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A role of PSA-NCAM in the survival of retinal ganglion cells (RGCs) after kainic acid damage

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ABSTRACT

Background: Neural cell adhesion molecule (NCAM) belongs to the immunoglobulin superfamily of adhesion molecules. Polysialic acid (PSA) is attached to NCAM post-translationally. PSA residues are considered to reduce the adhesive properties of NCAM and play an important role in the regulation of cell interactions. PSA-NCAM is largely expressed in the mature retina by glial cells adjacent to retinal ganglion cells (RGCs) but its functions remain unclear. The objective of this study was to explore the role of PSA-NCAM with respect to RGC survival following kainic acid (KA)-induced excitotoxicity.

Methods: Experiments were performed on C57BL/6NTac male mice. KA was injected intravitreally to induce RGC damage. RGCs were visualized using an anti-Brn3a antibody. Endoneuraminidase N (NA) was administrated intravitreally to cleave PSA chains from NCAM.

Results: KA induced an 80% reduction in the density of RGCs that was accompanied by a decrease in PSA-NCAM in the RGC layer. KA treatment induced a pronounced increase in the level of matrix metalloproteinase-9 (MMP-9) in the inner layers of the retina. Inhibition of MMP-9 reduced both RGC death and PSA-NCAM shedding in the retina. PSA-NCAM cleavage induced by NA abolished the protective action of the MMP-9 inhibitor and decreased RGC survival following KA-treatment.

Conclusions: A decrease in retinal PSA-NCAM levels following KA administration is due to the induction of active MMP-9, which removes extracellular PSA-NCAM from the surface of astroglial and Müller cells. The MMP-9 induced shedding of PSA-NCAM enhances KA-induced toxicity and at least in part contributes to the observed loss of RGCs following excitotoxic damage.

1. Introduction

The neural cell adhesion molecule (NCAM) is a cell-surface glycoprotein of the immunoglobulin superfamily of adhesion molecules. NCAM regulates cell-cell interactions and contributes to the cell adhesion (Edelman, 1986). The most prominent posttranslational modification of NCAM is the attachment of the polysialic acid (PSA) (Rougon, 1986). PSA is a long alfa-2,8 linked sialic acid polymer (Hoffman et al., 1982). It shows a high hydrated volume and a large negative charge density and when attached to NCAM, reduces NCAM adhesive properties, thereby modulating cell-cell interactions and playing a role in morphological or physiological plasticity (Rougon, 1986; Rutishauser and Landmesser, 1996). PSA-NCAM is highly expressed and plays a substantial role in the nervous system during embryogenesis. It has been shown that PSA facilitates precursor cell migration (Hu et al., 1996) and regulates cell differentiation until the place of destination is reached (Seki et al., 2007). PSA is important for hippocampal granule cell axonal outgrowth and pathfinding (Seki and Rutishauser, 1998; El Maarouf and Rutishauser, 2003a), and the maturation of neural circuits. In adulthood, PSA-NCAM expression is almost entirely limited to the brain regions where neurogenesis and plasticity take place (hippocampus, olfactory bulb) (Bonfanti, 2006; Sandi, 2004). In the developing and mature brain, PSA-NCAM influences a variety of CNS functions, including synaptic plasticity, integration of the immature neurons in the regions where neurogenesis occurs, and cortical network modification based on experience, learning and memory (Bonfanti, 2006; Rutishauser, 1998).

PSA-NCAM is strongly expressed in the mature retina by astrocytes and Müller cells in close proximity to the RGCs. It has been shown that PSA-NCAM is involved in RGC axonal outgrowth and pathfinding during development (El Maarouf and Rutishauser, 2003b). Despite diverse functions of PSA-NCAM in the adult brain, the role of PSA-NCAM in the mature retina remains to be elucidated. Some evidence demonstrated that PSA-NCAM plays a protective role in RGC survival (Murphy

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et al., 2007, 2009). Early studies suggested that PSA-NCAM interacts with an NMDA-type glutamate receptor subunit and that via this mechanism, it could influence survival of the neurons (Kochlamazashvili et al., 2010; Hammond et al., 2006). Excitotoxicity has been suggested to play a key role in RGC damage in glaucoma, retinal ischemia (Dreyer, 1998; Osborne et al., 1999). KA-induced excitotoxicity is widely used as a model of excitotoxicity and acute RGC damage. KA is potent agonist to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite class of ionotropic glutamate receptors, which are widely expressed in the retina (Hampson and Manalo, 1998). The activation of KA receptors produces membrane depolarization and results in alteration in intracellular calcium concentrations, which is required to trigger the neuronal death cascade. The excessive Ca^{2+} influx and overactivation of Ca^{2+}-dependent enzymes (proteases, kinases, endonucleases, phosphatases, phospholipases) can either directly or indirectly induce cell death. It was found that KA can also induce RGC death via activation of N-methyl-D-aspartic acid (NMDA) receptors, another subtype of glutamate receptor, likely by stimulating the release of endogenous glutamate(Glu)/aspartate(Asp)-like compounds that activate NMDA receptors (Sucher et al., 1991) or by directly opening NMDA channels (Michaels and Rothman, 1990). The impact of PSA-NCAM in RGC survival in the model of KA-induced excitotoxicity has thus far not been studied. Previous studies also demonstrated that excitotoxicity is accompanied by an increased expression of the active form of MMP-9 (Zhang et al., 2004a). Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that have the ability to degrade and remodel the extracellular matrix and have a broad spectrum of substrates: cell surface receptors, proteinases, signaling molecules (Birkedal-Hansen et al., 1993). Previously, a role of matrix metalloproteinase-9 (MMP-9) was demonstrated in RGC loss after KA damage (Zhang et al., 2004a)). Previous studies also showed that MMP-9 is involved in PSA-NCAM cleavage in neuroblastoma cells (Jaako et al., 2016).

Therefore, the purpose of our study was to investigate 1) whether active MMP-9 is involved in PSA-NCAM shedding after KA-induced RGCs damage and 2) whether PSA-NCAM has a protective role in the survival of RGCs after KA treatment.

2. Methods

2.1. Animals

Experiments were performed in agreement with the guidelines established in the Principles of Laboratory Animal Care (Directive 2010/63/EU). All experimental procedures conformed to regional guidelines on the ethical use of animals and were undertaken by persons who have a proper license. C57BL/6NTac (Taconic B6) male mice at age 3–4 months were group-housed (10 per cage) under standard conditions with a 12-h light/dark cycle.

2.2. Intravitreal injections

To inhibit MMP-9 activity, a specific MMP-9 inhibitor (2-(N-Benzyl-4-methoxyphenylsulfonamido)-5-((diethylamino)methyl)-N-hydroxy-3-methylbenzamide; Abcam, USA) was chosen for intravitreal injections which belongs to the hydroxamates. It was demonstrated that this compound inhibits MMP-9 in low concentrations (IC50 = 5 nM), whereas its inhibitory activity for MMP-1 is IC50 = 113 nM and for MMP-13, IC50 = 1050 nM (Gupta, 2012; Scozzafava and Supuran, 2000).

Endoneuraminidase N (NA), provided by Professor Rita Gerardin-Schahn, is an enzyme that selectively cleaves PSA chains from the extracellular domain of NCAM (Gerardy-Schahn et al., 1995). The dose of NA for intravitreal administration was selected on the basis of the series of preliminary experiments. The effectiveness of NA to remove PSA from NCAM was tested by using both Western blotting and immunohistochemistry 24 h following enzyme administration. It was found that 6.5 U of NA was able to complete remove PSA from NCAM in the retina. Lower doses provided only partial effect. Therefore, 6.5 U of NA was used for intravitreal injections, as soon as it was the lowest dose, which induced complete removal of PSA from NCAM in the retina. The dose of KA for intravitreal administration was 10 nmol. Each intravitreal injection was performed in a final volume of 2 μl. The mice were anesthetized with 1.5–2% isoflurane inhalation for 6–7 minutes for intravitreal administrations. Intravitreal injections were performed using an Agilent Manual Syringe, 5 μl (Agilent Technologies, Australia). The injections were made through the sclera into the outer upper segment of the eyeball. Twenty four mice received intravitreal injections of vehicle (0.9% NaCl) or KA (10 nmol) into the right and left eyes, respectively. Another twenty-four mice were injected intravitreally with KA + MMP-9 inhibitor (MMP-9i) or a mixture of NA + KA + MMP-9i into the right and left eyes, respectively. Animals were sacrificed 24 h following intravitreal injections by decapitation or during perfusion.

2.3. RGC staining and density analyses

Twenty-four hours following intravitreal injections, animals were perfused transcardially, retinas were gently removed, post-fixed for 25 min and wholemounted on slides. RGCs were visualized using goat anti-Brn3a (C-20) antibody (Santa Cruz Biotechnology, Germany). RGCs immunohistochemical staining and density evaluation was performed as described previously (Lobanovskaya et al., 2015).

2.4. Immunoblotting analysis

Lysate preparation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDA-PAGE) and transfer to the Immobilon-FL polyvinylidene difluoride membranes were all performed as previously described (Lobanovskaya et al., 2015). After blocking with Odyssey Blocking Buffer (PBS) (LI-Cor Biotechnology, USA), the membranes were incubated for 48 h, at 4 °C with primary antibodies ((monoclonal mouse anti-PSA-NCAM, IgM (1:1000; Millipore, clone 2-2B, MAB5324, USA); monoclonal mouse anti-active MMP-9 (4A3) (1:1000, Novus, USA); rabbit anti-β-actin (1:5000, Li-Cor, USA)). The membranes were then washed and incubated with goat anti-mouse IRDye 680 L T, IgM (for PSA-NCAM detection), goat anti-mouse IRDye 800CW (for active MMP-9 detection), goat anti-rabbit IRDye 800CW (for actin detection) or goat anti-rabbit IRDye 680 L T (for actin detection) (Li-Cor, USA). Membrane immunoreactivities were detected using the Odyssey Infrared Imaging System (Odyssey CLx, Li-Cor Biosciences, USA). To normalize immunoreactivity of the proteins, β-actin was measured on the same blots and the ratios of proteins were calculated and expressed as the mean optical density ratio in arbitrary units ± SEM.

2.5. DAB immunohistochemistry of PSA-NCAM, MMP-9, GFAP on retinal sections

At twenty-four hours following intravitreal injections, animals were anesthetized with intraperitoneal injection of chloral hydrate (300 mg/kg) 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, post-fixed in paraformaldehyde/PBS solution for 24 h, and incubated in 30% sucrose solution overnight. Retinal cryosections (18 μ m) were cut. For blocking of endogenous peroxidase activity, sections were incubated with 3% H_2O_2 for 15 min. Non-specific binding was blocked using 4% normal goat serum (Vector Laboratories, USA) in PBS containing 0.5% Triton X-100 and 0.25% Tween 20 for 1 h at room temperature followed by 24–48 hours of incubation with primary antibodies. Primary antibodies used were: monoclonal mouse anti-PSA-NCAM, IgM (1:400; Millipore, clone 2-2B, MAB5324, USA), monoclonal mouse anti-active MMP-9 (4A3) (1:800; Novus, USA), rabbit anti-GFAP (1:1000; Dako, Denmark). After washes

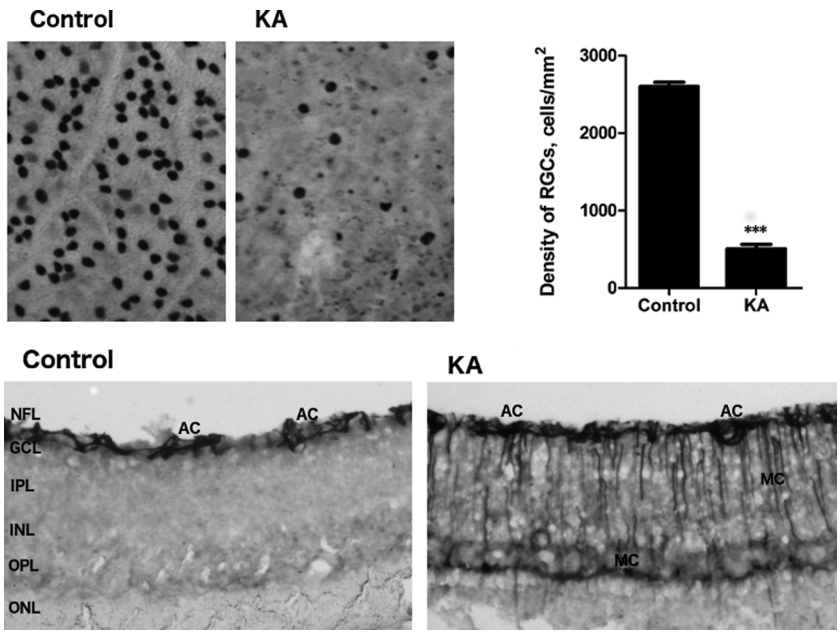

Fig. 1. The effect of intravitreal administration of KA (10 nmol) on the RGCs, retinal astroglial and Müller cells. Upper panel (left): representative microphotograph of RGCs after administration of vehicle (control) or KA. Upper panel (right): the density of RGCs following vehicle or KA administration. The results are expressed as mean ± SEM. ***p < 0.0001 (Tukey's test), n = 12 animals per group. Bottom panel: representative microphotograph of GFAP-immunoreactive astroglia and Müller cells in the retinal sections following intravitreal injection of vehicle (control) or KA. Magnification ×200. NFL: Nerve fiber layer; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer. AC – astrocytes, MC – Müller cells.

in PBS, sections were incubated in biotinylated anti-mouse (1:200; Vector Laboratories, Inc., USA) or anti-rabbit antibodies (1:200; Vector Laboratories, Inc., USA) for 1 h at room temperature. Immunoreactivities of proteins were visualized using the peroxidase method (Vectastain ABC kit and Peroxidase substrate kit DAB, Vector Laboratories, USA) and observed using an Olympus BX-51 microscope.

2.6. Statistical analysis

The results are expressed as mean ± SEM. Statistical analyses were performed using Student's *t*-test or one-way ANOVA followed by the Tukey's post-hoc test, where appropriate.

3. Results

3.1. Effect of KA on RGC survival, PSA-NCAM levels and MMP-9 expression

Intravitreal injection of KA (10 nmol) reduced the density of RGCs by approximately 80% at 24 h after its administration (Fig. 1, upper panel).

KA-induced retinal damage was accompanied by astrogliosis as evidenced by the increase in GFAP-positive astroglia. Immunohistochemistry revealed GFAP-immunoreactivity in the inner part of the retina close to the RGCs. Müller cell processes and soma showed increased GFAP-associated signal in the middle and outer retina layers following KA injection (Fig. 1, bottom panel).

Western blotting revealed that KA-induced loss of RGCs is also accompanied by a remarkable decrease in PSA-NCAM levels (Fig. 2). PSA-NCAM immunoreactive signal appeared as a high molecular weight smear at approximately 180–240 kDa on SDA-PAGE. Immunohistochemical detection of PSA-NCAM in the retinal sections demonstrated its selective loss in the inner part of the retina, near to RGCs (Fig. 2).

Our next task was to study the mechanisms by which KA induces loss of PSA-NCAM in the retina. Previous studies have demonstrated that KA-induced death of RGCs is associated with the induction of metalloproteinases (MMPs) (Zhang et al., 2004a), and MMP-9 can target and induce shedding of NCAM/PSA-NCAM in neural cell lines (Jaako et al., 2016). To investigate whether KA induces upregulation of metalloproteinase-9 (MMP-9), the active form of MMP-9 protein was evaluated using western blotting and immunohistochemistry using a specific monoclonal antibody labeling only active MMP-9. Western blotting revealed that the active form of MMP-9 protein was almost absent in control retinas. In contrast, a marked increase in the active form of the protein was observed after KA administration (Fig. 3). Immunohistochemistry demonstrated an increased expression of active form of MMP-9 protein primarily in the inner layers of the retina (Fig. 3).

To investigate whether MMP-9 upregulation in the inner part of the retina contributes to the cleavage of PSA-NCAM, a selective inhibitor of MMP-9 (MMP-9i) was used. Intravitreal administration of MMP-9i together with KA partially protected against both the loss of PSA-NCAM immunoreactivity (Fig. 4) and the loss of RGCs (Table 1) in the injured retina. The administration of MMP-9i alone did not affect PSA-NCAM levels (Fig. 4). These experiments demonstrated that activation of MMP-9 is important for KA-induced damage of RGCs.

Our next question was to clarify whether MMP-9 directly affects RGC survival or its action is realized via shedding of PSA-NCAM. To answer this question, we removed PSA resides using NA. The animals

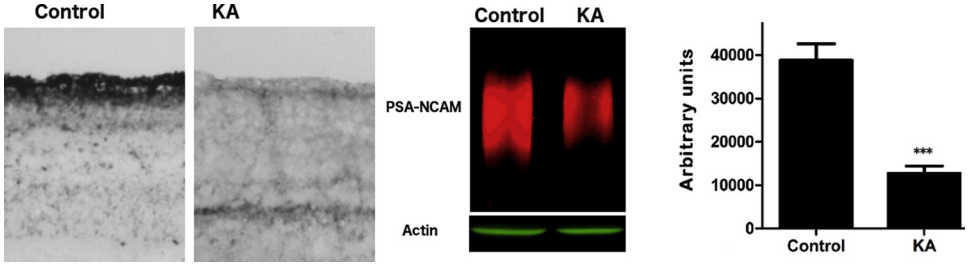

Fig. 2. Effect of intravitreal KA (10 nmol) administration on PSA-NCAM levels. Left panel: immunohistochemistry of PSA-NCAM in retinal sections following vehicle (control) or KA administration. Middle panel: western blot of PSA-NCAM protein in lysates from control and KA-treated retinas. Right panel: quantitative analysis of PSA-NCAM protein (western blot). The results are expressed as mean ± SEM. ***p < 0.0001 (Student's *t*-test), n = 6 animals per group.

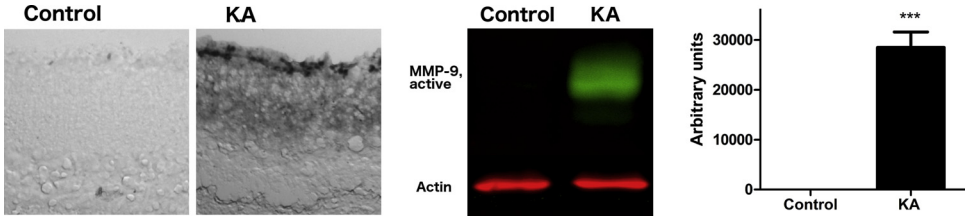

Fig. 3. Effect of intravitreal KA (10 nmol) administration on the level of active MMP-9 protein. Left panel: immunohistochemistry of active MMP-9 in retinal sections after vehicle (control) or KA administration. Middle panel: western blot of active MMP-9 protein in lysates from control or KA-treated retinas. Right panel: quantitative analysis of the MMP-9 protein. The results are expressed as mean ± SEM. ***p < 0.0001 (Student's *t*-test), n = 6 animals per group.

received an intravitreal injection of KA, MMP-9i and NA. Our previous study demonstrated that intravitreal administration of NA removes PSA from NCAM and does not affect RGCs (Lobanovskaya et al., 2015). Removal of PSA residues from the NCAM molecule reduced the protective action of MMP-9i on RGC survival (Table 1). These data demonstrated that the presence of PSA-NCAM plays a protective role and its loss reduces the survival of RGCs after KA administration.

4. Discussion

Intravitreal injection of KA induces time- and dose-depended RGC loss (Zhang et al., 2004a). Intravitreal injections of 10 nmol KA caused an approximate 80% reduction in the density of RGCs at 24 h after administration. Excitotoxicity has been shown to play a substantial role in the damage of RGCs in different retinal diseases (Dreyer, 1998; Osborne et al., 1999). It was demonstrated that approximately 60% loss of RGCs in rats at 3 months after elevation of intraocular pressure (Guo et al., 2005). It was found that retinal ischemia in rats induced progressive RGCs death reached approximately by 62% at day 21 following ischemia induction (Vidal-Sanz et al., 2001).

KA-induced loss of RGCs is accompanied by increased GFAP signal in all retina layers due to the activation of astrocytes and Müller cells. These data support previous observations demonstrating that KA-induced death is accompanied by retinal tissue inflammation and glial cell activation (Shin et al., 2000). Western blot analysis demonstrated that intravitreal injection of KA also induced a decrease in PSA-NCAM levels. Immunohistochemistry in the retinal sections confirmed a pronounced reduction of PSA-NCAM protein in the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL). The mechanism of PSA-NCAM reduction after KA administration in the retina has not previously been studied. However, there are observations to suggest that MMPs are involved in NCAM/PSA-NCAM degradation in neurons (Hübsmann et al., 2005; Shichi et al., 2011). Previous studies in our laboratory have demonstrated that MMP-9 is the major metalloproteinase involved in PSA-NCAM/NCAM shedding in neuroblastoma cells (Jaako et al., 2016). Our experiments revealed that KA administration induced an upregulation of the active form of MMP-9 protein in the inner layers of the retina close to RGCs, where PSA-NCAM is primarily expressed. We suggest that MMP-9 participates in PSA-NCAM shedding in the retina. Indeed, intravitreal administration of the selective MMP-9 inhibitor prevented both KA-induced PSA-NCAM shedding and RGC degeneration. Thus, MMP-9 upregulation in the inner part of the retina could contribute to the PSA-NCAM cleavage and RGC survival. MMPs belong to the zinc proteases superfamily and specialize in degrading the extracellular matrix, cytokines, growth factors, cell surface receptors, and cell adhesion molecules (Bonnans et al., 2014; Rodriguez et al., 2010). MMPs show low expression and activity in normal conditions but are increased during remodelling or repair and in disease, such as diabetic retinopathy (Kowluru et al., 2014), glaucoma (De Groef et al., 2014), retina ischemia (Zhang et al., 2002). Activated astroglia and Müller cells are the major sources of MMP-9 protein (Zhang et al., 2004b). It should be noted, however, that MMP-9 inhibition only partially prevented PSA-NCAM shedding and death of RGCs. Thus, an MMP-9-independent mechanism is also

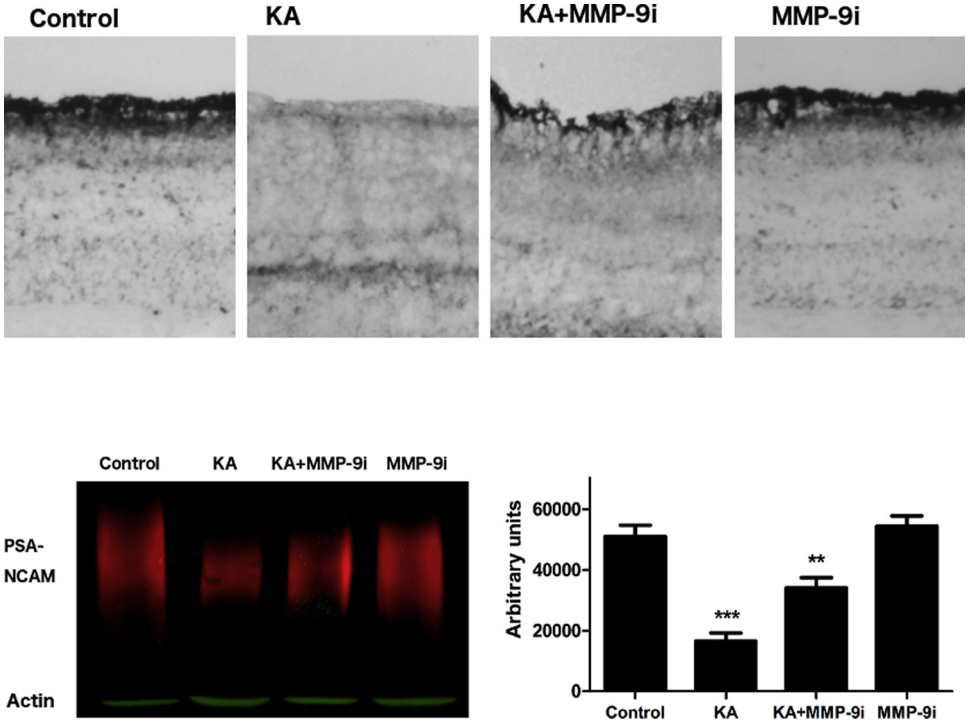

Fig. 4. The effect of MMP-9 inhibitor on the KA-induced reduction in PSA-NCAM levels. Upper panel: representative microphotograph of immunohistochemistry of PSA-NCAM protein in retinal sections after vehicle (control), KA, KA + MMP-9i or MMP-9i treatments, respectively (from left to right). Bottom panel (left): western blot of PSA-NCAM protein in lysates from vehicle (Control), KA, KA + MMP-9i or MMP-9i-treated retinas. Bottom panel (right): quantitative analysis of PSA-NCAM protein in western blot. Western blot demonstrated approximately 70% reduction in PSA-NCAM signal in the retinas treated with KA as compared with control. The results are expressed as mean ± SEM. ***p < 0.0001 (Tukey's test). Intravitreal administration of KA + MMP-9i induced approximately 30% reduction in PSA-NCAM signal as compared with control. The results are expressed as mean ± SEM. **p < 0.001 (Tukey's test), n = 6 animals per group.

Table 1
The effects of MMP-9 inhibition and its combination with Endoneuraminidase N (NA) on KA-induced RGC death. The data are expressed as the mean ± SEM. ***p < 0.0001 (Tukey's Multiple Comparison Test). In brackets: the number of retinas studied.

Group	RGC density (cell/mm^2)	RGC reduction in comparison to control (%)	Group comparisons
Control (Vehicle)	2533 ± 32 (12)		
KA	505 ± 57 (11) ***	80	KA vs Control
KA + MMP-9i	1327 ± 132 (12) ***	48	KA + MMP-9i vs KA
NA + KA + MMP-9i	815 ± 75 (12) ***	68	NA + KA + MMP-9i vs KA + MMP-9i

Abbreviations: KA – kainic acid, RGC – retinal ganglion cell, NA – Endoneuraminidase N, MMP-9 - matrix metalloproteinase-9, MMP-9i – matrix metalloproteinase-9 inhibitor.

activated following KA to induce PSA-NCAM degradation and RGC death. It has been shown that the ADAMs family of metalloproteinases are also involved in PSA-NCAM/NCAM degradation (Kalus et al., 2006). Furthermore, PSA-NCAM is degraded in the neonatal brain by plasmin, known as endopeptidase, and inhibitors of plasminogen activators (PAs) decrease PSA-NCAM shedding (Endo et al., 1998). Several studies showed that KA-induced hyperstimulation of non-NMDA receptors in the retina upregulated PAs and active plasmin, which was associated with RGC death and inhibition of PAs attenuated RGCs damage (Mali et al., 2005). Our next question was to clarify the importance of the observed PSA-NCAM degradation for the KA-induced loss of RGCs. NA was used to remove PSA chains from the NCAM in the retina. Our previous studies demonstrated that intravitreal administration of NA largely ablates PSA from the retina (Lobanovskaya et al., 2015). Administration of NA reduced the protective property of MMP-9i on RGC survival. Thus, we conclude that PSA-NCAM has a protective role for RGCs and its degradation decreases RGC survival following KA damage. The protective actions of PSA-NCAM on RGC survival has also been shown previously. Murphy et al., 2009 demonstrated increased death of RGCs after axotomy following NA injection (Murphy et al., 2009). It was also found that aged NCAM knockout mice have excessive loss of RGCs in contrast with the minimal loss of RGCs in aged control animals (Murphy et al., 2012). The mechanisms underlying the protective role of PSA-NCAM are remain unclear. Previous studies suggested that PSA-NCAM interacts with NMDA-type glutamate receptors. It was shown that PSA-NCAM inhibited NMDA receptors in hippocampus and deficits in NCAM/PSA-NCAM increased GluN2B-mediated Ca^{2+} influx in CA1 pyramidal cells in hippocampal slices (Kochlamazashvili et al., 2010). It was demonstrated that PSA prevented the activation of GluN2B – an NMDA receptor subunit – and decreased glutamate-induced cell death in the primary hippocampal culture (Hammond et al., 2006). The NMDA GluN2B subunit plays a critical role in RGC degeneration by glutamate excitotoxicity (Bai et al., 2013). Previously, it was shown that NCAM knockout mice demonstrated altered NMDA-induced Ca^{2+} dynamics in RGCs accompanied by an increased loss of RGCs (Murphy et al., 2012). Another possible mechanism by which PSA-NCAM is protective is the fact that PSA-NCAM modulates neurotrophic signalling. PSA-NCAM increases the sensitivity of neurons to brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (Hildebrandt et al., 2007). NCAM/PSA-NCAM also induces the activation of fibroblast growth factor receptor 1 (FGFR1) (Kiselyov et al., 2003). Prominent expression of FGFR1 was found in the RGC layer (Catalani et al., 2009). FGFR1 and FGFs play an important role in RGC survival (Blanco et al., 2008).

In conclusion, our study demonstrates that MMP-9-induced shedding of PSA-NCAM enhances KA-induced toxicity and at least in part contributes to the observed loss of RGCs following excitotoxic damage.

Conflicts of interest

None.

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