Endogenous Polysialylated Neural Cell Adhesion Molecule Enhances the Survival of Retinal Ganglion Cells

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Purpose. During development, all retinal cells express polysialylated neural cell adhesion molecule (PSA-NCAM). PSA is localized only on glia in the adult retina, but as Müller glial processes ensheathe most retinal neurons, PSA remains in the extracellular environment of adult neurons. The authors sought to investigate the influence of endogenous PSA on the survival of neonatal as well as adult normal and injured retinal ganglion cells (RGCs).

Methods. Endogenous retinal PSA was selectively degraded by application of endoneuraminidase. PSA presence and removal were confirmed by immunohistochemistry and levels were assessed by Western Blot analysis. Neonatal RGC survival after PSA removal was assessed in vitro in RGCs immunopanned from rat pups. Adult RGC survival was assessed in vivo in mice by investigating RGC densities after removal of PSA in normal retinas and after optic nerve transection.

Results. Virtually all neonatal RGCs express PSA-NCAM and survive well in vitro; however, removal of PSA resulted in a 42% loss of these cells 3 days after the treatment. Similarly, removal of PSA in the adult retina in vivo induced a loss of 25% of RGCs at 14 days, and significantly reduced RGC densities after optic nerve transection by an additional 27% (relative to injured retinas with a vehicle injection) at 7 days.

Conclusions. Together, these findings demonstrate that endogenous PSA supports the survival of neonatal as well as injured and normal adult RGCs and provide the first functional evidence of a role for PSA in the adult retina. (*Invest Ophthalmol Vis Sci.* 2009;50:861–869) DOI:10.1167/iovs.08-2334

Polysialic acid (PSA) is a carbohydrate composed of unbranched homopolymers of approximately 100 – 300 α-2, 8-linked sialic acid residues that are localized almost exclusively on the neural cell adhesion molecule (NCAM) in the vertebrate nervous system.[1,2] PSA functions in reducing contact-dependent interactions between cells by decreasing physical impedance and/or increasing intermembrane charge repulsion.[3,4] During development, NCAM is highly sialylated (PSA-NCAM) throughout many neural regions.[5,6] However, this widespread sialylation is downregulated extensively during the postnatal period and remains expressed only in selective neural regions in adulthood,[7,8] most of which are characterized by permanent plasticity or by neurogenesis.[9] It is well established that PSA functions in aspects of development and regeneration including axon growth, guidance and fasciculation,[10–12] synaptic plasticity,[13] sprouting,[14,15] and cell migration.[16,17] Recent studies have also implicated NCAM and PSA-NCAM in cell survival.[18,19]

Retinal ganglion cells (RGCs) of the adult mammalian retina degenerate and die by apoptosis after optic nerve (ON) transection in a predictable time-dependent manner. In rodents, cell loss begins at day 5, 50% die by 1 week, and 80% to 90% are lost by 2 weeks.[20–24] Intravitreal administration and genetic overexpression of various trophic factors[25–27] or anti-apoptotic agents,[28,29] have proven effective in delaying—but not preventing—RGC death.[30–32] Intracellularly, RGC death is mediated by an apoptotic pathway associated with caspase activation,[33,34] yet the extracellular mechanisms that influence this process are poorly understood.

While PSA-NCAM is located on cell bodies in all retinal layers in the developing rodent retina,[35] Müller glia and astrocytes are the only adult retinal cells that express the highly sialylated form of NCAM.[35,36] Since the end-feet of Müller glia ensheathe the somas of many retinal neurons,[37,38] PSA-NCAM remains in close proximity to neurons, including RGCs, in the adult retina. During development, PSA-NCAM participates in several critical functions.[12,39–42] In the adult, we have recently demonstrated that the onset of RGC loss after ON transection is earlier in mice lacking NCAM.[43] Nonetheless, despite its extensive expression, a functional role for PSA-NCAM in the adult retina has yet to be determined.

The presence of PSA on the surface of developing RGCs and in the extracellular environment of adult RGCs, along with the previously demonstrated role for PSA in neuron survival in the CNS, provide the rationale for postulating that endogenous PSA influences RGC survival. In the present study, we have specifically investigated the functional role of PSA in the survival of early postnatal RGCs in vitro, and of uninjured and injured adult RGCs in vivo.

Materials and Methods

Animals

All animals were cared for by the Dalhousie University Committee on Laboratory Animals in accordance with the Canadian Council on Animal Care and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For in vitro experiments, litters (6–10 pups/litter, $n = 3$ with each n representing all data for a single litter) of Long-Evans rats (Charles River, Montreal, Quebec, Canada) were used at postnatal day (P)7 to P8. In additional in vitro experiments, RGC cultures were generated from adult Long-Evan rats aged 10 to 15 weeks (3 rats per culture). Rats were killed by overexposure to halothane and decapitation. For in vivo experiments, adult female C57BL/6 mice (18–20 g and 8–13 weeks; Charles River Canada, St. Constant, PQ,

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Canada) or adult NCAM$^{-/-}$ mice generated from the C57BL/6 strain (8–14 weeks; Case Western Reserve University, Cleveland, Ohio) originally created as previously reported[17] were used. All surgical procedures on mice were performed under general anesthesia using a mixture of ketamine (135 mg/kg), rompun (7.2 mg/kg), and acepromazine (1.17 mg/kg) in 0.9% saline, administered by intraperitoneal injection.

Generation of RGC Cultures

RGCs were isolated and purified as previously described.[44,45] The eyes from the neonatal and adult rats were enucleated, the anterior segments and lenses were removed, and the posterior eyecups were immersed in dissection medium (2% B27 supplements and 10 μg/mL gentamicin in Hibernate-A medium; BrainBits, Springfield, IL)[46] until further retinal processing.

The neonatal retinal tissue was incubated for 30 minutes (60 minutes for adult retinas) at 37°C in a papain solution (165 U in 10 mL of Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline [DPBS]) containing 1 mM L-cysteine and 0.004% DNase. The papain-treated retinas were then triturated sequentially in DPBS containing 1.5 mg/mL ovomucoid (Roche Diagnostics, Laval, Quebec, Canada), 1.5 mg/mL bovine serum albumin (BSA), and 0.004% DNase, and the rabbit anti-rat macrophage antibodies (1:75; Accurate Chemical, Westbury, NY) required for the macrophage panning step. The suspension was centrifuged, rewashed in a high concentration ovomucoid/BSA solution (10 mg/mL each in DPBS), and the dissociated cells were resuspended in DPBS with 0.2 mg/mL BSA and 5 μg/mL insulin.

RGCs were isolated and purified using a two-step antibody-mediated plate adhesion (immunopanning) procedure, essentially as previously described.[47,48] To remove macrophages, the mixed retinal cell suspension was incubated on Petri dishes coated with affinity-purified goat anti-rabbit IgG (H + L) antibodies (Jackson ImmunoResearch, West Grove, PA). To purify RGCs, the remaining cells were then transferred to a Petri dish that had been first coated with affinity purified goat anti-mouse IgM (μ chain) antibodies (Jackson ImmunoResearch) and then with anti-Thy-1.1 monoclonal IgM antibodies (cell line T11D7e2; TIB-103; American Type Culture Collection, Manassas, VA). After 30 minutes, the dish was repeatedly rinsed with DPBS to remove any nonadherent cells. Adherent cells (RGCs) were released by first incubating the cells in a 0.125% trypsin solution and then manually pipetting an enzyme inhibitor solution (30% FBS in Neurobasal-A) along the surface of the dish.

Neonatal RGCs were plated onto poly-D-lysine/laminin-coated 16-well chamber slides (Nagle Nunc International, Rochester, NY) at a density of 6000 RGCs per well. The cells were cultured in 150 μL of serum-free culture medium consisting of Neurobasal-A with 2% B27 supplements, 1 mM glutamine, 50 ng/mL BDNF, 10 ng/mL CNTF, 5 μM forskolin, and 10 μg/mL gentamicin.[46,48] Cultures were maintained at 37°C in a humidified 5% CO_2-95% air atmosphere. Half the medium was replaced every 3 days and all subsequent experimental procedures on the isolated RGCs were performed at 3 days after the day of cell dissociation and panning.

In Vitro Survival Assay

At 3 days after RGC isolation and purification, neonatal cultures were treated with vehicle (1 μL of 50% PBS/glycerol) or endoneuraminidase (Endo-N; 0.5, 1, and 3 μL of 6.7 U/μL in 50% PBS/glycerol), or they were left untreated. One single stock supply of Endo-N was diluted and used for all in vitro and in vivo experiments. Endo-N is a phage enzyme that selectively degrades linear homopolymers of sialic acid with α-2, 8-ketosidic linkages with a minimum chain length of at least 7 to 9 residues, and spares other sialic acid-containing moieties.[10,49] The enzyme requires no cofactors, is effective in all species, is stable for weeks, and removes all PSA both in vitro and in vivo.[11,16,42,50] Furthermore, a previous study found that continuous infusion of Endo-N into the cell media had no effect on the survival of NCAM$^{-/-}$ cortical neurons or cortical astrocytes (which do not express PSA).[51] Initially we investigated the effectiveness of 0.5, 1, or 3 μL of Endo-N (at 6.7 U/μL) for removing PSA. PSA was absent from cultured RGCs 1 day after treatment with 1 or 3 μL but not with 0.5 μL of Endo-N (n = 2/dose). Therefore, 1 μL of Endo-N was used for all subsequent experiments with cultured RGCs, since this was the lowest dose which induced complete removal of PSA from these cells by 1 day.

RGC survival was assessed (LIVE/DEAD Viability/Cytotoxicity Assay Kit for animal cells; Molecular Probes, Eugene, OR) at 3 days after treatments. Briefly, the medium was removed, RGCs were washed gently with PBS for 1 minute, and incubated in a solution containing both calcein AM (2 μM) and ethidium homodimer (EthD-1; 4μM) in PBS (total volume of 150 μL) at room temperature (Rt) for 30 minutes. Reagents, chambers, and chamber adhesive were removed from the slides, a coverslip was gently placed over the slides and RGCs were visualized for fluorescence on microscope (CTR 6000B; Leica Microsystems, Richmond Hill, Ontario, Canada). Using an optical fractionator probe (Stereo Investigator 6; MicrobrightField Inc., Williston, VT), all RGCs that expressed calcein-AM (live cell marker, green) but did not label for EthD-1 (dying cell marker, red) in each well were counted. Counting was performed manually by a single observer who was masked to the experimental treatments. Counted RGCs were marked on the computer screen to ensure that RGCs were not missed and not counted twice. Within each litter, treatments were performed for each group (untreated, vehicle, or Endo-N) on 1 to 5 wells containing 6000 RGCs each, and a mean RGC count was taken for each group. RGC means were converted to a percentage of mean RGCs in untreated wells ± SEM. This procedure was repeated with two additional litters to yield an n = 3 for each of the three groups.

In Vivo RGC Labeling

In adult mice, the cell bodies of RGCs were labeled through retrograde transport by application of fluoro-gold (FG; 2% FG in 0.9% saline; Fluorochrome Inc., Denver, CO)-soaked gelfoam (UpJohn, Don Mills, ON, Canada) to both exposed superior colliculi as previously described for rats.[52,53] All subsequent surgeries (optic nerve transection) were performed at 7 days after FG labeling.

Intravitreal Injections

Intravitreal injections were performed as previously described.[25,54] Briefly, a 30-gauge needle was inserted through the sclera and retina into the vitreous chamber of the eye by a posterior approach. A unilateral injection of vehicle (1 μL of 50% PBS/glycerol) or Endo-N (1 μL of 6.7 U/μL in 50% PBS/glycerol) was slowly administered into the vitreous of the left eye through the opening created by the 30-gauge needle, using a 2 μL Hamilton syringe fitted with a drawn-out glass micropipette. Care was taken to ensure no injury was made to other structures of the eye, specifically the lens and the anterior portions of the eye, which both promote survival and regeneration when punctured.[25,55,56] For RGC survival experiments with ON transection, intravitreal injection was performed immediately after axotomy. Animals were killed at 6 hours, and at 1, 2, 4, 7, and 14 days after intravitreal injection.

ON Transection

The left ON was transected with microscissors at approximately 0.5 mm from the globe of the eye as previously described for rats.[25,26] Right eyes served as internal, non-surgical controls. Animals whose eyes showed evidence of ischemia or infection were killed and excluded from the analysis. Mice were killed at 4 and 7 days after ON transection.

SDS-PAGE, Western Blot Analysis, and Immunoblotting

PSA-NCAM immunoblotting was performed as previously described.[42,54] Briefly, mice were perfused with PB and the retinas were extracted and stored at −80°C. In one set of experiments, right and left retinas were dissected and separated using one mouse for each time. This procedure was repeated in two other experiments using pooled

tissue from two mice, instead of one mouse, for each time (n = 3/injury time). Tissue was homogenized in PSA extraction buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 100 μg/mL leupeptin, 0.2 TIU/mL aprotinin, 1% NP-40), placed on ice for 1 hour, and then centrifuged at 18,300*g* for 1 hour at 4°C. Supernatant was extracted and protein concentration determined by BCA. Tissue samples (0.5 μg) were incubated in sample buffer, boiled for 5 minutes at 100°C, separated on denaturing 6% SDS-polyacrylamide gels at 75 V, and transferred onto a polyvinylidene fluoride membrane at 120 V for 1 hour (Millipore, Mississauga, ON, Canada). Membranes were air-dried, wet with methanol, rinsed in Tris-buffered saline (TBS) for 5 minutes, blocked in 5% dry nonfat milk in TBS with 0.1% Tween 20 (TBS/T) for 1 hour, washed three times for 5 minutes each in TBS/T (all washes followed this protocol) and incubated overnight in monoclonal mouse anti-PSA (1:20; 5A5, Developmental Studies, Iowa City, IA)[57] in TBS/T with 5% BSA. Blots were then washed, incubated for 1 hour at Rt in peroxidase-conjugated goat anti-mouse IgM (for PSA; 1:2000; Chemicon International, Temecula, CA) and washed again. To visualize primary antibodies, membranes were reacted with Western blotting reagent (ECL-Plus; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol and scanned for fluorescence (PhosphorImager STORM-840; Amersham Biosciences). Images were edited for brightness and contrast only using an image-editing program (Adobe Photoshop 7.0; Adobe Systems Inc., San Jose, CA). Protein levels were quantified by applying densitometry methods to the scanned blots (Scion Image, Frederick, MD). Membranes were stained with amido-black to ensure equal amounts of protein were loaded into lanes on each gel.

Immunohistochemistry

To assess the presence of PSA-NCAM in vitro, RGCs were prepared for immunohistochemistry at 12 hours, and 3 and 5 days after treatments. The medium was removed and RGCs were incubated in 4% paraformaldehyde (PFA) in PB for 10 minutes (200 μL/well). Cells were washed in PB (3 times for 5 minutes each), and incubated in blocking solution (10% normal goat serum in PB) for 30 minutes at Rt followed by exposure to monoclonal mouse anti-PSA (1:5, 5A5) in PB overnight at 4°C. Next, RGCs were washed, incubated in rodamine-conjugated goat anti-mouse IgM (1:200 in PB; AP128R; Chemicon) for 60 minutes at Rt and washed again. The chamber wells and adhesive were then removed, slides were air-dried overnight and coverslipped with a mounting medium (Vectashield; Vectashield Laboratories, Burlington, CA).

For in vivo experiments, mice were perfused transcardially with 20 mL of PB followed by 20 mL of chilled PFA in PB. Retinas were dissected, post-fixed overnight at 4°C, cryo-protected in 30% sucrose, embedded in gelatin, post-fixed overnight at 4°C, and cryo-protected again in 30% sucrose. Radial sections (30 μm) through the optic disc were cut on a freezing microtome and stored in Millonig's solution (16.88 mg / ml $NaH_2PO_4H_2O$, 3.86 mg / ml NaOH, and 0.006% Na azide in distilled water) at 4°C. Sections were washed, blocked, incubated in primary (1:5, 5A5) and secondary antibodies (1:200), and washed again as described previously with the exceptions that incubations were performed on a shaker and that the blocking solution and primary antibody were dissolved in 0.1 M PBS/3% Triton X-100. After the last wash, sections were incubated in Hoechst nuclear stain (1:1000; Hoechst 33258 in 0.1 M PB; Sigma-Aldrich Corp., St. Louis, MO), washed again, floated onto gelatinized slides, air-dried overnight and coverslipped. Slides were coverslipped using 1-ounce micro cover glasses at #0 thickness. Immunoreactivity was visualized under fluorescence microscopy and differential interference contrast (DIC) microscopy.

RGC Counting In Vivo

Retinas were extracted after perfusion, sectioned in four quadrants, post-fixed for 1 hour, and wholemounted on gelatinized slides. RGC counting was performed as previously described.[20,21] Briefly, the entire retina was examined to ensure that FG labeling was uniform and retinas that did not meet this standard were not included in further data analysis. FG-labeled RGCs were counted manually (by a single observer who was blind to experimental treatments) in three counting regions in each of the four retinal quadrants (12 regions) for a total area of 0.75 mm^2. The total RGC number was then converted to a density ($RGCs/mm^2$) and expressed as mean ± SEM (n = 3 to 6/group).

Statistical Analysis

Statistical significance was assessed with a two-way analysis of variance (ANOVA), followed by a one-way ANOVA (SPSS 9.0 software; SPSS Inc., Chicago, IL). Values identified as outliers were removed from analyses. The Levene test was used to determine the homogeneity of variances. For parametric statistics, multiple comparisons within groups were performed using a Fischer's LSD post hoc test, and independent samples *t*-tests were performed for comparisons between groups using parametric statistics. For nonparametric statistics, individual comparisons were made with a Games-Howell post hoc test. Differences were considered statistically significant if $P \leq 0.05$.

Results

Enhanced Survival of Cultured Neonatal RGCs by Endogenous PSA

To determine whether PSA plays a role in the survival of cultured neonatal RGCs, we first set out to determine whether PSA is present on the surface of these cells. To investigate PSA immunoreactivity, RGCs were immunolabeled for PSA-NCAM and virtually all neonatal RGCs (P7 to P8) expressed PSA-NCAM in vitro (Figs. 1A, 1B). To remove PSA from RGCs, cultures were treated with Endo-N (or vehicle as controls) and immunolabeled for PSA-NCAM at various times after the treatment. PSA-NCAM was absent from cultured RGCs 12 hours after

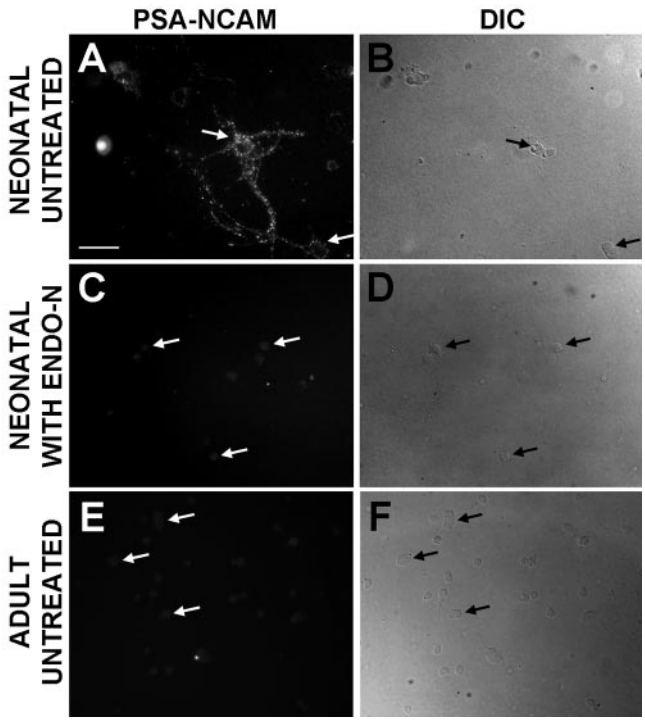

Figure 1. PSA-NCAM is localized on neonatal RGCs in vitro. RGCs were isolated and purified from neonatal retinas by an immunopanning procedure that yields a culture of ~97% pure RGCs. (**A**) Virtually all neonatal RGCs express PSA-NCAM. (**C**) PSA immunostaining is absent at 12 hours after exposure to Endo-N (*arrows*) and remained absent 5 days after treatment (data not shown). (**E**) Adult RGCs do not express PSA-NCAM. (**B**, **D**, **F**) DIC images of the paraformaldehyde-fixed RGCs. *Arrows* indicate examples of RGCs. Scale bar: 50 μm.

Endo-N exposure (Figs. 1C, 1D) and remained absent 5 days after the treatment (data not shown). To ensure the specificity of our antibody and the applicability of our findings to an in vivo setting, we investigated PSA-NCAM immunoreactivity on RGCs isolated from adult rat retinas. It is well established that PSA-NCAM is not on the surface of adult RGCs in vivo[35] and in agreement with these findings, we observed that RGCs cultured from adult retinas did not express PSA-NCAM (Figs. 1E, 1F).

To determine whether PSA influences the survival of neonatal RGCs, cells were treated with vehicle, Endo-N, or no treatment ($n = 3$ litters/group) and RGC survival was evaluated three days later. Figure 2 shows RGCs that were untreated (Figs. 2A–2C) or treated with Endo-N (Figs. 2D–2F) and exposed to the live cell marker calcein-AM (Figs. 2A, 2D) and the dead cell marker EthD-1 (Figs. 2B, 2E). RGCs that were stained by calcein-AM but not by EthD-1 were counted as surviving RGCs. There were significantly fewer RGCs (percentage of mean control RGCs ± SEM) in the Endo-N–treated group (54.0 ± 3.7) relative to vehicle group (96.1 ± 7.3; $P = 0.029$; Fig. 2G). Taken together, these findings demonstrate that neonatal RGCs express PSA-NCAM in culture, and PSA on their surface participates in maintaining the survival of these cells.

Removal of Retinal PSA by Endo-N

As a prelude to determining the effects of PSA removal on RGC survival in vivo, we first set out to demonstrate that retinal PSA could be removed by intravitreal injection of Endo-N and to verify the timing over which this removal occurs. Figure 3A shows a pattern of PSA-NCAM immunoreactivity in multiple layers of control retinas ($n = 6$), consistent with previously published results.[35,36] At 6 hr after Endo-N exposure, PSA was completely absent from the inner (the GCL and IPL) but not the outer retina (Fig. 3D). At 24 hours after Endo-N exposure, PSA was removed (Fig. 3G) and remained absent at 14 days (Fig. 3J) ($n = 2$ or 3/experimental group). Retinal PSA-NCAM immunostaining after vehicle injection was consistent with that of non-injected eyes, suggesting there was no injection or vehicle effect ($n = 2$ at 6 hours and 4 days; data not shown). Hoechst staining (Figs. 3B, 3E, 3H, 3K) and DIC imaging (Figs. 3C, 3F, 3I, 3L) of retinal sections demonstrate that all tissue remained intact and that there was no histologic disturbance that would indicate toxicity. These findings indicate that Endo-N removes retinal PSA in vivo from the inner retina, including the extracellular environment of RGCs, within 6 hours of exposure with no evidence of histologic disturbance.

Promotion of RGC Survival in the Normal Retina by PSA

Since PSA influenced the survival of cultured RGCs, we next examined whether PSA plays a role in RGC survival in the normal adult retina in vivo. RGCs were quantified in retinal wholemounts at 7 and 14 days after no treatment, after intravitreal injection of vehicle and after intravitreal injection of Endo-N. We found that PSA is involved in maintaining the survival of RGCs in the normal retina ($P = 0.003$, 0.001, and <0.001 for treatment effect, day after treatment effect, and treatment X days after treatment interaction respectively; Fig. 4A). Specifically, no significant differences in RGC densities occurred 7 days after treatment (3238 ± 49, 3348 ± 45, and 3320 ± 87 for no treatment, vehicle, and Endo-N groups respectively; $n = 3$/group; $P = 0.485$). However, at 14 days after Endo-N treatment, RGC densities were significantly reduced by approximately one-third (2132 ± 185; $n = 6$) compared with control groups (3068 ± 59 and 2856 ± 86 for no treatment and vehicle groups, respectively; $n = 5$/group; $P \leq 0.001$). To ensure differences in RGC densities after Endo-N treatment were due specifically to the removal of PSA and not a toxic effect of the Endo-N, RGC densities were quantified in NCAM$^{-/-}$ mice at 14 days after intravitreal injection of Endo-N. RGCs were evaluated at 14 days because there was a significant reduction in RGC densities in wild-type mice at this time after exposure to Endo-N (Fig. 4A). No differences in RGC densities in NCAM$^{-/-}$ mice were found 14 days after exposure to Endo-N (3429 ± 68 and 3360 ± 58 for no treatment and Endo-N groups, respectively, $n = 3$/group; $P = 0.414$; Fig. 4B), indicating that the loss of RGCs observed in Endo-N–treated wild-type retinas (Fig. 4A) was not a consequence of a potential cytotoxic effect of Endo-N. Together, these findings indicate that at least some RGCs require endogenous PSA in vivo to maintain their survival.

Promotion of the Survival of Injured RGCs by PSA

To directly test our hypothesis that PSA-NCAM plays an in vivo role in the survival of injured adult neurons, RGC densities

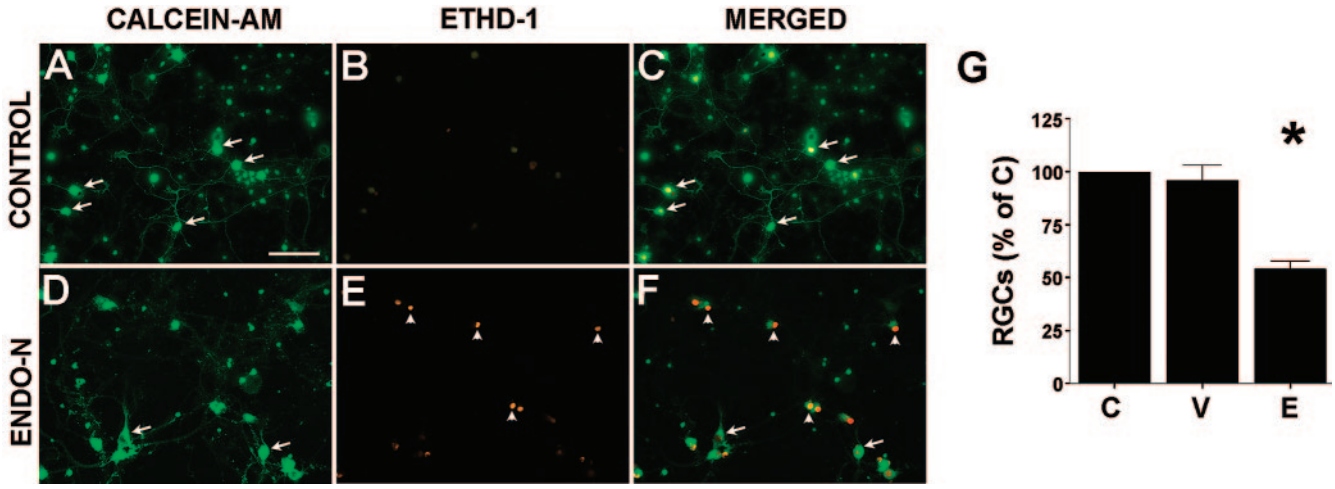

Figure 2. Endogenous PSA on the surface of neonatal RGCs influences their survival. Cultures were treated with vehicle or Endo-N, or left untreated ($n = 3$/group). After three days, cultures were exposed to both calcein-AM (*green*, *arrows*) and EthD-1 (*red*, *arrowheads*). RGCs that labeled for calcein-AM but did not express EthD-1 were counted as living cells, whereas those that labeled for EthD-1 were not counted. (**A–C**) Examples of untreated RGCs at 3 days: (**A**) calcein-AM; (**B**) EthD-1; and (**C**) merged image. (**D–F**) Examples of RGCs treated with Endo-N are also shown: (**D**) calcein-AM; (**E**) EthD-1; and (**F**) merged image. (**G**) At 3 days after PSA removal, 42% fewer RGCs survived compared with vehicle-treated groups in which PSA is present ($P = 0.029$). EthD-1, ethidium homodimer; C, untreated control; V, vehicle-treated control; E, Endo-N treatment. *$P \leq 0.05$, different from V. Scale bar: 50 μm.

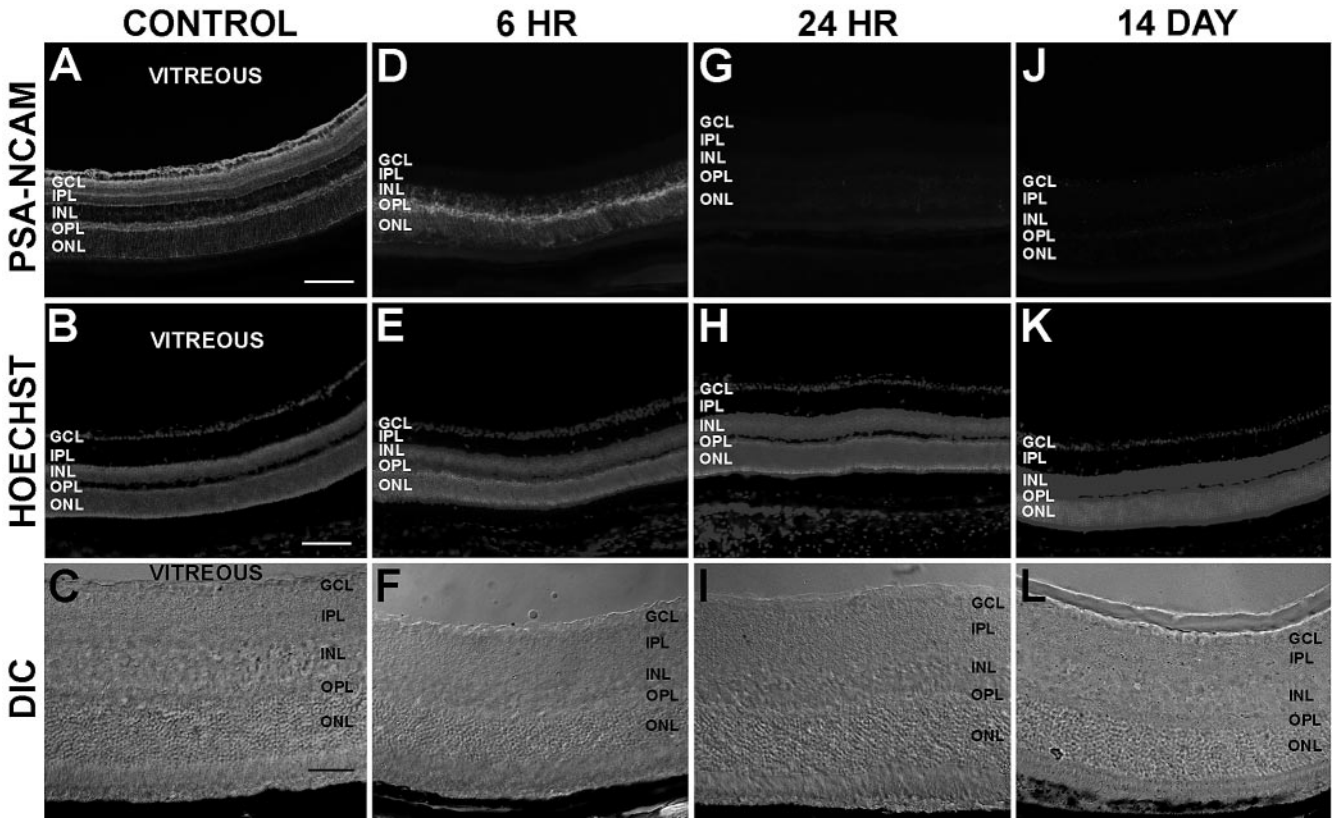

FIGURE 3. Endo-N removes retinal PSA from Müller glia and astrocytes in vivo early after intravitreal injection. Endo-N was injected into the vitreous of adult mice. Retinal sections were immunolabeled for PSA-NCAM, exposed to Hoechst nuclear staining, and visualized for fluorescence by DIC. (**A**) Control retinas displayed extensive PSA-NCAM immunoreactivity between the inner and outer limiting membranes of the retina. (**D**) At 6 hours after Endo-N injection, PSA was no longer present in the GCL and IPL, but remained expressed in the outer retinal layers. (**G**) At 24 hours after Endo-N exposure, PSA was absent from the entire retina and (**J**) remained absent 14 days after treatment. (**B**, **E**, **H**, **K**) Hoechst labeling and (**C**, **F**, **I**, **L**) DIC imaging demonstrate that the retinas showed no evidence of histologic disturbance. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bars: (**A**, **B**, **D**, **E**, **G**, **H**, **J**, **K**) 100 μm; (**C**, **F**, **I**, **L**) 50 μm.

(RGCs/mm^2 $\pm$ SEM) were quantified in retinal wholemounts from wild-type mice at 4 and 7 days after the following: (1) internal control with no intravitreal injection or injury (Fig. 5A); (2) intravitreal vehicle injection and axotomy (Fig. 5B); and (3) intravitreal Endo-N injection and axotomy (Fig. 5C). We found distinct differences in the timing of injury-induced RGC loss between groups ($P < 0.001$ for treatment effect, injury day effect and treatment X injury day interaction; Fig. 5D). At 4 days after treatment and injection, RGC densities did not differ between groups (3083 $\pm$ 53, $n = 4$; 3041 $\pm$ 100, $n = 4$; and 3174 $\pm$ 65, $n = 3$; for no treatment with no injury, vehicle with injury, and Endo-N with injury, respectively; $P = 0.531$). At 7 days after vehicle injection and injury, RGC loss was approximately 50% (1472 $\pm$ 36, $n = 4$ and 3139 $\pm$ 48, $n = 6$ for vehicle injection and injury, and no injury groups, respectively; $P < 0.001$), which is consistent with previous reports in rats[20,25] and mice.[24] Of note, RGC densities were significantly reduced an additional 27% (1073 $\pm$ 34; $n = 4$) at 7 days after axotomy in the Endo-N–treated group compared with retinas from axotomized animals that received only vehicle injection ($P < 0.001$).

It is well established that an intravitreal injection resulting in lens injury or communication with the anterior chamber can be neuroprotective for RGCs.[25,55,56] In NCAM$^{-/-}$ mice, RGC loss after ON transection occurs earlier than in wild-type mice[43]; however, results from the present study show that RGC loss is not earlier in wild-type mice with removal of PSA (Fig. 5D). To exclude the possibility that in the retinas treated with Endo-N the injection itself was neuroprotective, and that for this reason RGC loss after injury was not earlier in Endo-N–treated retinas compared with vehicle-treated retinas, we evaluated RGC survival in NCAM$^{-/-}$ retinas, which lack NCAM and PSA, at 4 days after ON transection to determine the effects of intravitreal injection of vehicle (Fig. 5E). Consistent with our previous studies showing earlier RGC loss after axotomy in NCAM$^{-/-}$ mice,[43] RGC densities at 4 days after axotomy and intravitreal injection were significantly lower (by 28%; 2773 $\pm$ 151), compared with uninjured NCAM$^{-/-}$ mice (3876 $\pm$ 66, $n = 3$/group, $P = 0.003$). Therefore, the injection itself does not have a significant neuroprotective effect on RGCs at 4 days. Consequently, we can conclude that the normal RGC density observed in wild-type mice 4 days after Endo-N treatment (Fig. 5D) is due to the fact that the removal of PSA does not influence the onset of RGC loss at this early time after injury. However, the increased death of RGCs at 7 days after PSA removal indicates that retinal PSA provides neuroprotective support for RGCs at this later time after injury.

Increase in Levels of Adult Retinal PSA-NCAM after ON Transection In Vivo

We have recently reported that after ON transection, PSA-NCAM levels increase significantly in the superior colliculus, the principal RGC target tissue of rodents.[54] In this study, Western blot analysis was used to investigate whether retinal PSA-NCAM levels change after ON transection at various times after injury (Fig. 6A). In the normal uninjured retina, PSA-NCAM is present (Fig. 6A, lane 2). Retinas from NCAM$^{-/-}$ mice

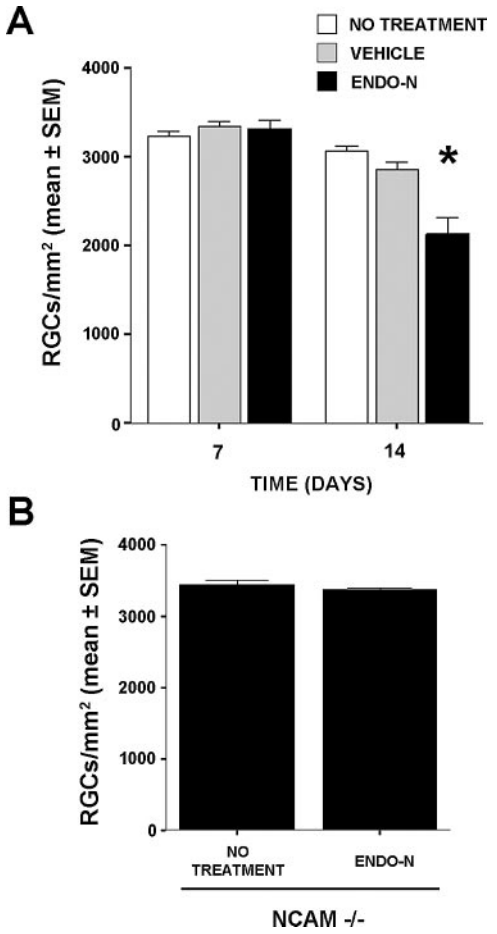

FIGURE 4. Endogenous PSA influences the survival of normal adult RGCs in vivo. RGCs were retrogradely labeled with FG and the eyes subsequently received an intravitreal injection of vehicle or Endo-N into the left eye (n = 3 to 6/group). (**A**) In uninjured retinas, there is no RGC loss at 7 days after Endo-N treatment (P = 0.485); however, a significant reduction in RGC densities occurs at 14 days after exposure to Endo-N compared with no treatment and vehicle-treated retinas (P = 0.001). (**B**) To ensure that differences in RGC densities after Endo-N treatment were due specifically to the removal of PSA and not a toxic effect of Endo-N, eyes of NCAM$^{-/-}$ containing FG-labeled RGCs received an intravitreal injection of Endo-N. At 14 days, there was no significant difference in RGC densities compared with untreated NCAM$^{-/-}$ retinas (P = 0.414; n = 3/group). *$P \leq 0.05$.

were used as a negative control, showing only a faint nonspecific line (~160 kDa) (Fig. 6A, lane 1) present at all experimental times. Right uninjured retinas were used as controls for all quantifications (mean arbitrary unit ± SEM = 1605.0 ± 135.5). After transection of the left ON, retinal PSA-NCAM levels increased significantly in injured retinas ($F_{6,14}$ = 3.34, P = 0.029 for injury day effect; n = 3/time after injury) at 10 days by 179% (4479.3 ± 1257.2, P = 0.043) and at 14 days by 285% (6176 ± 1579, P = 0.003) relative to control (Fig. 6B). There was no significant change in detectable PSA-NCAM levels during the first week after optic nerve axotomy (1, 2, 4, and 7 days post-transection; $P > 0.05$).

DISCUSSION

It is well established that PSA-NCAM is extensively downregulated in the nervous system after development; however, the retina is one location that maintains significant PSA expression into adulthood, specifically on glial cells. Despite these findings, no functional role has been determined for adult retinal PSA. We observed significant RGC death when PSA was removed from the surface of neonatal RGCs in vitro, and from neighboring glia in the adult injured and uninjured retina in vivo. Together, our findings demonstrate that PSA influences RGC survival in both developing and mature rodents and under both physiological and pathologic conditions. These results indicate for the first time that PSA supports cell survival when localized on the cell's surface or when localized on the surface of neighboring glia, and provide the first functional evidence of a role for PSA in the adult retina.

Mediation of RGC Survival by PSA

Our findings may provide insight into PSA's role in maintaining the viability of RGCs during retinal development and maturation. Interestingly, even though 50% of neonatal RGCs die by 3 days after PSA removal in vitro (Fig. 2), normal RGCs survive in vivo into adulthood despite endogenous downregulation of PSA from their surface.[35] This discrepancy may be explained by our findings that RGC loss occurs in vivo after removal of all retinal PSA (Fig. 4A), and indicates that the presence of PSA on neighboring glia[35] may be sufficient to mediate PSA's function in maintaining RGC viability in the adult. However, endogenous downregulation of PSA after postnatal development likely does not cause the programed cell death seen during the first week after birth since the majority of developmental RGC programed cell death is complete by P8,[58,59] and PSA remains expressed on the surface of mouse RGCs until post-natal week 3.[35]

It is important to point out that we used different species for in vitro (rat) and in vivo (mice) studies. Mice were used for in vivo experiments so that our findings could be compared directly with those obtained from our previous experiments using NCAM$^{-/-}$ mice.[43] We chose to isolate rat RGCs as opposed to mouse RGCs because we have extensive experience isolating RGCs from rat pups.[44,45] Furthermore, the generation of purified rat RGC cultures through immunopanning is a well-established technique,[47,48] whereas there have been few studies for comparison that have successfully isolated mouse RGCs.

Influence on the Survival of Injured RGCs by PSA

Adult RGCs do not express PSA in vivo under normal conditions.[35,36] Although we show increased retinal PSA after injury (Fig. 6), it is not clear whether PSA is upregulated specifically on RGCs in vivo in response to ON transection. However, RGCs immunopanned from adult rats fail to express PSA (Figs. 1E, 1F), which suggests that adult RGCs may not express PSA after injury in vivo. In addition, we observed normal retinal PSA levels at 7 days after ON transection (Fig. 6), when approximately 50% of RGCs have died (Fig. 5D). This finding suggests that adult injured RGCs do not express PSA in vivo at 7 days after ON transection; however, electron microscopy may be required to conclude with certainty whether injured RGCs express PSA in vivo. Regardless of the cell-specific localization of PSA after injury, we find no loss of uninjured RGCs in vivo until 14 days after removal of retinal PSA (Fig. 4), whereas ON transection and simultaneous removal of retinal PSA result in accelerated RGC loss at 7 days (Fig. 5). Together, these results indicate that the condition of the RGC (injured or not) independently influences the survival effect of PSA. It remains to be determined whether the retinal PSA that is upregulated at 10 and 14 days after injury is involved in the long-term viability of some RGCs that survive several weeks after ON transection.[20,60]

PSA Promotion of RGC Survival via Masking of NCAM Function

There are conflicting reports as to PSA's role in cell survival. Consistent with our findings for RGCs, PSA removal has been

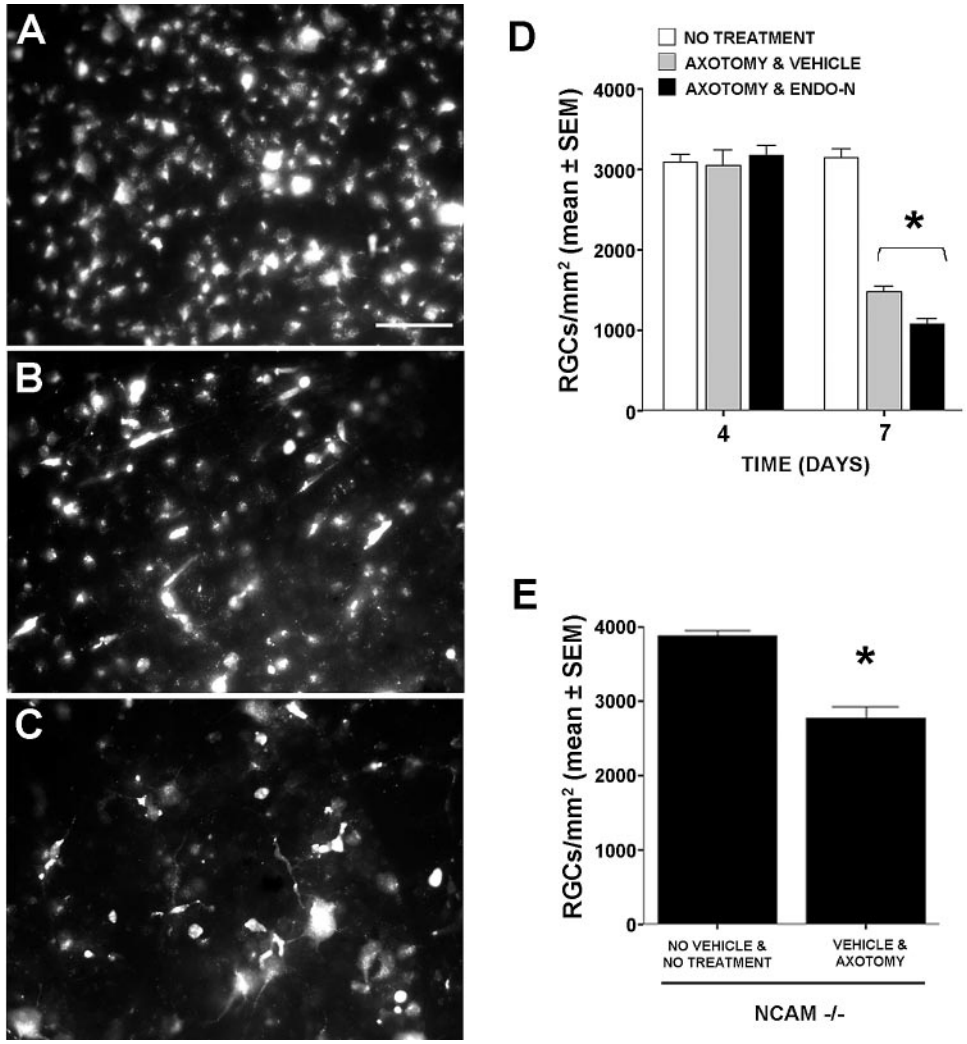

FIGURE 5. Endogenous PSA influences the survival of injured adult RGCs in vivo. (**A–C**) FG-labeled RGCs in retinal wholemounts: (**A**) no injection and no injury; (**B**) 7 days after axotomy and vehicle injection; and (**C**) 7 days after axotomy and Endo-N injection. (**D**) No RGC loss occurred 4 days after intravitreal injection of Endo-N and axotomy ($P = 0.531$); however, a significant further reduction in RGC densities resulted 7 days after ON transection and Endo-N injection, compared with vehicle-treated retinas ($P < 0.001$). (**E**) To demonstrate that the injection itself is not neuroprotective, RGC densities in NCAM$^{-/-}$ mice were examined after axotomy. At 4 days, there was a reduction (28%) in RGC densities (RGCs/mm^2 ± SEM) that was significantly different from control (uninjected and no injury) retinas ($P = 0.003$; $n = 3$/group) but which was not different from survival previously reported after axotomy alone.[43] *$P \leq 0.05$. Scale bar: 50 μm.

shown to induce apoptosis of cultured cortical[51] and hypothalamic neurons,[61] neuronal precursors in the subventricular zone in vitro,[62] and to prevent hyperthermia-induced neuroprotection of hippocampal neurons from kainate in vivo.[63] Furthermore, our in vivo results demonstrate that endogenous PSA is necessary for the survival of at least some uninjured neurons (Fig. 4A), and that removal of endogenous PSA with injury increases neuronal cell loss (Fig. 5D). In contrast to these reports, overexpression of PSA by polysialyltransferase transfection increases cell loss in hippocampal cultures[64] and enhances apoptosis in the embryonic chick retina,[65] whereas PSA removal from cultured neuroblastoma cells enhances cell viability during serum deprivation.[66]

These apparently contradictory effects of PSA removal on cell survival may be explained by PSA's ability to modulate NCAM signaling in at least two manners. First, NCAM-mediated signaling induced by exogenous NCAM agonists has been consistently shown to enhance the survival of different CNS neurons.[18] Consistent with this, removal of PSA promotes neuroblastoma cell viability by enhancing NCAM heterophilic interactions and promoting intracellular signaling.[66] By the same logic, overexpression of PSA, as seen in the embryonic chick retina and in hippocampal progenitors, may inhibit NCAM-heterophilic or homophilic interactions, which in turn would impede NCAM-mediated signaling. Alternatively, PSA may promote neuron survival by masking a harmful effect of NCAM.[67] This situation may explain our findings concerning RGC loss after PSA removal in developing, adult, and later (7 day) pathophysiological conditions. We previously reported that adult NCAM$^{-/-}$ mice have more RGCs which suggests that developmental absence of all NCAM, including PSA, does not result in increased RGC death.[43] In the present study, however, removal of PSA from either neonatal RGCs in vitro (Fig. 2) or from neighboring glia in the adult retina in vivo (Fig. 4) resulted in RGC loss. Furthermore, while RGC loss was significantly greater at 7 days after optic nerve injury with PSA removal compared with vehicle-treated controls (Fig. 5), we previously found no difference in RGC counts in NCAM$^{-/-}$ versus wild-type mice at the same time after injury.[43] Instead, RGC loss after optic nerve axotomy occurs earlier in adult NCAM$^{-/-}$ mice (4 days post-transection[43]; also see Fig. 5E), compared with wild-type mice with PSA either present or removed through Endo-N injection (Fig 5). These findings suggest that the absence of only PSA (and not NCAM) is more detrimental to the survival of RGCs than the loss of all NCAM (including PSA) during neonatal development, in the normal adult and later after injury; however, early after injury the loss of all NCAM (including PSA) is more harmful to RGCs than the absence of only PSA.

In agreement with our proposed mechanism for PSA's role in RGC survival in neonatal, normal adult and later after injury, knock-out mice lacking polysialyltransferases II and IV (the enzymes responsible for NCAM polysialylation; therefore, these mice have normal NCAM but lack PSA) exhibit several abnormalities not present in NCAM$^{-/-}$ mice including complete absence of the anterior commissure, severe hydroceph-

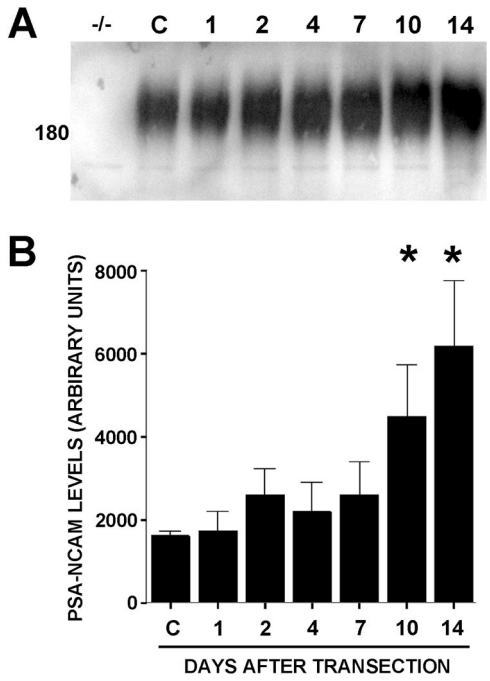

FIGURE 6. PSA levels increase in the adult retina after ON injury. (**A**) Retinas were collected at various times (in days) after ON transection, and Western blot analyses were performed for PSA. (**B**) PSA levels (n = 3/group) increased significantly at 10 (179% increase; $P = 0.043$) and 14 (285% increase, $P = 0.003$) days after axotomy. C, control; 1, 2, 4, 7, 10, and 14 are the number of days after axotomy; (−/−) represents $NCAM^{-/-}$ retina; and 180 = molecular weight in kDa; $^*P \leq 0.05$, different from C.

alus, reduced size of the internal capsule, post-natal growth retardation, and precocious death.[68] Furthermore, these deficits were restored by knocking out NCAM within the mice ($II^{-/-}$ $IV^{-/-}$ $NCAM^{-/-}$), indicating that a gain of NCAM function in the absence of PSA is more detrimental to certain aspects of nervous system development than the complete absence of NCAM. The exact mechanism by which a gain in NCAM function is detrimental to cell function is currently unknown.

Our findings demonstrate for the first time that PSA supports the survival of neonatal as well as injured and normal adult RGCs and provide the first functional evidence of a role for retinal PSA. Furthermore, these findings indicate that targeted upregulation of retinal PSA may be a rational treatment strategy for the protection of injured neurons.

Acknowledgments

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