Absence of galectin-3 promotes neuroprotection in retinal ganglion cells after optic nerve injury

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Summary. A trauma to the mature central nervous system (CNS) often leads to persistent deficits, due to the inability of axons to regenerate after being injured. Increasing evidence suggests that pro-inflammatory and pro-apoptotic genes can present a major obstacle to promoting neuroprotection of retinal ganglion cells and consequently succeed in axonal regeneration. This study evaluated the effect of the absence of galectin-3 (Gal-3) on retinal ganglion cells (RGC) survival and axonal regeneration/regeneration after optic nerve crush injury. Two weeks after crush there was a 2.6 fold increase in the rate of cell survival in Gal-3-/- mice (1283±79.15) compared to WT animals (495.4±53.96). However, no regeneration was observed in the Gal-3-/- mice two weeks after lesion. Furthermore, axonal degeneration presented a particular pattern on those mice; Electron Microscopy (EM) analysis showed incomplete axon degeneration while the WT mice presented an advanced stage of degeneration. This suggests that the removal of the nerve fibers in the Gal 3-/- mice could be deficient and this would cause a delay in the process of Wallerian degeneration once there is a decrease in the number of macrophages/microglia in the nerve. This study demonstrates how the absence of Gal-3 can affect RGC survival and optic nerve regeneration/regeneration after lesion. Our results suggest that the absence of Gal-3 plays an important role in the survival of RGC and thus can be a potential target for therapeutic intervention in RGC neuroprotection.

Key words: Retinal ganglion cells, Optic nerve, Galectin-3, Wallerian Degeneration.

Introduction

A lesion to the central nervous system (CNS) elicits a lower capacity for axon regeneration than a peripheral nervous system (PNS) lesion. The CNS non-permissive regenerative environment is due to the expression of oligodendrocyte-derived myelin inhibitors, glial scar formation and slow clearance of axonal debris (Gensel et al., 2012; Sharma et al., 2014). Besides, intrinsic neuronal regenerative capacity is not turned on as in the PNS (Skene and Willard, 1981; Kiryu-Seo and Kiyama, 2011). Regenerative failure is a critical endpoint of these destructive triggers, culminating in neuronal apoptosis, Wallerian degeneration (WD) and inhibition of morphological and functional recovery (Quigley et al., 1995; Narciso et al., 2001; Magharious et al., 2011; Monnier et al., 2011).

The optic nerve injury model is widely used to investigate retinal ganglion cell (RGC) survival and CNS axon regeneration (Berry et al., 2008). Lesions to the optic nerve lead to WD of the distal nerve stump (Marques et al., 2008) and retinal ganglion cell (RGC) death in both crush and transection lesions (Beirowski et al., 2008). These phenomena have been associated to loss of trophic support and neuroinflammation (Fischer...
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and Leibinger, 2012). Neutrophils, microglia and macrophages secrete reactive oxygen species, cytokines and proteases that lead to neuron and oligodendrocyte death (Mietto et al., 2015). On the other hand, intraocular inflammation leads to RGC survival and optic nerve regeneration due to neutrophil and macrophage infiltration and oncomodulin secretion (Yin et al., 2006; Kurimoto et al., 2013).

Although previous studies on CNS trauma models have investigated the mechanisms related to neuronal apoptosis and regeneration, gaps still exist for identifying neuroprotection and regeneration inducing targets (Agudo et al., 2008; Qu and Jakobs, 2013). Galectin-3, a member of the lectin family that binds β-galactoside (Leffler et al., 2004; Dumic et al., 2006) is upregulated after spinal cord and peripheral nerve injuries (Pajoohesh-Ganjii et al., 2012). In the CNS, Gal-3 is expressed in astrocytes, microglia, oligodendrocytes, neurons and has been functionally related to microglia/macrophages phagocytosis of myelin debris (Pesheva et al., 2000; Rotshenker, 2009; Shin, 2013), a phenomenon that is important for axon regeneration (Rotshenker, 2009). Interestingly, Gal-3 is not expressed in normal optic nerves and is only sporadically expressed after injury, an observation that was correlated with the slow myelin clearance observed during optic nerve Wallerian degeneration (Reichert and Rotshenker, 1996). Also, Gal-3 is upregulated in areas of myelin degeneration and demyelination of spinal cords and optic nerves during mouse Experimental Autoimmune Encephalomyelitis (EAE) and when EAE is suppressed, demyelination and Gal-3 expression is also suppressed (Reichert and Rotshenker, 1999). Our group has previously shown that Galectin-3-/- (Gal-3-/-) mouse presents faster WD and regeneration after peripheral nerve crush, with an increase in the numbers of Schwann cells and macrophages, as well as increased phagocytic activity on the distal stump (Narciso et al., 2009; Mietto et al., 2013). Within the CNS, our group has shown that Gal-3-/- mouse presents better functional recovery and white matter sparing after spinal cord lesion (Mostacada et al., 2015).

Therefore, we have speculated whether the absence of Gal-3 would affect RGC death and optic nerve degeneration/regeneration after crush. For that, we evaluated the effect of the absence of Gal-3/- on retinal ganglion cell survival, apoptosis rate, microglia / macrophage activation and axonal regeneration / degeneration after optic nerve crush injury.

Materials and methods

Animals, surgery and tissue processing

A total of 71 wild-type and galecitin-3 knockout C57/Bl6 mice were used in the present study. For each experimental condition 3-8 animals were utilized. Inbred C57/Bl6 and Gal-3-/- mice (backcrossed to c57/black6 for 10 generations) (Hsu et al., 2000) were obtained from the colony bred at the Universidade Federal do Rio de Janeiro and Fundação Oswaldo Cruz (Fiocruz), Brazil. All protocols for the use and care of animals were approved by the Commission of Animal Care of the Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro (protocol number DHICB061). Surgery was performed under general anesthesia using xylazine (15 mg/kg) plus ketamine (100 mg/kg) injected intraperitoneally. The left optic nerve was exposed and crushed with fine forceps (Dumont; WPI) at 1 mm behind the eye, for five seconds, and care was taken to avoid injury to the ophthalmic artery. The contralateral right optic nerves were used as normal controls. After periods of 96 hours (four days) or two weeks, the animals received an overdose of anesthesia and were perfused intracardially with a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). After perfusion, retinas and optic nerves were dissected and immersion-fixed in 4% paraformaldehyde for 24 h.

RGC survival

RGC survival was evaluated in WT (n=4 - lesioned; n=3 - unlesioned) and Gal-3-/- (n=9 - lesioned; n=5 - unlesioned) animals by whole mount retinas immunostained with a rabbit anti-βIII tubulin antibody (Abcam; 1:500) followed by an Alexa-488 antibody to rabbit IgG made in goat. Within the ganglion cell layer, βIII tubulin is expressed only in RGC. Immunopositive cells were counted under fluorescent illumination (400x) in 8 pre-specified areas (Fig. 1A) to quantify RGC survival per square millimeter in three to eight mice per group. Statistical analysis was performed (One-way ANOVA, followed by Tuckey’s multiple comparison test) using Graphpad Prism. Results were expressed as mean±SEM; the p value was considered significant when <0.05.

Apoptosis evaluation

We evaluated apoptosis rate at 4 days after crush in WT (n=5) and Gal-3-/- (n=5). In Situ Apoptosis Detection Kit (Millipore Corporation, USA) was used following the protocol provided by the manufacturer. The slides were washed in PBS solution 2 x 5 minutes, post-fixed in ethanol: acetic acid solution (75 microliters / cm 2) was applied directly onto the sample and incubated for 10 seconds at room temperature. Subsequently, sections were incubated with TdT enzyme diluted in 30% reaction buffer for one hour at 37°C. The TdT enzyme recognizes the free 3’OH ends and adds nucleotides labeled with a digoxigenin molecule. For negative controls, the slides were incubated in equilibration buffer replacing the TdT enzyme. Then, the sections were incubated in wash buffer and 1 mL wash buffer in 35 mL of MilliQ water.
for 10 minutes. After incubation, the tissues were washed in PBS and incubated with rhodamine-conjugated anti-digoxigenin antibody for 30 minutes. The cross sections were observed in a fluorescence microscope (Zeiss Axiocam 2 Plus) and photographed with Zeiss Axiocam camera MRC using Axiovision software, (version 4.5). Five sections of each retina were analyzed; from each section 5 images were acquired at 20X, giving a total of 25 images per retina. Images were processed using Adobe Photoshop and ImageJ software. The total number of stained cells was quantified. Statistical analysis was performed (unpaired t-test) using Graphpad Prism. Results were expressed as mean±SEM; the p value was considered significant when <0.05.

**Immunofluorescence analysis**

Immunofluorescence for GAP-43 (n=5) (14 days after crush) and F4/80 (n=5) (4 days after crush) were performed in WT. Optic nerves were transferred to 30% sucrose (12-24 h), embedded in OCT (optimum cutting temperature) and frozen. To analyze fibers by light microscopy, optic nerves were sectioned longitudinally at 14μm and mounted on coated glass slides. Optic nerve slides were washed in 0.1 M TBS (pH 7.4) three times. Shortly after, slices were incubated in blocking solution (2% BSA, 5% Donkey serum in TBS2T) for 1 hour. Slides were incubated in the primary antibody growth-associated protein (GAP-43, made in sheep, 1:1500) overnight at room temperature. Sections were washed with TBS, 3x30 min and incubated with secondary antibody (Alexa Fluor 488 Donkey anti-sheep, Invitrogen, 1:500). The sections were washed, coverslipped with Fluoromount and examined in a Leica confocal microscope.

For the identification of microglia and macrophages, two slides containing 10 nerves sections from each animal in both groups were washed in 0.1 M PBS (pH 7.4) and Triton X-100 (Wash Buffer 0.1 M) three times. Shortly after, the sections were washed in blocking solution (0.05% Triton, 2.5% BSA, normal goat serum to 15%) for 1 hour, and then using the same solution the primary antibody rat monoclonal anti-F4/80 was added (AbDSerotec 1:100), overnight, at room temperature. Then, the sections were washed in PBS solution 3x15 min, and incubated with secondary antibodies (Alexa 488 goat anti-rat, Molecular Probes, USA) for 2 hours at room temperature. After the reaction, the sections were washed with PBS. Nuclei were stained with DAPI (Molecular Probes, USA, 1:10,000) and sections were mounted with Fluoromount (EMS). The primary antibody was omitted for negative controls. The sections were observed in a fluorescence microscope Zeiss Axiocam 2 Plus and photographed with a Zeiss Axiocam MRC camera using Axiovision software (version 4.5). The quantification of the positive cells in serial longitudinal cuts was made. Statistical analysis was performed (unpaired t-test) using Graphpad Prism. Results were expressed as mean±SEM; the p value was considered significant when <0.05.

**Results**

**Absence of Gal-3 promotes neuroprotection of RGC**

In order to evaluate whether the absence of Gal-3 promotes RGC neuroprotection, we analyzed retina whole mounts immunolabeled for βIII tubulin, two weeks after optic nerve crush (Fig. 1). We observed that WT (3206±66.19) and Gal-3-/- (2838±195.8) animals present the same amount of RGC in normal conditions (Fig. 1B,C,F). Following optic nerve crush, we found an increase in the rate of RGC survival of Gal-3-/- mice (1283±79.15), when compared to WT (495.4±53.96) animals (Fig. 1D,E,F). These results show that the absence of Gal-3 favored the survival of RGC after lesion.

**Apoptosis dynamics of RGC is altered in the absence of Gal-3**

We evaluated the apoptotic process in RGC, using
apoptag cell death reaction staining in transverse sections of WT and Gal-3-/- retinas four days after optic nerve crush (Fig. 2A-F). We observed a decrease in the number of positive cells in Gal-3-/- animals (1.465±0.1466) when compared to WT (2.736±0.4944) (Fig. 2G). Apoptotic cells were confined to the RGC layer. These results indicate that there are less apoptotic RGC in the retinas from Gal-3-/- animals.

Gal-3-/- RGC axons do not regenerate after optic nerve crush

To study whether the increase in cell survival would also be related to regeneration of RGC axons, we performed GAP-43 immunostaining on longitudinal sections of optic nerves from both WT and Gal-3-/-, two weeks after crush (Fig. 3). No GAP-43 positive fibers in the WT or Gal-3-/- were observed after the lesion site. Insets show the GAP-43 positive fiber staining pattern proximal to the lesion site.

Wallerian Degeneration is not altered four days after crush

In order to compare the course of WD in the optic nerves of WT and Gal-3-/-, we analyzed the ultrastructure under TEM, four days after crush (Fig. 4). We observed that non-injured animals from both groups presented similar morphological features, showing myelinated fibers of various calibers, with regular contour and normal axoplasm (Fig. 4A-D). Four days after crush both WT (Fig. 4E,F) and Gal-3-/- (Fig. 4G,H) optic nerves presented disorganized ultrastructure, with thick and prominent astrocytic processes among the nerve fibers. However, well-preserved fibers can be found within the nerves, in both groups (Fig. 4F,H). Quantitative analysis revealed around 60% of axons undergoing degeneration in both WT (330.3±19.46) and Gal-3-/- (309.5±14.34) animals (Fig. 4I).

Wallerian degeneration is impaired in Gal-3-/- mice 14 days after crush

Ultrastuctural analyses showed, 14 days after crush, that WT animals exhibited intense astrogliosis and very few fibers, all degenerating (Fig. 5A,B). However Gal-3-/- animals presented less intense astrogliosis and the presence of many degenerating myelinated nerve fibers (Fig. 5G,D). Quantitative analysis of electron micrographs from the optic nerve of Gal-3-/- animals.
two weeks after optic nerve crush showed an increase in the number of fibers undergoing degeneration (777.6±17.71) when compared with WT group (463.8±21.6) (Fig. 5E).

Macrophage/Microglia activation is reduced in the absence of Gal-3

We also investigated the effects of the absence of Gal-3 on optic nerve inflammation after crush. We performed immunohistochemistry for F4/80 in longitudinal sections, four days after lesion (Fig. 6A-F).

Discussion

In this study we investigated the effect of the absence of Gal-3 on RGC survival and optic nerve
degeneration and regeneration after injury. Our results showed that two weeks after crush, there was a 2.6 fold increase in cell survival rate of Gal-3-/− mice (52.7±2.8% when compared to non-lesioned WT animals - 17.0±1.9%), indicating a promotion of RGC neuroprotection. Although using different methods and with different conditions, the increase in RGC cell survival found in the present work is higher than what was described elsewhere, when RGC PTEN deletion resulted in a survival rate of 45%, two weeks after crush (Park et al., 2008), and is comparable to the best treatment for this visual pathway so far, the combination of intraocular inflammation with PTEN deletion and increased levels of cAMP (Kurimoto et al., 2010; de Lima et al., 2012). Kurimoto and collaborators (2010), showed that two weeks after optic nerve injury the rate of cell survival was 54%, and 6 weeks after injury there were still 39% of total RGC. De Lima and co-workers (2012) showed that the same treatment induced 36% survival rate even 10 weeks after optic nerve crush.

Apoptosis assay showed that few RGC undergo apoptosis in Gal-3-/− animals when compared to WT, four days after optic nerve crush. Thus, our data demonstrated that the absence of Gal-3 favored a lower rate of cell death after optic nerve crush, suggesting that Gal-3 has a pro-apoptotic effect in this process and its absence could be inhibiting apoptotic events or stimulating anti-apoptotic signaling pathways in RGC. Opposed to this, it was shown that Gal-3 protects from neurotoxicity mediated by Abeta42 (Kim et al., 2010). It has been proposed in cancer research that intracellular Gal-3 stimulates cell-survival, whereas extracellular Gal-3 leads to cell death (Nakahara et al., 2005).

Since in the absence of Gal-3 there was an increased survival of RGC, it was of interest to investigate whether this phenotype could promote RGC regeneration. Regeneration aims to ensure the viability of committed neurons and to promote axon growth so that it crosses the site of injury, reaches distal regions, enters the brain and forms new functional synapses (Harvey, 2007; Benowitz and Yin, 2008). Our results showed no fibers crossing the lesion site two weeks after optic nerve damage in both groups, suggesting that regeneration capacity was not induced in Gal-3-/− animals. Similarly, bcl-2 gene overexpression stimulates RGC survival, but does not modulate optic nerve regeneration (Chierzi et al., 1999, Inoue et al., 2002). Previous findings of our

![Figure 4](image.jpg)

**Fig. 4.** Electron micrographs 4 days after optic nerve injury. A-B. Non-lesioned WT animals present normal nerve fibers of various calibers (arrows) in the optic nerve, showing appropriate myelin sheath thickness compared to the axon diameter. C-D. Nerve fibers of non-lesioned Gal-3-/− animals show profiles similar to that found in WT animals’ fibers (arrows). E-F. WT animals after injury present different profiles of fibers: apparently normal structured fibers (black arrows) and fibers that show structural disorganization with no well compact myelin sheath (white arrows). G-H. Lesioned Gal-3-/− animals present fibers with normal appearance (black arrows) and fibers with degenerative features (white arrows). I. Graph shows the percentage of nerve fibers present after lesion in WT and Gal 3-/− animals. There was no significant difference between groups.
group (Narciso et al., 2009; Mietto et al., 2013) showed that, in a model of peripheral nerve injury, Gal-3-/- animals present a favorable environment for sciatic nerve regeneration, and this result was explained by the faster WD process, reflecting greater efficiency in the removal of cell debris by macrophages and Schwann cells, allowing regeneration to begin earlier.

WD in the CNS has a slower course when compared to the PNS. This process generally does not occur properly or efficiently in the CNS. The removal of myelin debris by microglia is relatively low, leaving inhibitory molecules that can block axonal growth (Fawcett and Asher, 1999; Rotshenker, 2011; Svenningsen and Dahlin, 2013; Jung et al., 2014). The present work showed that the course of WD remained unchanged four days after optic nerve crush, but was significantly impaired 14 days after lesion. At this later time-point, Gal-3-/- animals presented less pronounced gliosis and more identifiable, although degenerating, fibers than WT animals. We found that the axoplasm

![Fig. 5. Electron micrographs 2 weeks after optic nerve injury. A-B. Electron micrograph of nerve fibers from WT animals 2 weeks after optic nerve crush showing hypertrophied astrocytes (white star) and few degenerating fibers (black arrows). C-D. Electron micrograph of optic nerve from Gal-3-/- animals after injury shows fibers in degenerative process. Note nerve fiber with empty axoplasm and no discernible organelles, typical of watery degeneration (white arrow) and others presenting electron dense axoplasm, typical of dark degeneration (black arrows), and presenting preserved myelin sheaths. E. Graph represents the mean number of degenerated fibers in Gal 3-/- animals compared to WT animals. ** represents p<0.01.](image-url)
was not preserved, but myelin was intact in many degenerating fibers of Gal-3-/- animals. Since WD is an inflammatory process, we addressed the question whether it would make any difference on the numbers of activated microglia/macrophages within the lesioned optic nerve, once we know that these cells are responsible for removing debris from degenerating axons. Our results showed that Gal-3-/- mice presented less activated microglia/macrophages within the optic nerve, four days after crush. The fewer number and/or weaker activation of macrophages/microglia found in the present work might be related to the slower clearance of degenerating axonal profiles found in the Gal-3-/- mice. Consistent with our results, Rotshenker (2009) showed that Gal-3 is a unique marker for microglia/macrophages that are able to engulf myelin and it has been described that microglia are deficiently activated and only sporadically express Gal-3 “in vivo” after transection of the mice optic nerve, and this observation was correlated to the slow myelin removal observed during optic nerve Wallerian degeneration (Reichert and Rotshenker, 1996). Thus, microglia/macrophages might not efficiently phagocyte myelin of Gal-3-/- mice, therefore leaving degenerating fibers for longer periods of time. In consonance with this, macrophages and Schwann cells of slow Wallerian degeneration mice do not express Gal-3 after peripheral nerve lesion (Reichert et al., 1994). Besides, we found higher astroglial reactivity within the optic nerves of WT animals, when compared to Gal-3-/- mice 14 days after crush (Fig. 5). Astrocytes have previously been described to phagocyte myelin after optic nerve crush (Narciso et al., 2001). Since Nguyen and coworkers (Nguyen et al., 2011) showed glial reactivity, followed by galectin-3 up-regulation in phagocyting astrocytes, we cannot rule out that the lack of Gal-3 can impair the proper activation of astrocytes and their phagocytic activity, leaving degenerating fibers for a longer period of time in our study. Thus, low activation or proliferation of microglia/macrophages and/or astrocytes might be involved in the slower degeneration observed in Gal-3-/- mice after optic nerve crush.

Optic nerve crush is a suitable model for the study of the patophysiological aspects of Glaucoma, the leading cause of irreversible blindness worldwide (Almasieh et al., 2012). Literature data has been controversial in the participation of glutamate excitotoxicity in the pathophysiology of glaucoma. One line of evidence suggests that optic nerve lesion induces loss of glutamate/aspartate transporters, leading to hyperactivation of Muller cells, which secrete TNF-alpha, inducing increased surface levels of calcium-permeable AMPA receptors in RGC, leading to apoptosis (Schuettauf et al., 2007; Lebrun-Julien et al., 2009; Almasieh et al., 2012). In addition, NMDA receptor containing GluN2B subunit activation has been related to glutamate excitotoxicity in diverse neurodegenerative diseases (Namekata et al., 2014). Besides, reactive oxygen species (ROS) are involved in RGC death in glaucoma (Tezel et al., 2005; Kanamori et al., 2010). It is widely accepted that activated microglia are the major source of ROS in the CNS. So, reducing microglial activation seems to be a key aspect to be targeted in glaucoma or ONC treatment (Almasieh et al., 2012). Since Gal-3 promotes microglia activation (Burguillos et al., 2015), its absence in the Gal-3 null
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mice should reduce ROS production and RGC death. Alternatively, it has been proposed that failures caused by the axonal transport system might impair the neurotrophins signalling machinery within the retinofugal pathway, leading to RGCs apoptosis (Pease et al., 2000; Quigley et al., 2000). Although recombinant BDNF intraocular injection enhances RGC survival after crush, it does not increase lens injury-induced regeneration (Pernet and Di Polo, 2006). Thus, strategies that enhance RGC survival via different mechanisms are required to test combinatorial therapies to promote both survival and regeneration of RGC after ONC or glaucoma. Since guanine exchange factor Dock-3 overexpression promotes RGC survival, reducing NMDA receptor mediated excitotoxicity, and regeneration, stimulating cytoskeleton remodeling after ONC (Namekata et al., 2012), it would be interesting to induce viral mediated retinal Dock-3 overexpression in Gal-3 knockout mice, to study if they present synergistic effects on RGC survival and regeneration after injury.

Given the increase in the survival of RGC in Gal 3/-/ mice, it would be important to understand the reasons for the delayed WD, and overcome it. Besides, the results presented here open up the possibility of investigating the association of the absence of Gal-3 with other pro-regenerative therapeutic strategies after traumatic injury or degenerative diseases.

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References


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