Research Report

PSA modification of NCAM supports the survival of injured retinal ganglion cells in adulthood

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A B S T R A C T

Neural cell adhesion molecule (NCAM) is known as the cell surface glycoprotein, and it belongs to the immunoglobulin superfamily of adhesion molecules. Polysialic acid (PSA) is a carbohydrate attached to NCAM via either of two specific sialyltransferases: ST8SiaII and ST8SiaIV. Polysialylated neural cell adhesion molecule (PSA-NCAM) mediates cell interactions, plays a role in axon growth, migration, synaptic plasticity during development and cell regeneration. Some evidence has shown that PSA-NCAM supports the survival of neurons. It was demonstrated that PSA-NCAM is present in abundance in the retina during development and in adulthood. The aim of this study was to investigate whether PSA-NCAM promotes retinal ganglion cell (RGC) survival in transgenic mice with deficiencies in sialyltransferases or NCAM or after the administration of endon neuraminidase (Endo-N). RGC injury was induced by intravitreal administration of kainic acid (KA). These studies showed that injection of Endo-N after 14 days enhances the toxicity of KA to RGCs in wild-type (WT) mice by 18%. In contrast, in knockout mice (ST8SiaII−/−, ST8SiaIV−/−, NCAM−/−), survival of RGCs after KA injury did not change. Deficiencies of either ST8SiaII or ST8SiaIV did not influence the level of PSA-NCAM in the adult retina, however, in neonatal animals, decreased levels of PSA-NCAM were observed. In knockout ST8SiaII−/− adults, a reduced number of RGCs was detected, whereas in contrast, increased numbers of RGCs were noted in NCAM−/− mice. In conclusion, these data demonstrate that PSA-NCAM supports the survival of injured RGCs in adulthood. However, the role of PSA-NCAM in the adult retina requires further clarification.

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1. Introduction

NCAM is expressed on the membranes of neurons and glial cells. The extracellular fragment of NCAM contains five Ig-like modules followed by two fibronectin type III modules (Cunningham et al., 1987). NCAM is considered to be a mediator of cell-cell interactions that can promote cell adhesion through a homophilic binding mechanism (rev. by Edelman, 1986). NCAM functions may be regulated by the addition of long linear homopolymers of alpha 2-8-linked...
sialic acids (PSA). PSA has a large hydrated volume and a high negative charge density and thus, reduced adhesion forces (Rougon, 1986; Rutishauser and Landmesser, 1996). Two transferases, called ST8SiaII and ST8SiaIV, synthesize PSA on NCAM. ST8SiaII functions primarily during embryogenesis, and its expression decreases shortly after birth. In contrast, ST8SiaIV is the primary in adulthood, and is almost absent during development (Livingston and Paulson, 1993; Eckhardt et al., 1995). PSA is widely expressed in the central nervous system during embryogenesis and is remarkably down-regulated after birth (Rothbard et al., 1982; rev. by Seki and Arai, 1993). However, its expression persists in brain regions with ongoing plasticity and neurogenesis, such as the olfactory bulb, piriform cortex, hippocampus, hypophysis and hypothalamus (rev. by Seki and Arai, 1993; Eckhardt et al., 2000). During development, PSA-NCAM is expressed abundantly in the retina, and it continues to be expressed in the adult optic nerve and retina by astrocytes and Müller cells (Bartsch et al., 1990; Sawaguchi et al., 1999; Canger and Rutishauser, 2003; Murphy et al., 2009). Müller cells are a type of glial cell, which extend processes throughout the retina. During embryogenesis, Müller cells promote the growth of RGCs axons towards the optic disk, which subsequently remains in close contact with the endfeet of Müller glia (Stuermer and Bastmeyer, 2000). PSA-NCAM has been shown to play a role in cell migration (Wang et al., 1994; Rousselot et al., 1995), synaptic plasticity (Lüth et al., 1994; Müller et al., 2000), is implicated in the regulation of axon growth, guidance, and fasciculation (Doherty et al., 1990; Tang et al., 1992; Rutishauser, 1996), and promotes the survival of cortical neurons (Vutskits et al., 2001), subventricular zone neurons (Gascon et al., 2007), and RGCs (Murphy et al., 2009).

Previously, it was shown that PSA on the surface of cultured neonatal RGCs supports the survival of these cells and that removal of PSA from the retina in vivo reduced RGC density following optic nerve transection by a further 27% (Murphy et al., 2009). In NCAM−/− mice, RGC loss following optic nerve transection occurs earlier than in WT mice (Murphy et al., 2007).

Knockout mice missing the sialyltransferases ST8SiaII, ST8SiaIV and NCAM−/− are used widely to explore PSA-NCAM functions (Murphy et al., 2007; Aonurm-Helm, 2010). Transgenic ST8SiaII−/− and ST8SiaIV−/− animals have normal NCAM, but lack PSA during the prenatal period and in adulthood, respectively. NCAM−/− mice do not have any NCAM or PSA during life. Knockout animals exhibit a number of morphological and functional abnormalities in the central nervous system and also behavioral abnormalities (Wood et al., 1998; Weinhold et al., 2005; Hildebrandt et al., 2007; Aonurm-Helm, 2010). In previous studies, PSA was removed from the eye via the application of Endo-N (Murphy et al., 2009). In this current study we wanted to explore the impact of retinal PSA-NCAM on the survival of injured and uninjured RGCs in vivo using transgenic animals or via the intravitreal administration of Endo-N.

![Fig. 1](representative microphotograph of PSA-NCAM expression in retinal sections from WT, NCAM−/−, ST8SiaII−/− or ST8SiaIV−/− mice. Arrowheads show PSA-NCAM expression. Magnification x 200.)

![Fig. 2](representative microphotograph of PSA-NCAM expression in hippocampal sections from WT, ST8SiaII−/− or ST8SiaIV−/− mice. Abbreviations: gcl – granular cell layer; sgl – subgranular layer; hil – hilus. Magnification: x 200 (inset: x 400).)
Immunohistochemical detection of PSA-NCAM in the retinal sections from adult WT mice revealed abundant expression of PSA-NCAM. Immunohistochemistry revealed that PSA-NCAM immunopositive cells were located primarily in the inner part of the retina in close proximity to the RGCs (Fig. 1). No PSA-NCAM was detected in the retina of NCAM−/− animals (Fig. 1). Mice with constitutive deficiencies in ST8SiaII−/− or ST8SiaIV−/− did not demonstrate any changes in the level of PSA-NCAM in retina compared with WT animals (Fig. 1). In contrast, PSA-NCAM staining of the hippocampal sections demonstrated clear differences between genotypes. In WT mice, both hilus (hil) and granular cell layer (gcl) demonstrated positive signal for PSA-NCAM. ST8SiaII deficient mice had positive signal only in hilus, but no in gcl, whereas in ST8SiaIV deficient mice, only newly generated cells located in gcl expressed PSA-NCAM (Fig. 2). Western blotting analysis also failed to demonstrate a reduction in the level of PSA-NCAM in the retinal tissues of ST8SiaII−/− or ST8SiaIV−/− animals (Fig. 3). For comparison, we measured the level of PSA-NCAM in the brain tissues of these animals and noted a considerable reduction of PSA-NCAM in the brain of ST8SiaIV−/− mice but not in the brain of ST8SiaII−/− mice (Fig. 3). In WT mice, we also assessed the effect of the enzyme Endo-N, which removes PSA residues from the NCAM molecule at the level of PSA-NCAM. Intraocular administration of Endo-N induced a long-lasting reduction in the level of PSA-NCAM at 2, 14 and 28 days following administration (Fig. 4).

To determine the cellular localization of PSA-NCAM in the retina, we performed co-localization studies of PSA-NCAM with Brn3a (RGC marker) and GFAP (astrocyte marker) in the retinal sections. Consistent with previous studies (Sawaguchi et al., 1999), PSA-NCAM, although located close to RGCs bodies, did not co-localize with Brn3a (Fig. 5, upper panel). The majority of PSA-NCAM co-localized with GFAP (Fig. 5, bottom panel). PSA-NCAM/GFAP-positive cells were observed under the inner basal membrane, intimately located to axons of the nerve fiber layer and the intraocular portion of the optic nerve. The processes of PSA-NCAM/GFAP-positive cells were restricted to the RGCs and to the nerve fiber layer.

2.2. The level of PSA-NCAM expression in newborn (P1) WT and ST8SiaII−/− or ST8SiaIV−/− mice

It is well established that PSA-NCAM levels are extensively downregulated in the nervous system after development (Rothbard et al., 1982; Nakayama et al., 1995). We therefore, measured the level of PSA-NCAM in the retina of newborn transgenic animals with a deficiency in ST8SiaII or ST8SiaIV. In contrast to adult mice, PSA-NCAM was almost absent in the retina of neonatal ST8SiaII mice, whereas the level of PSA-NCAM in the retina of ST8SiaIV−/− mice was approximately two-fold lower compared with WT animals (Fig. 6). A one-way ANOVA demonstrated a significant effect of genotype \( F = 191; p < 0.0001 \).

2.3. Effect of intravitreal injection of kainic acid on the survival of RGCs in adult WT, ST8SiaII−/−, ST8SiaIV−/− and NCAM−/− mice or in mice administered with Endo-N

One of the putative roles of PSA-NCAM in the adult retina is its protective function for RGCs (Murphy et al., 2009). To examine whether a constitutive deficiency in NCAM or its sialylation affects the density of RGCs, we counted Brn3a immunoreactive cells in wholemounted retinas of ST8SiaII−/−, ST8SiaIV−/− or NCAM−/− mice and their WT littermates. The density of Brn3a-positive cells (RGCs) was significantly lower \( p < 0.05 \) in ST8SiaII−/− mice compared with their WT littermates, whereas no differences in the density of Brn3a-positive cells was observed.

Fig. 3 - Western blots demonstrating PSA-NCAM protein levels in adult retinas and in the mature brain of WT, ST8SiaII−/−, ST8SiaIV−/− mice.

Fig. 4 - Western blots demonstrating the removal of PSA from the retina at 2, 14 and 28 days following exposure to Endo-N. Control retinas displayed pronounced bands of PSA-NCAM immunoreactivity.
in ST8SiaIV−/− animals (Table 1). In contrast, in NCAM−/− mice, the density of Brn3a-positive cells was significantly higher (p<0.001) compared with their WT littermates (Table 1).

Next, we examined whether Endo-N had any influence on the survival of RGCs in the adult retina. The densities of RGCs were quantified in wholemounted retinas at 2 and 14 days after intravitreal injection of vehicle or Endo-N. No significant differences in RGC densities were noted at 2 or 14 days following Endo-N treatment compared with vehicle (Table 2). It appears that a temporary reduction in PSA-NCAM in adult retinas by Endo-N does not affect the survival of RGCs in the retina.

To assess whether a deficiency in sialyltransferases or NCAM genes affects the viability of RGCs, we performed intravitreal injections of the excitotoxin KA at a dose of 5 nmol in WT, ST8SiaII−/−, ST8SiaIV−/− and NCAM−/− mice. In all groups of animals, a reduction in the density of RGCs was already observed at 24 h after the administration of KA. At this time point, KA induced a reduction in the density of RGCs by approximately 60% in all groups of mice (Table 1). Thus, a constitutive deficiency in ST8SiaII−/− or ST8SiaIV−/− or in NCAM−/− does not affect the toxicity of KA (Table 1). A two-way ANOVA demonstrated a significant effect of genotype (F3,63=20.98; p<0.0001), a significant effect of KA (F1,63=1029; p<0.0001) and a significant effect of interaction (F3,63=4.68; p=0.0046).

To assess whether the temporary reduction in PSA-NCAM levels induced by Endo-N affects the viability of RGCs after KA administration, we injected Endo-N, and at 2 days and 14

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell density, cells/mm²</th>
<th>% Reduction</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>3064 ± 73 (13)</td>
<td>1170 ± 76 (13)</td>
</tr>
<tr>
<td>NCAM−/−</td>
<td>3703 ± 121*** (6)</td>
<td>1379 ± 51 (6)</td>
</tr>
<tr>
<td>ST8SiaII−/−</td>
<td>2772 ± 47* (9)</td>
<td>1009 ± 82 (9)</td>
</tr>
<tr>
<td>ST8SiaIV−/−</td>
<td>2944 ± 97 (9)</td>
<td>1172 ± 97 (6)</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± SEM. Abbreviations: KA – kainic acid, RGC – retinal ganglion cell.

* p<0.05 .
*** p<0.001 (two-way ANOVA, followed by Bonferroni post-hoc test).

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Fig. 5 – Laser scanning images of sections double-labelled for PSA-NCAM (red) and a marker for RGCs – Brn-3a (green, upper panel) – or a marker for astrocytes – GFAP (green, bottom panel). Magnification × 400.

Fig. 6 – PSA-NCAM protein levels in the newborn (P1) retina from WT, ST8SiaII−/− or ST8SiaIV−/− knockout mice. The data are expressed as the mean ± SEM. *p<0.0001 (one-way ANOVA, followed by Bonferroni post-hoc test).
days later, we administered KA. Our experiments demonstrated that in Endo-N-treated animals, KA was considerably more toxic at 14 days ($p<0.001$) but not at 2 days following Endo-N exposure compared with control animals (Table 2). A two-way ANOVA demonstrated a significant effect of Endo-N ($F_{2,45} = 5.9; \ p = 0.0053$), a significant effect of KA ($F_{1,45} = 617; \ p < 0.0001$) and a significant effect of interaction ($F_{2,45} = 5.366; \ p = 0.0081$).

2.4. Expression of ST8SiaII and ST8SiaIV in the retinas of WT, ST8SiaII$^{-/-}$, ST8SiaIV$^{-/-}$ knockout mice

In order to get more information whether sialyltransferases are able to compensate each other we measured the expression levels of ST8SiaII and ST8SiaIV proteins in the retinas of transgenic animals with a deficiency in ST8SiaII or ST8SiaIV genes using western blot. In ST8SiaIV$^{-/-}$ mice, an increased level ($p<0.05$) of ST8SiaII compared with WT was observed (Fig. 7). In contrast, the level of ST8SiaIV was not changed in the retina of ST8SiaII$^{-/-}$ mice (Fig. 7).

3. Discussion

Our observation that PSA-NCAM is expressed at a high level in the adult retina of WT mice supports the previous studies (Bartsch et al., 1990; Sawaguchi et al., 1999; Murphy et al., 2009). In the adult retina, the highest signal of PSA-NCAM expression was observed in the inner part of the retina: predominantly closely to the RGCs bodies, followed by the inner plexiform layer where a laminated PSA-NCAM signal was detected. PSA-NCAM co-localized with the astrocyte marker GFAP but not with neuronal markers, which suggests a predominant astroglial localization of PSA-NCAM. Our data confirm previous studies, which revealed a multistratified distribution of PSA primarily in the inner plexiform layer, on the membrane of Müller cells (Sawaguchi et al., 1999), and astrocytes (Bartsch et al., 1990), which represent glial-derived cells in the retina.

Table 2 – Effect of intravitreal injection of KA (5 nmol) following the administration of Endo-N on the survival of RGCs in adult wild-type mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell density, cells/mm$^2$</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>KA</td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>3001 ± 82 (11)</td>
<td>1144 ± 67 (11)</td>
</tr>
<tr>
<td>Endo-N, 2 days</td>
<td>2958 ± 110 (6)</td>
<td>1113 ± 115 (10)</td>
</tr>
<tr>
<td>Endo-N, 14 days</td>
<td>2967 ± 139 (7)</td>
<td>545 ± 20*** (6)</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± SEM. In brackets: the number of retinas studied. Abbreviations: KA – kainic acid; Endo-N – endoneuraminidase, RGCs – retinal ganglion cells. *** $p<0.001$ (two-way ANOVA, followed by Bonferroni post-hoc test).

Fig. 7 – Western blotting analysis demonstrating ST8SiaIV protein levels (upper panel) and ST8SiaII protein levels (bottom panel) in the adult retinas of WT, ST8SiaII$^{-/-}$ and ST8SiaIV$^{-/-}$ mice. The data are expressed as the mean ± SEM. * $p<0.05$ (Student’s t-test).
This study also demonstrates that PSA-NCAM is present in the retina of adult ST8SiaI/+/-- and ST8SiaIV/+/-- mice at the same level as in WT animals. These data contrast with data obtained from the brain tissue of adult ST8SiaIV/+/-- mice where a marked reduction in PSA-NCAM levels was observed (Eckhardt et al., 2000).

In contrast to adult animals, in neonatal (P1) animals deficient for ST8SiaI, a marked reduction in retinal PSA-NCAM levels was noted. Newborn (P1) mice deficient for ST8SiaI also showed a two-fold lower expression of PSA-NCAM in the retina. These data contrast with those derived from adult retinas of the transgenic mice. It was previously demonstrated that in the adult retina, ST8SiaI and ST8SiaIV mRNAs are expressed at a ratio of 2:2.5, whereas in the developing retina, the ratio is 10:1.25 (Kurosawa et al., 1997). In the developing retina, this compensatory capacity of sialyltransferases appears to be much lower, particularly of ST8SiaI. It was shown that in the newborn brain deficient for ST8SiaI, 45% of NCAM is not polysialylated. In contrast, in animals deficient for ST8SiaIV, only 6% of NCAM is not polysialylated (Galuska et al., 2006).

Our study demonstrated that both sialyltransferases are expressed in the retina of WT mice. Further analysis demonstrated that in ST8SiaIV/+/-- mice the level of ST8SiaI was about two times higher as compared with WT animals. It appears that in the adult retina, deficiency in ST8SiaIV is easily compensated for by the other sialyltransferase. In contrast, in the retina of ST8SiaI/+/-- mice, the level of ST8SiaIV was not changed.

Despite the presence of a high level of PSA-NCAM in the retina of adult ST8SiaI/+/-- mice, a decreased density of RGCs in the retina of ST8SiaI/+/-- mice was observed. It appears that developmental deficiency of ST8SiaI plays an important role in the survival of RGCs in adulthood. A decrease in survival of RGCs in ST8SiaI/+/-- mice was predicted because previous in vitro and in vivo studies have demonstrated increased retinal cell death after enzymatic removal of PSA by neuroaminidase (Bartsch et al., 1990; Murphy et al., 2009). It is proposed that the survival of RGCs is dependent on the availability of trophic factors produced by their targets – tectal neurons – upon the establishment of their synaptic contacts (Vecino et al., 2004). It appears that PSA-NCAM ensures the proper formation of these synaptic contacts between RGCs and tectal neurons during development (Yin et al., 1995).

As expected, no PSA-NCAM was detected in the retina of NCAM−/−/− animals. Because NCAM is the major, if not the exclusive carrier of PSA in vertebrates, this outcome is predictable. Interestingly, NCAM knockout mice have a significantly higher density of RGCs compared with WT mice. The higher density of RGCs in NCAM−/−/− mice may be explained partly by the ability of NCAM to inhibit the proliferation of neural progenitor cells (Amoureux et al., 2000; Seidenfaden et al., 2003), and the deficiency in NCAM results in an increased production of progenitors without significant alteration to their survival. Moreover NCAM−/−/− animals have increased trophic support derived from their target tissue (Murphy et al., 2007).

Despite the observed differences in the density of RGCs in transgenic mice, the excitotoxic effect of KA did not differ between genotypes. The survival of RGCs after intravitreal administration of KA was similar in WT, NCAM−/−/−, ST8SiaI/+/-- and ST8SiaIV−/−/− animals. These data indicate that a constitutive deficiency of PSA or NCAM or both does not affect the sensitivity of RGCs to the excitotoxic effects of KA. Nevertheless, it was reported that in NCAM−/−/− mice, RGCs are more sensitive to the effects of axotomy (Murphy et al., 2007).

Consistent with previous studies (Murphy et al., 2009), intravitreal administration of Endo-N in adult mice induced a long-lasting (approximately 28 days) reduction in PSA-NCAM. However, no decrease in the density of RGCs, was observed following Endo-N treatment. These data contradict previous observations where a reduction in the density of RGCs following Endo-N exposure was noted (Murphy et al., 2009). Apparently, the survival of RGCs following PSA elimination depends on the age of the animals. In our experiments, we used animals that were 5–6 months old, whereas in the previous study (Murphy et al., 2009), younger (2–3 months old) animals were used. It may be that RGCs in younger animals are more sensitive to trophic support.

In contrast to data obtained in retinas of transgenic mice, Endo-N increased the toxicity of KA. In the Endo-N-treated retinas, the survival of RGCs following KA administration was significantly lower compared with control retinas. This effect of KA was observed at 14 days after Endo-N injection but not at 2 days, when KA toxicity was similar in both control and Endo-N-treated groups. Previous studies have shown that a loss in RGCs induced by axotomy was also higher following Endo-N administration (Murphy et al., 2009).

The mechanisms by which PSA-NCAM contributes to the survival of neurons remain unknown. Some findings point to the fact that PSA-NCAM may mediate neurotrophic signaling. It was demonstrated that in the presence of PSA, neurons are more sensitive to brain derived neurotrophic factor (BDNF) or ciliary neurotrophic factor (CNTF) (Hildebrandt et al., 2007).

Fibroblast growth factor receptor 1 (FGFR1) is known to be one of the main interaction partners of PSA-NCAM (Kiselyov et al., 2003). Significant expression of FGFR1 was detected on RGCs and Müller cells (Quan et al., 1999; Catalani et al., 2009). It was shown that FGFR1 and FGFs play an important role in the survival and regeneration of RGCs in adult mammals (Quan et al., 1999; Blanco et al., 2008). Therefore, reduced FGF signaling due to the removal of PSA residues by Endo-N may play a role in the maintenance of RGCs survival following KA-induced damage.

4. Experimental procedures

4.1. Subjects

All experiments were performed in agreement with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC). Moreover, all experiments conformed to regional guidelines on the ethical use of animals. Experimental procedures were conducted by persons who have an appropriate license, and all efforts were undertaken to minimize the number of animal and their suffering. NCAM−/−/− mice and NCAM+/+/+ mice used for experiments were obtained by crossing C57BL6-Ncamtm1Cgn–/– heterozygous mice that were purchased from Jackson Laboratories, US. The generation of ST8SiaI (Angata et al., 2004) and ST8SiaIV (Eckhardt et al., 2000) knockout mice
has been described previously. ST8SialII and ST8SialIV knockout mice were back-crossed to C57BL/6J mice for six generations. Male and female F5 generation knockout mice (ST8SialII−/−, ST8SialIV−/−, NCAM−/−) mice and their wild-type littermates at age 5–6 months and at an average weight of 24.0 g were used. All animals were housed under standard housing conditions: mice were group-housed (five mice per cage) under a 12-h light/dark cycle. All mice had free access to food and water.

4.2. Immunoblotting analysis

Eyes were enucleated, retinas were extracted and stored at –80 °C until further processing. Tissues were lysed in 30 vol RIPA lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate), Na3VO4, and a protease inhibitor cocktail, homogenized manually, incubated for 20 min on the ice and centrifuged (13,000 rpm for 20 min at 4 °C). The supernatants were extracted and resolved by electrophoresis on 6% and 10% SDS-polyacrylamide gels. Proteins were transferred onto Hybond™-P PVDF Transfer Membranes (Amersham Biosciences, UK) in 0.1 M Tris–base, 0.192 M glycine and 20% (w/w) methanol using an electrophoretic transfer system. The membranes were blocked with 0.1% (w/w) Tween-20/TBS containing 0.5% (w/w) non-fat dried milk powder or with Odyssey Blocking Buffer (PBS) (LI-COR Biosciences, Germany) for 2 min to visualize proteins. The membranes were incubated overnight with primary antibodies: monoclonal mouse anti-PSA-NCAM antibody (1:1000; AbCys S.A., Paris), polyclonal rabbit anti-ST8SiaII (1:400; Proteintech, USA), polyclonal rabbit anti-ST8SiaIV (1:250; Thermo Scientific, USA), followed by incubation with a secondary antibody: goat anti-mouse HRP (1:400; L, Thermo Scientific, US), goat anti-rabbit HRP secondary antibody (1:2000; Pierce, USA) or mouse anti-PSA-NCAM antibody (1:400, AbCys S.A., Paris) and one of the following antibodies: goat anti-Brn3a (C-20) (1:100, Santa Cruz Biotechnology, Germany) or mouse anti-GFAP (1:800, 1 mg/ml; MAB 3402, Lot 24111346, Chemicon, US). Secondary antibodies were: DyLight (1:300, DyLight™ 594 – conjugated AffiniPure goat anti-mouse IgG+IgM (H+L), Thermo Scientific, US), Alexa-488 (1:300, Alexa Fluor® 488, goat anti-mouse IgG, 2 mg/ml; A21121, Molecular probes Inc., US) or anti-goat biotinylated (1:200; DAKO, Denmark), Streptavidin, Alexa Fluor® 488 conjugate (1:1000; Invitrogen, US). Fluorescent signals were detected using a confocal microscope (MRC-1024, Olympus/Biorad, Germany). Three-dimensional images were created using a × 40 (water) objective and further analyzed for the colocalization of PSA-NCAM signal with RGC or glial markers.

4.3. PSA-NCAM, Brn3a, GFAP immunohistochemistry

The mice were anesthetized deeply with chloral hydrate (300 mg/kg, i.p.) and perfused transcardially using 0.9% saline and then with 4% paraformaldehyde in phosphate buffered saline (PBS, 0.1 M, pH =7.4). Eye cups were removed, postfixed in paraformaldehyde/PBS solution for 24 h followed by 30% sucrose solution overnight and then sectioned into sections of 16 µm thick using a cryomicrotome. Sections were collected into PBS and maintained at 4 °C until further processing. After a post-fixation of the brain in paraformaldehyde/PBS solution for 24 h, sections of 40 µm thick were cut on a vibrimicrotome (Leica VT1000S, Germany), collected in PBS and kept at 4 °C until further processing.

For PSA-NCAM immunohistochemistry, the sections were dried onto glass slides and incubated in 3% H2O2 in PBS for 15 min. Incubation in blocking solution (0.25% Triton X-100, 0.5% Tween-80, 5% goat serum in PBS) for 1 h was followed by a 24-h incubation with mouse anti-PSA-NCAM antibody (1:400, AbCys S.A., Paris), at 4 °C. Further sections were incubated with a biotinylated goat anti-mouse antibody (1:200; Vector Laboratories, UK) for 1 h. PSA-NCAM was visualized using the peroxidase method (ABC system and diaminobenzidine as the chromogen, Vector Laboratories, UK). The sections were dried and coverslipped with mounting medium (Vector Laboratories, UK). Immunoreactivity for PSA-NCAM was observed using an Olympus BX-51 microscope. Retinal sections were analyzed for coexpression of PSA-NCAM and RGC (Brn3a) or astrocyte (GFAP) markers. For immunofluorescent double-labeling, sections were incubated with a mixture of mouse anti-PSA-NCAM antibody (1:400, AbCys S.A., Paris) and one of the following antibodies: goat anti-Brn3a (C-20) (1:100, Santa Cruz Biotechnology, Germany) or mouse anti-GFAP (1:800, 1 mg/ml; MAB 3402, Lot 24111346, Chemicon, US). Secondary antibodies were: DyLight (1:300, DyLight™ 594 – conjugated AffiniPure goat anti-mouse IgG+IgM (H+L), Thermo Scientific, US), Alexa-488 (1:300, Alexa Fluor® 488, goat anti-mouse IgG, 2 mg/ml; A21121, Molecular probes Inc., US) or anti-goat biotinylated (1:200; DAKO, Denmark), Streptavidin, Alexa Fluor® 488 conjugate (1:1000; Invitrogen, US). Fluorescent signals were detected using a confocal microscope (MRC-1024, Olympus/Biorad, Germany). Three-dimensional images were created using a × 40 (water) objective and further analyzed for the colocalization of PSA-NCAM signal with RGC or glial markers.

4.4. RGCs staining and cell density analyses

Retinas were extracted after perfusion, sectioned into four quadrants, post-fixed for 20 min, and wholemounted on gelatinized slides. For RGC density quantification, the Brn3a marker of RGCs was used. Retinas were incubated in 3% H2O2 in PBS for 15 min, followed by permeabilization in 3% Triton X-100 at –80 °C for 15 min. Incubation in blocking solution (0.25% Triton X-100, 0.5% Tween 80, 4% rabbit serum in PBS) for 1.5 h was followed by incubation with anti-Brn3a antibody (1:100, goat polyclonal anti-Brn3a (C-20), Santa Cruz Biotechnology, Germany), diluted in 4% rabbit serum 2% Triton X-100 for 48 h. Further sections were incubated in biotinylated rabbit anti-goat IgG antibody (1:200, Vector Laboratories, UK), diluted in 2% Triton X-100 and 4% rabbit serum for 2 h. Brn3a-positive cells were visualized using the peroxidase method (ABC system and diaminobenzidine as chromogen, Vector Laboratories, UK). Next, the retinas were dried and coverslipped with mounting medium (Vector Laboratories, UK). Immunoreactivity of Brn3a-positive cells and their density quantification was quantified using a stereology system comprising an Olympus BX-51 microscope and then Visiopharm Integrator System (Version 3.6.5.0, Denmark).
4.5. Intravitreal injections

Intravitreal injections of KA and Endo-N were performed using a Hamilton syringe. The needle was inserted through the sclera and retina into the vitreous chamber of the eye. A bilateral injection of vehicle (2 μl of 0.9% NaCl) or KA (5 nmol/2 μl in 0.9% NaCl) or Endo-N (6.5 U/2 μl in 0.9% NaCl) was administered into the right and left eyes of one animal. Attention was taken to prevent lens injury as it may promote survival and regeneration after puncture (Pernet and Di Polo, 2006).

4.6. Statistical analysis

Statistical analyses were performed using one-way ANOVAs or two-way ANOVAs followed by the Bonferroni post-hoc test. Two group comparisons were made using Student’s t-test. The results are expressed as the mean ± SEM.

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