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Research article

Changes in parvalbumin immunoreactive retinal ganglion cells and amacrine cells after optic nerve injury

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ABSTRACT

Parvalbumin (PARV) is a Ca^{2+}-binding protein that may offer resistance to cell death as it primarily functions to maintain Ca^{2+} homeostasis. The purpose of this study was to investigate whether PARV expressing retinal ganglion cells (RGCs) would be more resistant to cell death than RGCs that do not express PARV. RGCs in Sprague–Dawley rats were retrogradely labeled with Fluorogold (FG). After 2–28 days following an optic nerve crush (ONC) injury immunohistochemistry was performed on the sections using antibodies against PARV and markers of RGCs. The proportion of retinal ganglion cell layer cells labeled with PARV colocalized with FG or Brn3a and labeled only with PARV (displaced amacrine cells; dACs) were analyzed. PARV staining intensity was measured in ACs, dACs, and RGCs. Double labeling studies revealed that 49% of RGCs and 22% of dACs expressed PARV. There was an immediate reduction in RGC PARV staining after ONC but the overall rate of cell death after 28 days was similar in PARV and non-PARV expressing RGCs. There was no change in PARV AC or dAC number or staining intensity. Although this study suggests that there is no selective survival of the subpopulation of RGCs that contain PARV, there is down-regulation of PARV expression by these RGCs. This suggests that down-regulation of PARV may contribute to RGC death due to a compromised Ca^{2+} buffering capacity. Maintaining PARV expression after injury could be an important neuroprotective strategy to improve RGC survival after injury.

1. Introduction

Parvalbumin (PARV) is an EF-hand calcium-binding protein that is found throughout the central nervous system including the retina (Celio, 1990; Sanna et al., 1990). It is thought to play a variety of critical roles that range from intracellular calcium (Ca^{2+}) buffering to regulation of enzymes in Ca^{2+} signaling pathways (Schäfer and Heizmann, 1996). Changes in the expression of PARV has been demonstrated in a number of neurodegenerative disorders, suggesting that PARV may have a role in determining cell survival (Heizmann and Braun, 1992; Schäfer and Heizmann, 1996; Leuba et al., 1998; Cicchetti et al., 2000; Reynolds et al., 2001; Eyles et al., 2002).

A relationship between PARV expression and ocular neurodegenerative disorders has also been documented. In experimental glaucoma and following optic nerve transection, GABA and glycine, which are markers for 90% of amacrine cells (ACs) and displaced amacrine cells (dACs), do not change significantly (Kielczewski et al., 2005). However, it has been shown that PARV expression in AII AC sub-types is down-regulated in a model of diabetic retinopathy (Park et al., 2008), retinal ischemia (Kim et al., 2010; Huang et al., 2013), and in a rat model of glaucoma (Hernandez et al., 2009). In a mouse model of glaucoma, both calretinin and PARV expression was reduced in both the inner nuclear and ganglion cell layers (Gunn et al., 2011). These findings suggest that subpopulations of ACs down-regulate Ca^{2+} binding proteins after injury but the cells do not die by apoptosis because AC numbers do not change and are not labeled by markers of cell death (Huang et al., 2013).

Although both RGCs and ACs express PARV (Sanna et al., 1990), many studies examining changes in PARV expression after injury have concentrated on the AII ACs. Examining expression changes in other ACs, dACs, or RGCs is hampered by the ability to unequivocally differentiate between cell types, especially those that reside in the same layer. In the mouse retina, it was estimated that 86% of the PARV immunoreactive cells were RGCs and that 29% of RGCs were immunoreactive for PARV (Kim and Jeon, 2006). At least eight

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different types of mouse RGCs have been shown to express PARV (Kim and Jeon, 2006) and all mouse RGC types are affected in the DBA/2J mouse model of glaucoma (Jakobs et al., 2005), so it is not known if the Ca^{2+} buffering capacity afforded by PARV expressing RGCs increases their survival after injury. The purpose of the present study was to retrogradely label RGCs with Fluorogold (FG) to definitively label RGCs and subsequently label the retinas using PARV immunohistochemistry to differentiate between PARV expressing dAC and RGCs. We then examined the change in number and staining intensity of these cell populations over a 4 week period following optic nerve crush (ONC) injury. It was our hypothesis that PARV expressing RGCs would be more resistant to cell death than RGCs that did not express PARV.

2. Materials and methods

2.1. Animals

Adult Sprague–Dawley rats (n = 32; female; 225–250 g; 6–8 months old; Charles River, Wilmington, MA) free of common pathogens were used in all experiments. The study was approved by the McMaster Animal Research Ethics Board (AUP 09-11-47). The animals were cared for following the guidelines of the Canadian Council on Animal Care and according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. The animals were kept on a 12:12 h light:dark cycle and permitted food and water *ad libitum*.

2.2. Retrograde labeling of retinal ganglion cells

Rats were anesthetized with an intraperitoneal injection of 7% chloral hydrate (Thermo Fisher Scientific, Ottawa, ON; 0.42 g/kg) before surgical procedures. Animals were placed in a Kopf stereotaxic frame (David Kopf M900, Tujunga, CA) and an ophthalmic eye lubricant (Lacri-Lube; Allergan, Markham, ON) was applied to the eyes to prevent corneal desiccation. RGCs were retrogradely labeled with Fluorogold (FG; Fluorochrome LLC, Denver, CO) by bilateral injection into the superior colliculus. This procedure is known to label more than 98% of RGCs (Nadal-Nicolás et al., 2009). Briefly, a mid-sagittal incision was made in the skin to expose the sagittal suture and the lambda suture. Two 1 mm diameter holes were then drilled into the skull 2.5 mm rostral to lambda and 1.2 mm lateral to the sagittal sutures. Four microliters of FG was injected 3 mm deep into the parenchyma of the brain using a 10 μl Hamilton syringe (Hamilton M701, Reno, NV) with the aid of a microinjector (WPI UltraMicroPump, Sarasota, FL) (Koeberle and Ball, 1998). After all surgical procedures, the animals were allowed to recover on a heating blanket (38 °C) after a subcutaneous injection of Anafen (Merial Canada, Baie D'Urfé, QC; 5 mg/kg) to minimize discomfort, and 5 ml normal saline to maintain hydration.

2.3. Optic nerve crush injury

ONC injury is a useful acute injury model that is reproducible and results in 50% loss of RGCs within 2 weeks after injury (Kalesnykas et al., 2012). One week after FG injection the animals were prepared for surgery as previously described. An incision was made in the skin around the rim of the orbital bone. The orbital contents were retracted away until the optic nerve was reached. To expose the optic nerve, the eye was rotated temporally and the intradural optic nerve was compressed for 3 s using Dumont SS fine forceps (Fine Science Tools 11203-23; North Vancouver, BC). ONC injuries were done unilaterally and the non-injured eye served as a control. Eyes that were not immediately re-perfused after injury, or on rare occasion developed a cataract, were excluded from analysis. A total of 32 animals were used in this study (post-injury day 0 n = 3; day 2 n = 3; day 4 n = 3; day 5 n = 3; day 7 n = 6; day 14 n = 9; day 28 n = 5).

2.4. Tissue processing

The rats were killed at intervals between 2 and 28 days after ONC by a lethal dose of 7% chloral hydrate. The eyes were enucleated and the cornea and lens were dissected away. The remaining eyecup was fixed using phosphate buffered (Sørensen's; 0.1 M, pH 7.3) 4% paraformaldehyde containing 2% sucrose for 2 h at room temperature. The fixed eye cups were washed three times in sodium phosphate buffered saline (PBS; 0.1 M, pH 7.3, 0.9% NaCl) and immersed in a 30% PBS sucrose solution overnight at 4 °C. The eye cups were embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and sections were cut on a cryostat microtome (Leica Microsystems CM1900, Concord, ON) at −20 °C. Transverse sections of 12 μm thickness were collected on SuperFrost Plus slides (Thermo Fisher Scientific, Ottawa, ON) beginning 1 mm from the ora serrata and ending at the optic nerve head. The remaining hemi-eyecup was thawed and the retina dissected from the sclera and choroid. The vitreous was removed and flatmounted in Vectashield mounting medium (Vector Labs H1000 or H1200 containing DAPI, Burlington, ON).

2.5. Immunohistochemistry and imaging

After sectioning, retinal sections were washed 3 times in PBS for 10 min. A blocking solution containing 3% BSA, 1% Normal Donkey Serum, 0.1% TX-100, and 0.1% DMSO was then put on the slides for 1 h. The sections were incubated in rabbit anti-parvalbumin (PARV; 1:200; Pierce PA1-933; immunizing protein α-PARV purified from rat skeletal muscle; Cedarlane Laboratories, Burlington, ON) in blocking solution overnight at room temperature. The sections were then washed three times in PBS and incubated in donkey anti-rabbit Alexa 568 (1:200; Molecular Probes A10042, Life Technologies, Burlington, ON) in blocking solution for 5 h. After washing the sections three times in PBS, they were mounted in VectaShield (Vector Labs H1000 or H1200 containing DAPI, Burlington, ON) for epifluorescence microscopy (Zeiss Axioplan 2, Carl Zeiss, Toronto, ON). For double labeling experiments with PARV antisera and antisera directed against an antibody specific for RGCs, Brn3a (Nadal-Nicolás et al., 2009), retinal sections were washed as above, then incubated in a blocking solution containing 3% BSA, 1% Normal Goat Serum, 0.1% TX-100, and 0.1% DMSO for 1 h. The sections were incubated in rabbit anti-PARV (1:200) and goat anti-Brn3a (1:200; Santa Cruz SC-31984 (C-20); immunizing protein human class IV POU domain protein; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in blocking solution overnight at room temperature. The sections were then washed three times in PBS and incubated in donkey anti-rabbit Alexa 568 and donkey anti-goat Alexa 488 (A10042, A11055; Molecular Probes) in blocking solution for 5 h. After washing the sections they were mounted and visualized as described above.

2.6. Measurements and statistical analyses

Images were captured using an AxioCam MRm camera and AxioVision 4 software (Carl Zeiss Canada, Toronto, ON) and the numbers of different cell types in the retina (i.e. PARV-expressing RGCs, non-PARV-expressing RGCs, ACs) were counted manually. The counter was blinded to the identity of the sample treatment. RGC counts in flatmounts were made by counting all RGC somas in a 92,500 μm^2 sample randomly selected in each of the two remaining quadrants of the hemi-retina. Samples were taken from the mid-periphery, which was a distance of between 2 and 2.8 mm