Biochemistry and Molecular Biology

Polysialylated Neural Cell Adhesion Molecule Protects Against Light-Induced Retinal Degeneration

Margaret Po-shan Luke,[1] Terry L. LeVatte,[1] Urs Rutishauser,[2] François Tremblay,[3,4] and David B. Clarke[1,4,5]

[1]Department of Medical Neuroscience, Dalhousie University, Life Science Research Institute, Halifax, Nova Scotia, Canada
[2]Cellular and Developmental Neuroscience, Department of Cell Biology, Sloan–Kettering Institute, Memorial Sloan–Kettering Cancer Center, New York, New York, United States
[3]Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada
[4]Department of Ophthalmology & Visual Sciences, Dalhousie University, Halifax, Nova Scotia, Canada
[5]Department of Surgery (Neurosurgery), Dalhousie University, Halifax, Nova Scotia, Canada

Correspondence: David B. Clarke, Departments of Surgery (Neurosurgery), Medical Neuroscience, and Ophthalmology & Visual Sciences, Dalhousie University, Life Science Research Institute, 1348 Summer Street, Halifax, NS B3H 4R2, Canada; d.clarke@dal.ca.

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Purpose. We previously demonstrated that neural cell adhesion molecule (NCAM) plays an important role in supporting the survival of injured retinal ganglion cells. In the current study, we used light-induced retinal degeneration (LIRD) as a model to investigate whether NCAM plays a functional role in neuroprotection and whether NCAM influences $p75^{NTR}$ signaling in modulating retinal cell survival.

Methods. Retinas from wild-type (WT) and NCAM deficient (−/−) mice were tested by electroretinogram before and after LIRD, and changes in the protein expressions of NCAM, polysialic acid (PSA)-NCAM, $p75^{NTR}$, and active caspase 3 were measured by immunoblot from 0 to 4 days after light induction. The effects of NCAM and PSA-NCAM on $p75^{NTR}$ were examined by intraocular injections of the $p75^{NTR}$ function-blocking antibody and/or the removal of PSA with endoneuraminidase-N prior to LIRD.

Results. In WT mice, low levels of active caspase 3 activation were detected on the first day, followed by increases up to 4 days after LIRD. Conversely, in $NCAM^{-/-}$ mice, higher cleaved caspase 3 levels along with rapid reductions in electroretinogram amplitudes were found earlier at day 1, followed by reduced levels by day 4. The removal of PSA prior to LIRD induced earlier onset of retinal cell death, an effect delayed by the coadministration of endoneuraminidase-N and the $p75^{NTR}$ function-blocking antibody antiserum.

Conclusions. These results indicate that NCAM protects WT retinas from LIRD; furthermore, the protective effect of NCAM is, at least in part, attributed to its effects on $p75^{NTR}$.

Keywords: neural cell adhesion molecule (NCAM), polysialic acid (PSA), light-induced retinal degeneration (LIRD), mice, retina

The retina has the ability to capture photons of light efficiently and enact visual transduction, but excessive or continuous light exposure has been shown to result in cumulative oxidative stress, photo-transduction impairment, and photoreceptor cell death, leading to retinal damage, vision impairment, and blindness.[1] Extensive research in light-induced retinal damage (LIRD) has been conducted in rodents,[2] a model that shares many characteristics of human retinal degenerative diseases, including those caused by environmental insult.[3] The cleavage of the neural cell adhesion molecule (NCAM) has been shown to be involved in cortical neuronal death under oxidative stress,[4] and previous results from our laboratory have demonstrated that NCAM is important in retinal ganglion cell (RGC) survival and age-related deterioration in vision.[5–7] However, the molecular basis of NCAM's role in retinal degeneration remains elusive.

NCAM is a transmembrane protein that is involved in axonal fasciculation, cell migration, neurite outgrowth, synaptic plasticity, and the formation and stabilization of synapses during development.[8,9] It contains five immunoglobulin-like and two fibronectin type III repeats. NCAM is differentially expressed in two major transmembrane isoforms (180 and 140 kDa) and a glycophosphatidyl inositol-linked isoform (120 kDa).[10] Using immunohistochemical and electron microscopic techniques, NCAM was found to be present on all retinal neurons and in all layers in the developing and adult mouse retinas.[11,12] All major NCAM isoforms can be modified by polysialic acid (PSA), inserted as chains into N-glycosylation sites of the fifth immunoglobulin-like domain.[13] NCAM is the most abundant PSA carrier in mammals,[14] and the removal of NCAM abolishes almost all of the PSA in the nervous system.[15] PSA-NCAM is expressed throughout all retinal layers during development.[11] However, in adulthood, it is located exclusively on astrocytes and Müller glial cells of the mouse retina and on astrocytes of the optic nerve.[11]

$p75^{NTR}$ is an important neuronal type I transmembrane signaling protein that interacts with numerous ligands and coreceptors to regulate cellular survival and apoptosis, neurite outgrowth and repulsion, myelination, and long-term depression.[16] In the retina, $p75^{NTR}$ is found in the Müller cell processes,[17] which provide an environment for neurons to regulate synaptic activity and facilitate neuronal plasticity.[18]

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p75^{NTR} and sortilin have been shown to form a cell surface receptor complex for the proform of nerve growth factor (pro-NGF) to induce the death of RGCs in adult rodents.[19–21] Furthermore, the absence, or blockade, of p75^{NTR} promotes structural and functional photoreceptor cell survival after LIRD.[22]

Using light-induced injury to the retina as a model, we investigated whether NCAM plays a functional role in neuroprotection and whether NCAM influences p75^{NTR} signaling in modulating retinal cell survival. Our results in wild-type (WT) retinas after LIRD show an upregulation of PSA-NCAM and enhanced expression of p75^{NTR}. NCAM deficient (–/–) mice had diminished electroretinography (ERG) amplitudes and, as also observed in WT mice treated with endoneuraminidase-N (Endo N), exhibited earlier onset of retinal cell death. Blockade of p75^{NTR} in Endo N–treated WT and NCAM$^{-/-}$ retinas altered retinal apoptosis, suggesting that NCAM plays an important role in the modulation of the death-inducing effect of p75^{NTR} after LIRD.

Materials and Methods

Subjects

In this study, 2- to 4-month-old WT and homozygous NCAM$^{-/-}$ mice on a C57Bl/6J background were obtained by crossing heterozygous male and female animals (generously provided by Victor Rafuse, PhD).[23] The primer sequences were reported by Cremer et al.,[23] and the genotyping polymerase chain reaction conditions were 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute, then 72°C for 10 minutes.[23] The animals were subjected to in-house breeding and were cared for according to the Guide and Use of Experimental Animals of the Canadian Council on Animal Care. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Light Treatments

Mice ($n = 5$ to 10 per group; the exact number is provided in each figure legend) were anesthetized with a mixture of ketamine (100 mg/kg), acepromazine (0.62 mg/kg), and xylazine (15 mg/kg). Their pupils were dilated with 0.5% cyclopentolate hydrochloride (HCl) drops (Alcon, Fort Worth, TX, USA). Animals were dark adapted overnight, then exposed to 18,000 lux of white fluorescent light in a well-ventilated, air conditioned room for 6 hours. During exposure, a drop of normal saline was applied on the cornea every 20 minutes, and an additional one fifth of the anesthetic mixture would be given if the animals awakened during the photic injury procedure. Mice were euthanized at specific times from 0 to 4 days after photic injury.

Immunoblot

Protein lysates were prepared by gently pipetting the retinal tissues up and down in a 50-mM (hydroxymethyl)aminomehane(Tris)-HCl (pH 7.5), 5 mM EDTA, 300 mM sodium chloride (NaCl), 0.1% nonyl phenoxypolyethoxylethanol (NP-40), and 1X protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) several times and incubating for an hour in ice. Protein concentrations were quantified by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 20 µg for each of the WT and NCAM$^{-/-}$ proteins were adjusted with an equivalent volume of distilled water and mixed with 4x SDS-sample buffer (0.08% SDS, 250 mM Tris-HCl [pH 8.0], 40% glycerol, 20% β-mecaptoethanol, and a trace amount of bromophenol blue), boiled for 5 minutes, loaded in a polyacrylamide gel, ran for 2.5 hours at 80 V, then transferred to a polyvinylidene fluoride (PVDF) membrane at 0.25 A for 1.5 hours. Anti-PSA-NCAM (MAB5324, 1:2000; Millipore, Billerica, MA, USA), anti-NCAM (MAB310, 1:500; Millipore), anti-p75^{NTR} (8238, 1:1000; Cell Signaling Techology, Inc., Danvers, MA, USA), anti-Neurotensin Receptor 3 (Sortilin; 612100, 1:500; BD Transduction Laboratories, San Jose, CA, USA), anti-pro-NGF (AB9040, 1:2000; Millipore), and anti-actin (A2066, 1:5000; Sigma-Aldrich Corp., St. Louis, MO, USA) antibodies were used to determine protein expressions by standard SDS-polyacrylamide gel electrophoresis (PAGE) immunoblotting.[5] To capture active caspase 3 signals, protein lysates were loaded in a 15% polyacrylamide gel, separated via electrophoresis at 55 V for 5 hours and then transferred onto a PVDF membrane for 1 hour at 50 V. Anti-caspase 3 (cleaved) (AB3623, 1:100; Millipore) and anti-actin antibodies were used to determine protein levels. The chemiluminescent signals were detected by the Pierce ECL 2 Western Blotting Substrate (80196, Thermo Scientific, Rockford, IL, USA) and captured by exposure to X-ray films or by scanning with the Typhoon Variable Mode Imager (Amersham Biosciences, Sunnyvale, CA, USA). The levels of proteins were then quantified using Image J (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and the data obtained were converted to percentages of the controls.

Immunohistochemistry

Wild-type and NCAM$^{-/-}$ mice ($n = 5$ per group) were anesthetized and transcardially perfused with chilled 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The eyes were removed, postfixed for 30 minutes, and cryoprotected in 30% sucrose overnight at 4°C. Retinal sections (16 µm) were obtained by cutting along superior-inferior orientation of the eye with a cryostat apparatus (Leica CM1850; Leica Biosystems, Wetzlar, Germany). PSA-NCAM (MAB5324, 1:500; Millipore), Bassoon (SAP7F407, 1:500; Enzo Life Sciences LTD., Exeter, UK), p75^{NTR} (REX antibody: A gift from Louis Reichardt, University of California, San Francisco, San Francisco, CA, USA), and Hoechst (33258, 1:50,000; Sigma-Aldrich Corp.) immunostaining techniques, previously described,[6,24] were performed on sections from both light-induced and non-induced retinas.

Apoptosis Assay

After LIRD, the 16 µm cryostat sections were prepared as described previously. The WT retinal samples were fixed in 4% paraformaldehyde for 15 minutes, postfixed in precooled ethanol to acetic acid (2:1) for 5 minutes, permeabilized with proteinase K (20 µg/ml) for 10 minutes, and then stained with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Catalog #S7100, Millipore) according to the manufacturer's instructions.

Electroretinography (ERG)

Wild-type and NCAM$^{-/-}$ mice were used for visual electrophysiology examinations, which were performed 3 days before and 1 day after LIRD using the method described previously.[25] Briefly, mice were dark-adapted overnight and anesthetized. Pupils were dilated with 0.5% cyclopentolate HCl drops (Alcon). ERGs were recorded using a silver-impregnated nylon fiber electrode (Diagnosys, Littleton, MA, USA), which was placed on the mouse's corneal surface and hydrated with 2.5% hydroxypropyl methylcellulose solution. The protocol consisted of recording ERGs from a series of strobe flash visual stimuli

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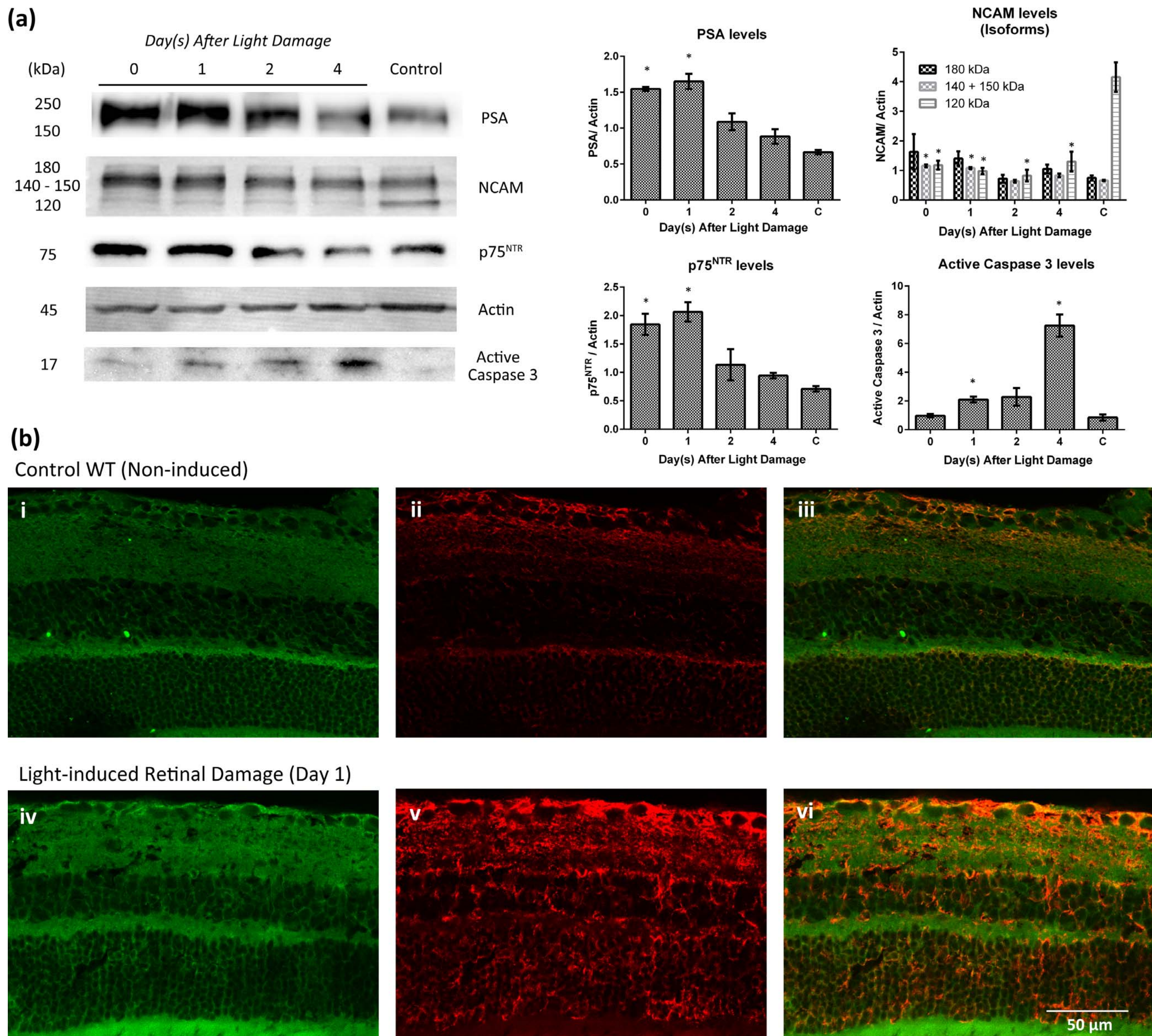

FIGURE 1. Western blot analysis (**a**) shows high levels of PSA-NCAM and p75^{NTR} proteins expressed in the WT retinas ($n = 5$ per group) shortly after light exposure. Active caspase 3 was detected at low levels on day 0, followed by increases up to 4 days after LIRD. Representative immunofluorescence staining (**b**) shows increased p75^{NTR} (*green*) and PSA-NCAM (*red*) expression in the WT retinas after light-induced injury: (i) noninduced, p75^{NTR} staining; (ii) noninduced, PSA-NCAM staining; (iv) induced, p75^{NTR} staining; (v) induced, PSA-NCAM staining; and (iii and vi) merged images of p75^{NTR} and PSA-NCAM staining. *Asterisk* (*) denotes significantly different from control (C).

with increasing intensity from −5.8 to 1 log cd·s/m^2 in scotopic and photopic conditions.

Surgery

Animals were anesthetized with isofluorane, and intravitreal injections were performed under an operating microscope using a microliter syringe (7102; Hamilton, Reno, NV, USA) with a glass micropipette tip. The WT mouse eye was injected with 1 µl of vehicle (50% PBS/glycerol solution), or vehicle containing Endo N (6.7 U/µl), Endo N and anti-p75^{NTR} REX antibody (1 mg/ml), or EndoN and immunoglobulin G (IgG) antibody (1 mg/ml). For NCAM$^{-/-}$ mice, their eyes were injected with the REX or control IgG antibody. A drop of antibiotic solution (Polysporin, Johnson and Johnson, New Brunswick, NJ, USA) was applied to the cornea of the anesthetized mouse immediately after the intraocular injection. Animals were subjected to LIRD 4 to 5 days after the intravitreal injection.

Data Analysis

The differences in Western blot densitometry from the three or four independent experiments were quantified by 2-sample *t*-tests. Data from electrophysiology experiments were analyzed with a customized program from Matlab (Mathworks, Natick, MA, USA). To analyze amplitudes and implicit times of the ERG responses, the a-wave, which represents the function of photoreceptor cells, was measured from the baseline to the trough. The b-wave, which reflects mainly on ON-bipolar activity, was measured from the trough of the a-wave to the peak of the b-wave. The maximum response amplitude Vmax, the sensitivity parameter log K (the intensity of the stimulus at

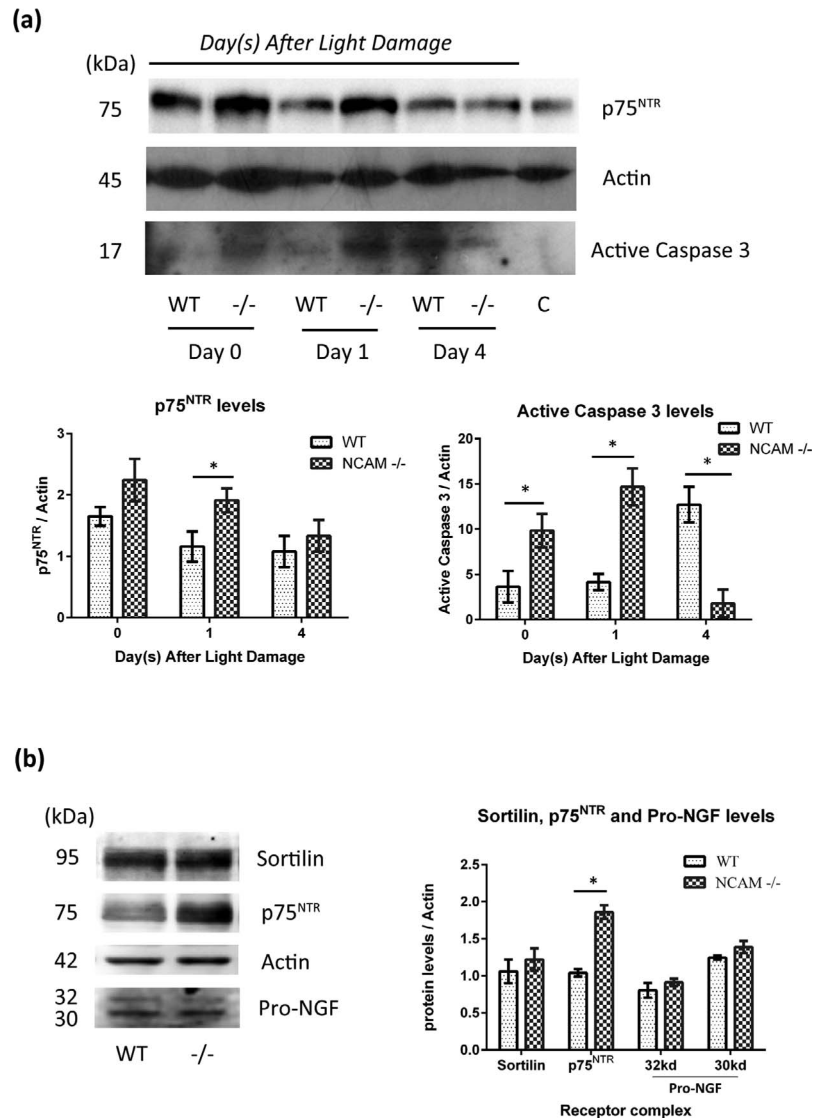

FIGURE 2. (**a**) Western blot analysis of p75^{NTR}, actin, and active caspase 3 protein expressions in WT and NCAM$^{-/-}$ retinas ($n = 5$ per group) after LIRD. Higher level of active caspase 3 expressed in NCAM$^{-/-}$ at days 0 and 1 after light exposure suggests photic injury induces earlier cell death in these retinas. The control sample (C) was prepared from noninduced NCAM$^{-/-}$ retinas. (**b**) Immunoblot analysis of p75^{NTR}, sortilin, pro-NGF, and actin protein expressions in uninjured mice shows elevated levels of p75^{NTR} expressed in NCAM$^{-/-}$ retinas. *Asterisk* (*) denotes the significant difference between WT and NCAM$^{-/-}$ protein expression.

half Vmax), and the exponent n (slope of the intensity-response function) of the Naka-Rushton hyperbolic equation were derived from the measurement values based on the curve-fitting procedure.[26] Differences (Vmax, log K, and n) were evaluated with Student's *t*-tests to compare NCAM$^{-/-}$ with the WT mice. Statistics were expressed as mean percentages ± standard error of the mean (SEM). Differences were considered statistically significant if $P < 0.05$. All analyses were performed using Minitab (Minitab, Inc., State College, PA, USA).[16]

RESULTS

Increased PSA and p75^{NTR} Levels After Photic Injury

To investigate whether NCAM plays a role in LIRD, we first assayed NCAM and PSA expression in WT retinas 0, 1, 2 and 4 days after 6 hours of photic injury (Fig. 1a). When compared with the noninduced control, we observed notably decreased levels of the 120 kDa, and increased levels of the 140 to 150 kDa, NCAM isoforms in the LIRD samples. NCAM-180 (180 kDa) expression did not appear to change over time. High levels of PSA (150–250 kDa) were expressed in the retinas immediately after light-induced damage, and their levels remained elevated for at least 2 days. An upregulation of p75^{NTR} (75 kDa) was found in the light-damaged retinas, and these findings were consistent with previous studies.[22,27] Interestingly, the patterns of p75^{NTR} and PSA expression following LIRD were similar; furthermore, immunostaining showed coexpression of p75^{NTR} and PSA in the retinas after LIRD (Fig. 1b). Cleaved caspase 3 (17 kDa), an indicator of apoptosis, was detected at low levels on day 0, followed by an increase up to 4 days after LIRD, results that were consistent with a TUNEL assay (Supplementary Fig. S1).

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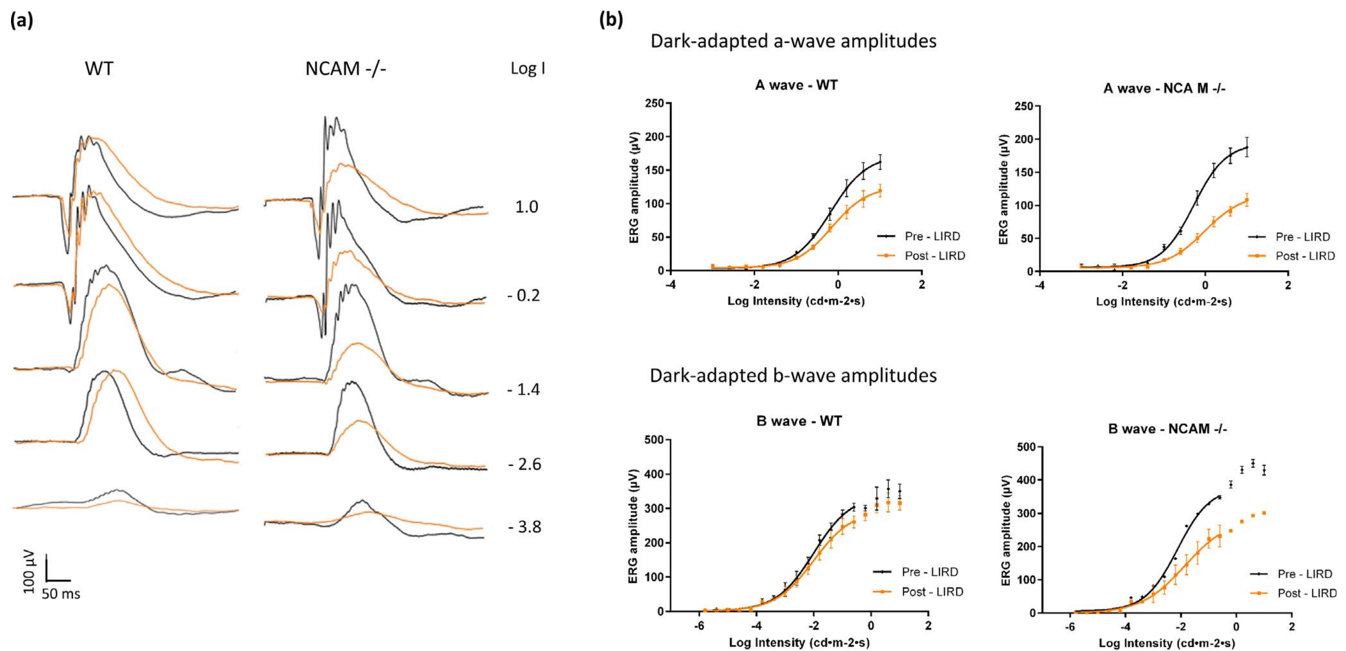

FIGURE 3. Analysis of pre- and post-LIRD ERG responses ($n = 6$ per group). (**a**) Representative dark-adapted ERGs recorded from WT and NCAM$^{-/-}$ mice showing 3 days pre- (*black*) and one day post- (*orange*) LIRD ERG waveforms. Flash intensity, increasing from −3.8 to 1 log cd*s/m^2, is marked on the right on each pair of the waveforms. (**b**) Mean dark-adapted a- and b-wave amplitudes plotted against log flash intensity for NCAM$^{-/-}$ and WT mice. NCAM$^{-/-}$ mice have significantly lower a- and b-wave amplitudes 1 day after LIRD.

Early Apoptosis in the NCAM$^{-/-}$ Retina

We next examined whether the timeline of retinal cell death we observed in LIRD was altered by the absence of NCAM (Fig. 2a). Relatively thinned photoreceptor cell layers were observed in WT and NCAM$^{-/-}$ retinas 4 days after LIRD (Supplementary Fig. S2). However, in NCAM$^{-/-}$ mice, higher baseline cleaved caspase 3 levels were found several days earlier at day 0 and day 1 when compared with WT retinas; by day 4, the cleaved caspase 3 levels were reduced in NCAM$^{-/-}$ retinas, whereas they were high in WT retinas. This earlier onset of cell death in NCAM$^{-/-}$ retinas correlated with higher levels of p75^{NTR} expression on days 0 and 1 after LIRD.

Higher p75^{NTR} Levels in the NCAM$^{-/-}$ Retina

As higher levels of p75^{NTR} were expressed in the NCAM$^{-/-}$ retinas after LIRD, we next investigated whether p75^{NTR} and its associated proteins were differentially expressed in the knockout's retinas. The pro-NGF, sortilin, and p75^{NTR} complex has been shown to participate in light-dependent photoreceptor degeneration,[21] so we compared their expressions in the retinas of WT and NCAM$^{-/-}$ mice using immunoblot analysis (Fig. 2b). Interestingly, although no difference was found in sortilin (95 kd) and pro-NGF (30 and 32kd) levels, p75^{NTR} expression in NCAM$^{-/-}$ retinas was significantly higher than in controls.

Decreased ERG Amplitudes in NCAM$^{-/-}$ Retinas After LIRD

In addition to examining the rate of apoptosis in WT and NCAM$^{-/-}$ mice, we also assessed retinal function before and after photic injury. Figure 3a shows a representative ERG intensity–response series recorded from WT and NCAM$^{-/-}$ mice. When compared with pre-LIRD values, ERG results showed significant drops of a- and b-wave amplitudes in both groups of mice a day after LIRD (Fig. 3b). Similar findings were observed in the b-wave light-adapted amplitudes (Supplementary Fig. S3). There were no differences in response times to the light stimuli for both groups of animals (Supplementary Fig. S4). Furthermore, our baseline results

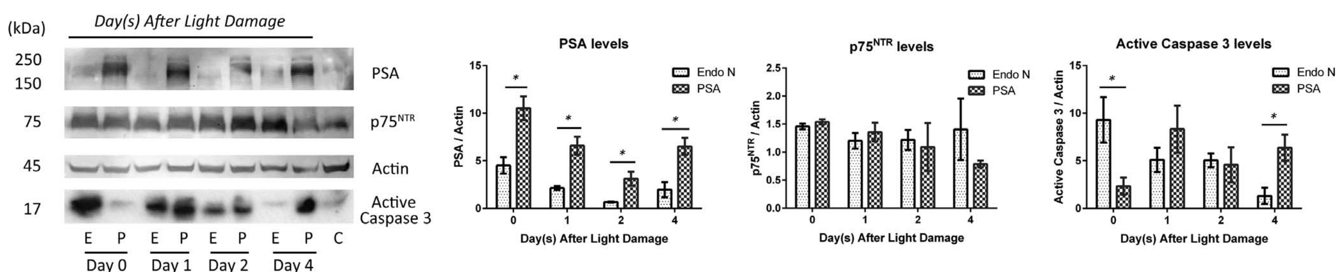

FIGURE 4. Immunoblot analysis shows that the removal of PSA induces the early onset of retinal cell death; active caspase 3 levels are elevated early after LIRD when PSA is removed by Endo N. At 4 to 5 days prior to light-induced retinal damage treatment, WT animals received an intraocular injection of Endo N (E) in the right eye and PBS (P) injection in the left eye. Mice ($n = 10$ per group) were euthanized 0, 1, 2, and 4 days after photic injury. The noninduced, PBS-injected WT retinas served as control (C). *Asterisk* (*) denotes the significant differences between Endo N- and PBS-injected retinas.

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Table 1. Vmax parameters Derived From Scotopic ERG a- and b-Wave Intensity-Response Function for NCAM$^{-/-}$ and WT Animals Show That Both Groups of Mice Have Lower Than Normal Vmax Levels 1 Day After LIRD

	Vmax		*t*-Test		
			Pre-LIRD		
	WT	NCAM$^{-/-}$	WT	NCAM$^{-/-}$	Between Group
a wave					
Pre-LIRD	173.73 ± 4.98	210.34 ± 6.07			0.001
Post-LIRD	129.07 ± 6.18	110.59 ± 4.54	0.000	0.000	0.039
b wave					
Pre-LIRD	343.97 ± 7.22	435.94 ± 13.51			0.000
Post-LIRD	300.27 ± 9.97	255.92 ± 23.41	0.006	0.000	ns

ns, not significant.

are similar to what has been recently reported: young adult NCAM$^{-/-}$ mice have significantly higher a- and b-wave Vmax than WT mice.[7] Using the Naka-Rushton equation, intensity–response functions indicated that the Vmax of the mean a- and b-waves were reduced by 47% and 41%, respectively, in NCAM$^{-/-}$ mice after LIRD (Table 1); in contrast, we observed a smaller 26% and 13% reduction in the Vmax of a- and b-waves for the WT animals. The parameter K reflects retinal sensitivity to a light stimulus; pre- and post-LIRD values of the mean a- and b-waves log K remained unaffected in WT and NCAM$^{-/-}$ animals (Table 2). There was a significant decrease in the slope (n) parameters in the b-wave of the NCAM$^{-/-}$ mice, suggesting that the animals' responses to light decline 1 day after photic injury (Table 3).

Removal of PSA-Induced Early Apoptosis

To determine whether the presence of PSA found in WT retinas protects against LIRD, PSA was selectively removed by Endo N 4 to 5 days before photic injury (Fig. 4). For eyes that received Endo N, the onset of extensive cell death commenced broadly and immediately (day 0) after LIRD, followed by decreasing active caspase 3 expression by days 2 and 4. However, for eyes that received a PBS intraocular injection, the onset of massive retinal apoptosis was delayed until 1 day after LIRD, with strong cleaved caspase 3 activity also seen at day 4. Furthermore, although elevated levels of p75^{NTR} appeared immediately after LIRD, there were no major differences in the p75^{NTR} expression between Endo N– and PBS-injected retinas on the first 2 days (days 0 and 1), times when we observed significant differences in the activated caspase 3 levels.

Blockade of p75^{NTR} Delays Retinal Cell Death in Endo N–Treated WT Mice

To determine whether p75^{NTR} has an effect on the premature onset of apoptosis in Endo N–treated WT retinas, we applied the REX antiserum, which inhibits p75^{NTR} function by binding to its extracellular domain (Fig. 5). The coadministration of Endo N and REX antibodies delays the onset of retinal apoptosis by 1 day when compared with controls (IgG–Endo N). Delaying apoptosis by blocking p75^{NTR} was independent of p75^{NTR} levels, which remained similarly elevated in both groups.

Blockade of p75^{NTR} Delays Retinal Cell Death in NCAM$^{-/-}$ Mice

The absence of NCAM removes almost all PSA in the null mutant mice. Based on our findings in WT retinas, we also investigated the effect of elevated p75^{NTR} levels in NCAM$^{-/-}$ retinas on the photic injury response (Fig. 6). For the controls (IgG), as expected, robust active caspase 3 levels (17 and 19 kDa) appeared immediately (day 0) after light-induced damage, whereas the administration of REX antiserum delayed the onset of apoptosis (day 1). Again, delaying apoptosis by the blockade of p75^{NTR} was independent of p75^{NTR} expression levels.

Discussion

Summary

In this study, we have investigated the role of NCAM and its polysialylated derivative, PSA-NCAM, in retinal apoptosis result-

Table 2. The Intensity-Response Function of the Scotopic ERG a- and b-Wave Shows No Difference in log *k* Values Before and After LIRD for Both NCAM$^{-/-}$ and WT Mice

	log *k*		*t*-Test		
			Pre-LIRD		
	WT	NCAM$^{-/-}$	WT	NCAM$^{-/-}$	Between Group
a wave					
Pre-LIRD	−0.22 ± 0.06	−0.20 ± 0.06			ns
Post-LIRD	−0.12 ± 0.09	−0.13 ± 0.10	ns	ns	ns
b wave					
Pre-LIRD	−2.01 ± 0.09	−1.95 ± 0.05			ns
Post-LIRD	−1.89 ± 0.15	−1.89 ± 0.15	ns	ns	ns

ns, not significant.

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TABLE 3. The Scotopic ERG Intensity–Response Function Shows That NCAM$^{-/-}$ Mice Have a Lower Slope (n) Value in the b-Wave 1 Day After LIRD

	n		*t*-Test		
			Pre-LIRD		
	WT	NCAM$^{-/-}$	WT	NCAM$^{-/-}$	Between Group
a wave					
Pre-LIRD	1.06 ± 0.09	0.95 ± 0.04			ns
Post-LIRD	0.93 ± 0.07	1.03 ± 0.12	ns	ns	ns
b wave					
Pre-LIRD	0.70 ± 0.05	0.87 ± 0.06			0.043
Post-LIRD	0.74 ± 0.07	0.57 ± 0.04	ns	0.002	ns

ns, not significant.

ing from phototoxicity. Our results show that an upregulation of PSA was expressed in the WT retinas immediately after 6 hours of photic injury. Retinal cell death occurred sooner than normal with the removal of PSA as well as in the absence of NCAM. The magnitude of retinal apoptosis in NCAM$^{-/-}$ mice was correlated with a marked reduction of the mean Vmax in a- and b-waves in ERG analysis. We specifically investigated the expression of the low-affinity neurotrophin receptor p75^{NTR}, known to promote retinal cell death caused by intense light illumination.[22] When compared with the WT, we detected higher levels of p75^{NTR} in young adult NCAM$^{-/-}$ retinas. The blockade of p75^{NTR} delayed retinal cells from degeneration in NCAM$^{-/-}$ mice and in WT retinas where PSA was removed, suggesting that the presence of NCAM protected retinas from p75^{NTR}-induced apoptosis after photic injury. Despite this, the blockade of p75^{NTR} by a single injection of blocking antibody only postpones the onset of retinal cell death, implicating involvement of alternate pathways independent of p75^{NTR} activation. Taken together, our findings strongly support the notion that NCAM plays an important role in protecting retinal cells from photic injury–induced death, at least in part through the modulation of p75^{NTR}.

Light Damage as a Model for Retinal Degeneration

Excessive light can cause retinal degeneration experimentally and may be a contributing factor in the progression of human retinal dystrophies and age-related retinal diseases.[2] Since its first use in rodents by Noell et al.,[28] LIRD has been used in many studies to examine the effects of white or visible light of different wavelengths and intensities on inducing photoreceptor cell damage.[3,29,30] Animal models of inherited retinal degeneration such as the retinal degeneration 1 mouse, have used LIRD to study cellular, molecular, and biochemical events associated with the regulation of photoreceptor cell death.[31–33] LIRD can be applied by long-term exposure (days) with low illumination or by short-term exposure (hours) with high intensity[2]; the former results in a slow progression of photoreceptor cell death, whereas the latter induces robust cell degeneration, causing at least 90% of photoreceptor cell loss over 10 days.[34] The induction of photoreceptor cell death by light exposure has also been used to identify new compounds or to evaluate the effectiveness of pharmacologic treatments such as the neuroprotective agent minocycline.[35–37]

The Association Between PSA-NCAM and p75^{NTR}

PSA-NCAM and p75^{NTR} in the Rodent Retina. The highly sialylated form of NCAM and the p75^{NTR} protein are multifaceted receptors capable of fulfilling a wide number of biological functions.[38,39] They are expressed widely during development in the nervous system, but decline dramatically by adulthood. During retinal development, PSA-NCAM is expressed in neuroblasts, young postmitotic neurons, astrocytes, and Müller cells of the mouse retina, but the levels progressively diminish in retinal neurons during the third postnatal week.[11] Likewise, high levels of p75^{NTR} are expressed in the inner nuclear layer, inner plexiform layer, and ganglion cell layer of the postnatal rat retina.[17,40] In adulthood, the expression of PSA-NCAM and p75^{NTR} are markedly reduced and expressed only in Müller glia, which have structural and functional roles for neurons in the retina.[11,17] Injury to the adult nervous system reactivates PSA-NCAM and p75^{NTR} expression, as observed in the visual system after RGC injury and in models of glaucoma.[6,17,41–44] We now know, based on the work of the Wada group,[22] as well as our current study, that intense and prolonged light exposure on the retina results in the activation of p75^{NTR}.

The Interplay of PSA-NCAM and p75^{NTR} in Neuronal Survival. The polysialyated form of NCAM has been shown to be a prosurvival molecule in numerous studies,[6,41,42,45–47] but

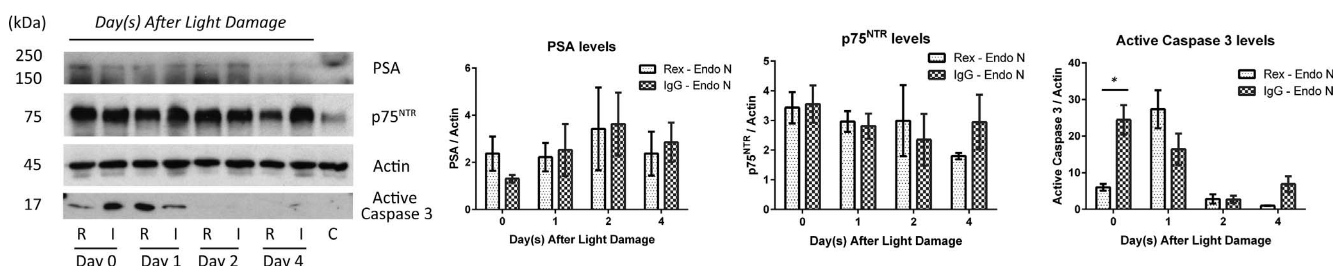

FIGURE 5. Immunoblot analysis shows that the blockade of p75^{NTR} binding site, as well as the removal of PSA, delay the early onset of retinal cell death. At 4 to 5 days prior to light-induced retinal damage treatment, WT animals received coadministration of Endo N and REX antiserum (R) in the right eye and Endo N and IgG (I) in the left eye. Mice ($n = 10$ per group) were euthanized 0, 1, 2, and 4 days after photic injury. The noninduced, Endo N–IgG-injected WT retinas served as control (C). *Asterisk* (*) denotes the significant differences between Endo N–REX-injected and Endo N–IgG-injected retinas.

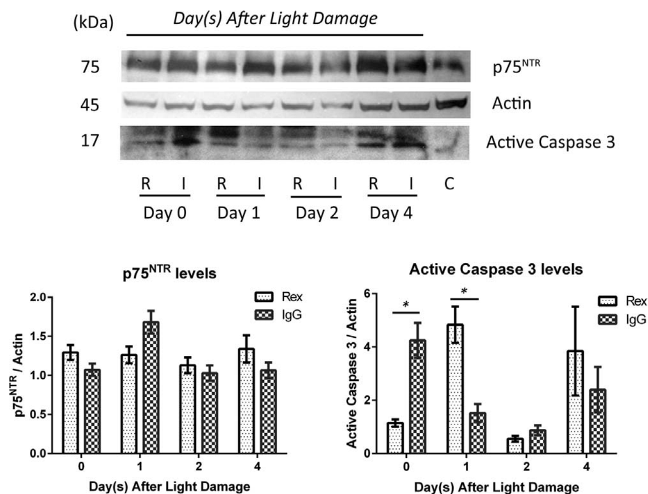

Figure 6. Immunoblot analysis shows a blockade of p75^{NTR} delayed early onset of retinal cell death in NCAM$^{-/-}$ mice. At 4 to 5 days prior to light-induced retinal damage treatment, NCAM$^{-/-}$ mice received an intraocular injection of REX (R) in the right eye and IgG (I) injection in the left eye. The animals ($n = 8$ per group) were euthanized 0, 1, 2, and 4 days after photic injury. The noninduced, IgG-injected NCAM$^{-/-}$ retinas served as control (C). *Asterisk* (*) denotes the significant differences between the REX- and IgG-injected retinas.

the mechanisms by which PSA-NCAM contributes to the survival of neurons remain unclear. There are a few studies that have examined the regulation of neurogenesis and its association with p75^{NTR}. The absence of NCAM, as well as the removal of PSA, results in enhanced apoptosis in postnatally generated new neurons of the olfactory bulb.[46] These changes are accompanied by an elevated level of p75^{NTR}, and pharmacologic blockade of the p75^{NTR} signaling pathway enhances the survival of these neurons.[46] Furthermore, the administration of LM11A-31, which selectively inhibits the binding of NGF and proNGF to p75^{NTR}, also increases PSA-NCAM expression and promotes the survival of progenitor neuronal cells in the subgranular zone.[48] Here, following light-induced retinal injury, we provide further evidence that NCAM and its polysialyated moiety PSA-NCAM modulate the activity of p75^{NTR} to promote retinal cell survival.

The Role of NCAM in RGC Survival

NCAM plays an important role in supporting neuronal survival in the mouse retina. Our previous reports have shown that NCAM and its PSA moiety influence RGC survival. We have previously demonstrated that NCAM influences the survival of RGCs during development and following injury: (1) NCAM$^{-/-}$ mice have more RGCs in the retinas and have higher levels of brain-derived neurotrophic factor (BDNF) in the superior colliculus than WT mice[5]; (2) following optic nerve transection, the onset of RGCs loss is earlier in mice lacking NCAM[5]; (3) and removal of PSA from the surface of neonatal RGCs in vitro, as well as from the adult injured and uninjured retina in vivo, promotes significant RGC death.[6] How NCAM influences RGCs survival in these models remain speculative but may involve interaction with the BDNF-induced tropomyosin receptor kinase (Trk)B cell surface receptor.[49] PSA has been shown to present BDNF to TrkB, thus concentrating the neurotrophin close to its site of action.[47] Numerous studies have demonstrated that neurotrophins activate the phosphatidylinositol-3-kinase-Akt signaling cascade through their corresponding receptor tyrosine kinases to promote neuronal survival.[50–53] Therefore, although little is known concerning how NCAM deficiency influences RGCs death, a loss of prosurvival BDNF–TrkB signaling in NCAM-deficient mice may be an important mechanism.

p75^{NTR} in Response to Retinal Injury and Degeneration: Molecular Basis

p75^{NTR} does not have intrinsic catalytic activity. Depending on its binding partners or the physiological state of the cell, p75^{NTR} associates selectively with a unique array of proteins, including sortilin, Trk, and Nogo receptors, to influence a wide range of cellular functions. The activation of p75^{NTR} results in distinct and even opposing actions, including the promotion of cell survival, the activation of apoptotic pathways, or support for the growth cone retraction. Numerous studies have examined the effect of p75^{NTR} activation in the retina. During light-induced retinal damage, the presence of p75^{NTR} in Müller glial cells suppresses the release of fibroblast growth factor, bFGF, which supports the survival of retinal neurons, and that the blockade of p75^{NTR} protects photoreceptor cells from apoptosis.[22] However, the absence of p75^{NTR} does not protect photoreceptor cells against light-induced injury, suggesting that alternative cell death pathways exist in p75^{NTR}-deficient mice.[54] Using chronic (glaucoma) and acute (optic nerve axotomy) injury models to induce RGC degeneration, the activation of p75^{NTR} has been shown to trigger the release of tumor-necrosis factor α and α2-macrogloblin neurotoxic proteins, which act against the protective effect of TrkA receptor and lead to neuronal cell death.[55] Furthermore, proNGF activates a noncell autonomous signaling pathway in response to central nervous system (CNS) injury; p75^{NTR} as well as its coreceptors Neurotrophin Receptor Interacting MAGE Homlog and sortilin are required to stimulate the release of tumor-necrosis factor α in Müller glial cells to induce RGC death.[20] Similar findings have been reported using glutamate-induced excitotoxicity in the mouse retina.[56] Taken together, in a variety of cells and in response to various stimuli, retinal apoptosis can involve an upregulation of p75^{NTR}, which associates selectively with specific receptors to activate a cascade of cell death signaling events.

Conclusions

We have demonstrated that NCAM protects WT retinas from LIRD through a mechanism that is, at least in part, a result of modulation in p75^{NTR} signaling. This greater understanding of the molecular mechanisms involved in LIRD may provide therapeutic opportunities for treatment in this injury model and may also be relevant to other diseases characterized by retinal degeneration.

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