas while NCAM-120 is the predominate isoform. NCAM was localized in the whole embryonic cornea including the corneal epithelium and stroma, and only in the epithelium and endothelium of the adult chick cornea. Both NCAM and polySia were detected on embryonic pericorneal and corneal nerves. In full, the expression, regulation, and localization of NCAM and polySia suggest their possible biological functions in sensory nerve innervation and development of the chick cornea.

Acknowledgments

The authors thank Stella Lee for the access to an MZ16F Leica epifluorescent microscope.

References

9. Schwend T, Lwigale PY, Conrad GW. Nerve repulsion by the lens and cornea during cornea innervation is dependent on Robo-Slit during development between stages E7 and E9 (Fig. 4A) when the sensory nerves start to invade the cornea. Thus, the soluble NCAMs may be involved in nerve growth cone invasion into cornea.

Also significant in this work, is the demonstration of localization of NCAM to the embryonic corneal stroma and corneal nerves. Prior reports revealed that NCAM was expressed in both the postnatal corneal epithelium and endothelium in our work, NCAM was also detected in embryonic corneal stroma and its distribution in stroma is developmentally regulated from anterior stroma to posterior stroma (Fig. 5). Particular attention should be paid to the developmental stages between E9 and E14 during which NCAM is highly expressed in anterior stroma, coincident with the location of initiation of significant corneal innervation and stromal development.

The NCAM isoforms have been reported to be associated with different biological functions. NCAM-120 is the most adhesion form, whereas NCAM-180 is associated with cell motility due to the interaction of its long intracellular domain with the cytoskeleton. The high amounts of NCAM-120 may indicate the important functions of cell adhesion in cornea. Soluble NCAMs are also involved in the peripheral nervous system development. In the present work, we did not determine the source of the soluble NCAMs in the chick cornea. However, we noticed that there were different soluble NCAM isoforms during development between stages E7 and E9 (Fig. 4A) when the sensory nerves start to invade the cornea. Thus, the soluble NCAMs may be involved in nerve growth cone invasion into cornea.

The authors thank Stella Lee for the access to an MZ16F Leica epifluorescent microscope.


56. Angata K, Suzuki M, McAuliffe J, Ding YL, Hindsgaul O, Fukuda M. Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct alpha 2,8-sialyltransferases, ST8Sia IV (PST), ST8Sia II (STX), and ST8Sia III. *J Biol Chem.* 2000;275:18594–18601.


