Analysis of optic nerve stroke by retinal Bex expression

Steven L. Bernstein,1,2 Jae Hyung Koo,2 Bernard J. Slater,1 Yan Guo,1 Frank L. Margolis2

Departments of 1 Ophthalmology and 2 Anatomy and Neurobiology, School of Medicine, University of Maryland-Baltimore, Baltimore, MD

Purpose: Few proteins are known to be selectively expressed in retinal ganglion cells (RGCs), the neurons directly affected by optic nerve stroke and glaucoma. In addition, subsets of RGCs are reported to project to various CNS areas via the retinohypothalamic pathway in rodents and primates. Many of these areas exhibit immunoreactivity for the brain-expressed X-linked (Bex) proteins Bex1 and Bex2. This prompted us to evaluate expression of these proteins in retina.

Methods: We utilized rats and transgenic mice, coupled with a new rodent model of isolated optic nerve stroke (rodent anterior ischemic optic neuropathy, rAION). An anti-Bex1 antibody was reacted to retinal tissue extracts. To evaluate short term effects of rAION on RGC Bex expression, a double transgenic mouse strain was employed expressing cyan fluorescent protein (CFP) under control of the Thy-1 protein promotor, and beta-galactosidase (lacZ) under control of the immediate early stress c-fos gene promotor. Positive identification of rat RGCs was performed by retrograde fluorogold labeling via stereotactic CNS injection. Retinas were analyzed using both diaminobenzidine (DAB)-linked immunohistochemistry and confocal microscopy.

Results: Bex immunoreactivity is present at high levels in the retina. Bex1 and Bex2 are selectively expressed in RGCs and differentially expressed in a subset of large RGC neurons. Bex signal levels are lower in small RGC neurons, which preferentially express high levels of the transcription factor Brn3b. Post-stroke, Bex accumulates in the RGC cytoplasm, consistent with the optic nerve edema produced by clinical AION.

Conclusions: Bex immunoreactivity can be used to evaluate, ex vivo, the distribution of RGC cell bodies and their axons in the retina and rAION effects on RGC axonal loss. Thus, Bex can be utilized to evaluate both long- and short-term effects of optic nerve stroke and may play a significant role in regulating RGC functions in both the axonal and cell body components of RGC neurons.

Retinal ganglion cells (RGCs) are the neurons responsible for transmitting the summed information obtained from phototransmission and other stimuli from the retina to the brain [1]. RGC axons are present as a separate layer (the nerve fiber layer, NFL), that collects centrally to form the optic nerve (ON) [1]. RGC axons are unmyelinated while in the retina, but become myelinated in the ON [1]. In most mammals, notably rodents, RGCs are present in a monolayer (the RGC layer), but only 65-75% of all cells in the RGC monolayer are actually RGCs [2]. These cells are defined by the presence of an axon within the ON and have a number of subtypes delineated by differences in morphology, size, and function [2,3].

Mature RGCs express no RGC-specific proteins. Two proteins that are expressed in other tissues, but selectively expressed in the retina only in RGCs include Brn3b [4], and Thy 1 [5]. These two proteins, along with retrograde labeling techniques utilizing fluorogold or other fluorescent dyes [6,7], are used to identify RGCs. In mice, transgenic strains incorporating reporter gene constructs under the Thy-1.2 gene promotor enable robust RGC cell and axonal identification in this species [8]. In other species however, in situ immunochemical reactivity to these protein is not robust, making in situ RGC identification difficult [9]. Thus, analysis of total RGC loss following different types of damage has typically relied upon quantitative axonal counts utilizing transmission electron microscopy [10].

Brain-expressed X-linked protein-1 (Bex1) is an X-linked protein expressed in a variety of tissues, but seen at highest levels in brain, testis, and the olfactory system [11,12]. Bex1 [1] is one of a family of at least six members, that are widely distributed in mammals [13,14]. The Bex genes are separate loci although Bex1, 2, 3, and 4 are close together on the X-chromosome at XF1 (X57.5 cM) in mouse and Xq22.1-2 in human [14]. The remaining members of this family are autosomally located. Bex 3 is reported to function in NGF mediated neuronal apoptosis and has been named NADE [15,16] but the function(s) of the other members of this family are not known. While many of the members have widely diverged, Bex1 and Bex2 are evolutionarily close. They share 87% amino acid homology [13], and antibodies to Bex1 cross-react with Bex2 [13]. The functions of Bex1 and 2 are currently unknown.

Initial evaluation of NEIBank rat and mouse retinal libraries (NEIBank) indicated that Bex1 is expressed in mouse, with Bex2 expressed in rat retinas. Initial histological analysis [13] using a rabbit polyclonal antibody to Bex1 generated from purified recombinant mouse Bex1 revealed that, similar to the POU domain transcription factor Brn3b [17], retinal Bex1 and Bex2 expression was present at highest levels in rat and mouse RGCs. As we will show in this work, Bex immunoreactivity (Bex-ir) from this antisera reacts well with both Bex1 and
Bex2. Except where specifically blocked antisera are used to discriminate the two cross reactive proteins, Bex-ir is intended to refer to the combined immunoreactivity of Bex1 and Bex2. Unlike antibodies to Brn3b and Thy-1, the Bex1 antibody identifies both RGC soma and their axons, and the immunohistochemical reaction is robust, and specific. We wanted to further evaluate the immunoreactivities of Bex1 and Bex2 (Bex-ir) in RGC types and its response in affected neurons following ON stroke. We also wanted to evaluate the possible application of Bex1 antibody in stereological analysis of RGC loss following optic nerve damage.

We recently reported on anterior ischemic optic neuropathy (AION) models generated in rat and mouse [18,19]. Rodent AION (rAION) utilizes laser-induced superoxide radical formation from rose Bengal (RB) dye to selectively damage the vascular endothelium of ON capillaries, producing highly predictable levels of isolated ON ischemia. rAION resembles the natural pathophysiology of human AION [18], enabling direct analysis of in vivo isolated CNS axonal ischemia. Using rat and the Thy1-CFP transgenic mouse strains, we evaluated short-term Bex1 expression in normal RGCs, and its expression in retina affected with rAION. We also applied Bex immunohistochemistry to perform the first direct analysis of the long-term effect of axonal stroke on the two dimensional distribution pattern of RGC axons present on the retinal surface.

**METHODS**

**Animals:** All animal procedures were approved by the University of Maryland-Baltimore (UMB) Institutional Animal Care and Use Committee. Normal male Sprague-Dawley albino rats (120-150 g) were obtained from Charles River (Wilmington, MA). Animals were kept in the UMB animal facility and were given food and water ad libitum. Tg-Thy1-CFP/c-fos/lacZ (Wilmington, MA). Animals were kept in the UMB animal facility and were given food and water ad libitum. Tg-Thy1-CFP mice on a C57BL6/6J background were obtained from Jackson Laboratories (Bar Harbor, ME). These animals expressed only Bex2.


preabsorbed with recombinant Bex2 protein (AB2b) was used, only Bex1 protein (arrowhead) was detected. B: Rat and mouse retinas express high levels of Bex proteins. Immunoprecipitated extracts of adult mouse (mRet) or rat (ratRet) retinas (5 mg each) were analyzed by immunoblotting with anti-Bex1 antiserum (Ab). Mouse retina gave strong signals for both Bex1 and Bex2. By contrast, rat retina apparently expresses only Bex2.
For ex vivo analysis, six rat retinas were removed and cut in a four-quadrant cloverleaf pattern. Retinas were frozen in 0.5 ml 1% Triton X-100-PBS at -70 °C for 30 min, and thawed at 25 °C. Thawed retinas were washed in three changes of 0.5% Triton-PBS, blocked with 2% donkey serum, and reacted with Bex1 (1:5000) or anti-fluorogold antibody (1:10,000; Chemicon, Temecula, CA) at 4 °C for 3 days. Post-reaction, retinas were reacted with biotinylated goat-antirabbit secondary antibody, washed with PBS, reacted with ABC reagent (Vector labs, Burlingame, CA) for 4 h at room temperature and developed using Diaminobenzidine (DAB) reagent (Vector Labs). Because both Bex1 and fluorogold antibodies were raised in rabbit, tissues used for double antibody confocal localization were reacted first with antibody to fluorogold, reacted with Cy3 labeled donkey-antirabbit antibody F(ab) fragment, and then blocked overnight at 4 °C with unlabeled 1:100 donkey antirabbit antibody. Tissue was sequentially reacted with fluorogold antibody, and incubated with Cy5-labeled donkey-antirabbit IgG antibody. Negative controls included Fluorogold-Cy3 secondary antibody treated control sections, pre-adsorbed with unlabeled donkey antirabbit antibody. These showed no detectable Cy5 channel fluorescence when subsequently incubated with Cy5-labeled secondary antibody without additional primary antibody.

**Bex/Brn3b/c-fos-β-galactosidase immunostaining:** Four enucleated eyes were post-fixed in 4% PF-PBS. Retinas were dissected free and washed in 1% Triton X-100 in PBS. For standard immunostaining, globes were dehydrated in ethanol, embedded in paraffin and 6 µm retinal cross-sections were analyzed using anti-Bex1 (1:10,000) and anti-Brn3b antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA: 1:500). Bex antibody immunohistospecificity was confirmed by pre-adsorbing the antibody with either Bex1 or Bex2 recombinant protein. Adsorbing the antibody with Bex1 protein resulted in complete loss of antibody reactivity. Tissue was treated for antigen retrieval using boiling citrate buffer (pH 4.7). Retinas were also flat-mounted using fluorescent mounting medium and examined via confocal microscopy. Confocal histological analysis was performed using an Olympus Fluoview 400 4-channel laser scanning microscope (Melville, NY).

---

**Figure 2. Confocal analysis of Bex and Brn3b expression in rat retina.**

**A:** Negative control (no primary; Cy3 and Cy5 labeled secondary antibodies only). Photoreceptor autofluorescence is seen. **B:** Primary antibody to Brn3b. Brn3b preferentially intensely labels small retinal ganglion cells (RGCs, arrow and arrowhead), while large RGCs are not as intensely labeled. A few RGCs with strong Brn3b signal co-label with Bex1 antibody (B-D, arrowhead). **C:** Retinal Bex-ir signal. Bex signal is strongest in large RGCs (arrow), while weaker expression is also found in small RGCs (arrowhead). **D:** Confocal localization of Brn3b and Bex-ir. Both proteins are expressed in RGCs. The scale bar represents 50 µm. The inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and photoreceptor layer (Prc) are identified.
**LacZ** expression was analyzed using a β-galactosidase monoclonal antibody (Promega, Madison, WI) at 1:1000. A donkey antimouse secondary antibody labeled with Texas Red was used for detection. Simultaneous Bex-ir expression was detected using a donkey antirabbit Cy5-labeled secondary antibody. Simultaneous CFP detection was performed using the 405 nm emitting diode laser-confocal channel/450 nm filter.

Identification of Bex1, and Bex2 in mouse, and rat retinae: Characterization of Bex protein expression in mouse central nervous system (CNS) and P14 mouse and adult rat ocular tissues was performed following immunoprecipitation of tissue extracts with Bex1 polyclonal antiserum as previously described [13] For immunoprecipitation each P14 retinal extract (5 mg of total protein), olfactory bulb extract (0.5 mg) and cerebellum extract (0.5 mg) were incubated with 5 ml of anti-Bex1 serum in a final volume of 1000 ml of 20 mM Hapes buffer containing 0.125% Triton X-100 on ice for 1 h. Protein A-Sepharose beads (10 ml, Amersham Pharmacia, Piscataway, NJ) were then added and incubated at 4 °C overnight with gentle mixing. The beads were washed 5 times with incubation buffer and the drained beads were then incubated at 95 °C in SDS sample buffer and used for western blot. Denatured immunoprecipitate supernatants were electrophoresed on 10-20% SDS-PAGE at low voltage to separate Bex1 from Bex2. Western blotting was performed using Ab (intact), AB1b (Bex1-blocked), or AB2b (Bex2-blocked) antibody as previously described [13]. A comparison of Bex1, and Bex2 expression in adult mouse (mRet), and rat retina (ratRet) was similarly performed, followed by immunoblot analysis with Bex1 antibody [13].

**RESULTS**

**Mouse retinas express Bex1 and Bex2 proteins while rat retina primarily expresses Bex2 protein:** Bex1 antiserum pre-adsorbed with Bex2 protein (Figure 1A, AB2b) still recognizes Bex1. Pre-absorption with Bex1 protein (AB1b) eliminates immunoreactivity to both Bex1 and Bex2 (Figure 1A, Ab). The anti-Bex1 antibody therefore cross-reacts with the Bex2 isoform.

A doublet immunoreactive banding pattern (representing Bex1 and Bex2 proteins) was seen when the antibody to Bex1 was reacted against extracts of P14 brain regions (Figure 1A) previously immunoprecipitated with anti-Bex1 (Figure 1A, Ab Ret, OB, CB). Bex1 antiserum pre-adsorbed with Bex2 protein (Figure 1A, AB2b) still recognizes Bex1. We conclude that the tissues examined express both Bex1 and Bex2 protein.

---

Figure 3. Bex-ir expression co-localization with fluorogold-labeled retinal ganglion cells in rat. Two weeks after superior colliculus injection with fluorogold, retinas were reacted with primary antibodies to Bex1 (A) and fluorogold (B). C: Merged confocal image. Retrograde transported fluorogold signal is seen in RGCs (B, double arrows), while Bex expression is seen in RGC axons (Ax; A), and in RGC cell bodies (A, double arrows). Some Bex positive cells in the layer which do not react with antifluorogold antibody are also present (A-C; single arrows). The scale bars represent 100 µm.
**Bex-ir and Brn3b expression patterns in rat retina:** In retina, Brn3b is selectively expressed in RGCs [17,22]. We performed confocal microscopy on rat retina using Bex1 and Brn3b antibodies following secondary antibody labeling (Figure 2). Brn3b expression is almost exclusively in the RGC layer (Figure 2B). The strongest Brn3b labeling was found in RGCs with small cell bodies (Figure 2B, arrow and arrowhead). Lower Brn3b expression was seen in RGCs with large cell bodies (Figure 2B, double arrow). The high level of fluorescence in the photoreceptor layer (Prc) is likely due to background autofluorescence or secondary antibody cross-reactivity, since signal was also seen in the IgG control section (Figure 2A, Prc).

Retinal Bex-ir immunolocalization reveals that Bex expression is highest in the RGC layer with low signal levels in the inner nuclear layer (INL). Bex expression was strongest in large RGCs, with lower levels in small RGC cell bodies (Figure 2C, arrows). A few small RGCs were seen with intermediate levels of Bex-ir and Brn3b (Figure 1D, arrowhead). Interestingly then, Bex and Brn3b antibodies distinguish two different RGC populations within the rat retina.

**RGC colocalization with retrograde fluorogold labeling and Bex-ir:** To determine whether Bex-ir can be used to delineate RGC cell loss and axonal patterns in toto in the rat retina, we compared the pattern of Bex-RGC immunoreactivity and fluorogold signal. Fluorogold accumulates only in
RGCs following CNS injection into the superior colliculus, by retrograde RGC axonal transport (Figure 3). Postinjection, only RGCs are labeled, seen as a retinal monolayer (Figure 3B, double arrow). The same cells are also visible after Bex immunostaining (Figure 3A,C, double arrow). While the patterns of staining are similar using both Bex1 and fluorogold antibodies, they are not identical. RGC axons (Figure 3A,C, Ax) are also visible using Bex1 Ab, enabling evaluation of RGC cell and axonal distribution patterns within the retina. Bex-ir is also expressed in large cells in the RGC layer that do not fill with fluorogold (Figure 3A,C, single arrow). Thus, antibodies to Bex1 and Bex2 protein recognize a large subset of cells within the retinal ganglion cell layer that also include retinal ganglion cell neurons.

Co-localization of Bex with Thy-1 promotor-driven CFP in transgenic mice: Thy-1-(CFP) transgenic mice express cyan fluorescent reporter protein in the majority of cells in the RGC layer under control of the Thy 1.2 gene promotor [8]. Retinal co-localization of CFP and Bex-ir is shown in Figure 4. CFP protein is expressed in both RGC cell bodies and axons (Figure 4A). A similar pattern of expression is seen for Bex1 protein in this cell layer (Figure 4B). Both CFP and Bex proteins are present in the same cells and structures in these retinas (Figure 4C). Analysis of CFP and Bex-ir expression in these cells at high power reveal that CFP is strongly expressed in most cells in the RGC layer and at lower signal levels in RGC axons (Figure 4D) consistent with previous reports [8]. Bex-ir is also present in these cells (Figure 4E), but signal levels are

Figure 5. Expression of Bex-ir and β-galactosidase in postaxonal stroke transgenic mice. Control sections (untreated eye) are shown in A-C: Low power images of rAION-affected regions are shown in D-F: High power images of the same area are shown in G-I. **A.D.G:** Bex-ir expression. Axons are indicated with an arrow, while Bex-ir positive cell bodies are indicated with arrowheads. **B.E.H:** β-galactosidase expression. Retinal vasculature (Vs; **B**) is lacZ positive, due to endogenous lacZ expression by RBCs, and indicated with an arrow. lacZ expression in ganglion cells is indicated by arrowheads. **C.F.I:** merged confocal images (Bex+ lacZ expression). lacZ expression is absent in retinal neurons in control eyes (**B**). lacZ expression is apparent in retinal cells three days post-rAION (**E.F, arrowheads**). Bex-ir signal is also present in the same cells expressing lacZ (**D, arrowhead**) and in the merged image (**F**). At high magnification, Bex-ir signal is increased in a number of cells (**G, arrowheads**), revealing that ischemia-affected, lacZ-expressing RGCs (**H.I, arrowheads**) have a relative transport block resulting in cytoplasmic Bex-ir accumulation at the cell soma. The scale bars represent 50 μm in **A.D** and 20 μm in **G**.
greatest in RGC axon bundles, with variable amounts in different cells of the RGC layer (Figure 4E). Both CFP and Bex are expressed in the same cells and structures, when seen at high magnification (Figure 4F). Thus, proteins are expressed in RGC cell bodies and in high concentrations in RGC axons.

Early Bex expression changes in RGCs following optic nerve stroke: c-fos/lacZ transgenic mice express β-galactosidase under control of the immediate early stress gene promoter c-fos. The expression of retinal Bex-ir and lacZ expression was compared in control mice and mice 3 days post-rAION induction (Figure 5). Retinal Bex-ir is identical to the pattern seen in CFP transgenic animals (Figure 5A) with Bex-ir present at low signal intensities in many RGCs. C-fos-lacZ-transgenic animals are positive for β-galactosidase protein in the RBCs and retinal vasculature of control animals (Figure 5B) with no overlap of lacZ and Bex-ir expression in the control animal retinas (Figure 5C). Three days after ON stroke induction, lacZ expression is increased in specific retinal regions, consistent with a regional pattern of RGC stress induced by optic nerve stroke. Bex-ir signal levels increase in individual cells (Figure 5D), in the axonal stroke-affected region, revealed by increased lacZ expression in RGC layer cells (Figure 5E,H). Higher magnification reveals that Bex-ir signal is increased in the cytoplasm of specific RGCs, by the appearance of outlined RGC nuclei (Figure 5G). RGCs with increased cytoplasmic Bex-ir also express β-galactosidase (Figure 5I, arrowheads). Thus, RGC-Bex-ir is at least partially cytoplasmic with axonal blockade resulting in cytoplasmic Bex accumulation.

Bex-ir analysis of RGC cell body and axonal pattern changes after ON stroke: Analysis of Bex-ir in the rat RGC intraretinal axon layer (the NFL) and RGC layer was performed in control and 21 days post-rAION retinas, using flat-mount immunoreactivity for Bex. Bex-ir immunostaining of the NFL in control retina shows axons distributed radially around the

Figure 6. Distribution of Bex-ir in rat retina following optic nerve stroke. Bex-ir immunoreactivity in control (A) and rAION-affected (C) flat mounted retinas. Antibody to Bex1 was reacted against whole retina, developed using DAB-immunohistochemistry, and examined at low and high magnification. A: Control and quarter of the retina flat mounted, low magnification. Axon fibers are distributed evenly and densely within bundles (Axb) to supply the entire retina. Bex positive cell bodies are visible between axons. B: Control retina flat mount, high magnification. Both Bex-ir positive axons and cell bodies are present. A blood vessel is also visible (white arrowhead). C: One-quarter of a retina flat mount 21 days post-rAION. Retinal ganglion cell axonal loss is visible as a decreased density of axon fiber bundles. D: Bex-ir in rAION retina, higher magnification. There is decreased axon fiber density, and decreased density of Bex-ir labeled cells. Large Bex-ir positive cells (Large black arrow) with long processes typical of axons (double arrows) are present in the stroke-affected tissue, which are not readily seen in the control section (arrows). The optic nerve (ON) is identified. The scale bars represent 40 µm.
ON (Figure 6A, control retina), extending out to the retinal periphery. RGC soma are distributed evenly across the retinal surface between the axon bundles (Axb; Figure 6A). Higher magnification reveals individual axon bundles, with RGCs closely packed between the bundles. At 21 days post-rAION, there is a decreased density of axons and RGCs consistent with intraretinal axonal loss (Figure 6C, arrows). At higher magnification, rAION-affected retinal regions show a loss of RGC axons (Figure 6D, double arrow), decreased thickness of axon bundles (Figure 6D, Axb) and a reduced density of Bex positive cell bodies (Figure 6D) compare to the control retina (Figure 6B). rAION-affected retinas can have either sectoral or global loss of RGC axons and cell soma (data not shown).

Interestingly, large Bex-ir cells are also visible in the RGC layer post-rAION (Figure 6D, large arrow). Morphologically, these resemble large RGCs of the G11-off variety. G11 cells are defined by their large cell bodies, any dendritic branches of equal diameter, and acute branching pattern [3]. Thus, Bex whole mount retinal immunostaining reveals that, similar to clinical AION, rAION produces regional RGC damage and loss.

**DISCUSSION**

In the CNS, Bex1 and Bex2 are proteins of unknown function, expressed only in neuronal subsets. The high degree of homology between Bex1 and Bex2 enables Bex2 detection by the Bex1 antibody, in both mice and rats, and is useful because of the largely Bex2-specific expression in rat retina. The expression of Bex 1 and 2 in mice, and Bex2 in rats, suggests that the functions of these two Bex isoforms are conserved in these mammalian species.

Numerous studies have implicated Bex proteins as modulators of signal transduction cascades. Bex1 and Bex2 have both been characterized as OMP partners by biochemical, commounoprecipitation, and structural studies [11,12,23]. Bex1 is also expressed in several other tissues [14,24-27] and was previously identified as a gene whose expression is suppressed by retinoic acid signaling in teratocarcinoma cells [28]. Bex3 interacts with the low-affinity neurotrophin receptor p75NTR in the presence of NGF, resulting in programmed cell death in a variety of cell lines [15].

While the exact role of Bex1 is unknown, it complexes with olfactory marker protein (OMP) and may regulate the activity of specific components of olfactory transduction signaling [11]. Since Bex1 is found in the nucleus along with OMP, it may play a role in nucleo-cytoplasmic signal transfer [11]. The previously determined association of Bex1 with OMP, a protein known to be involved with olfactory signal pathway regulation [12], suggests that Bex1 and Bex2 function may be in some way related to RGC intracellular signaling, both intraretinally, and intra-axonally. Large RGCs have large receptive fields [6,7], and their increased membrane surface area may require greater Bex1 production, which is initially localized at the soma. The largest RGCs have the strongest Bex signal. Interestingly, the strongest signal levels for Brn3b are seen in a subset of small RGCs. This dichotomy may be due to the functional differences between the two markers: While Bex-ir is primarily present throughout the cytoplasm, enabling higher signal levels in larger cells, Brn3b is a transcriptional factor that concentrated within the nuclear compartment. The increased signal density present in small RGCs could simply be due to a relatively higher concentration of Brn3b protein in a smaller compartment.

By utilizing transgenic mice, we demonstrate that, as in rats, Bex-ir is prominent in mouse RGC axons and soma. Bex-ir co-localizes in cells with Thy-1 promoter-driven CFP expression. As with rats, Bex-ir is seen in most, but not all, RGC layer nuclei, with variable Bex-ir levels in different RGCs. While these differences could be artificial, RGC Bex activity may also be linked in some way with specific functional demands that may vary both by cell morphology, dendritic receptive field, and time.

The ability to identify intracellular Bex 1 and 2 enables the visualization of early and late RGC morphological changes that may correlate with functional changes. The ON stroke model results in RGC stress and intraretinal axonal edema, and an axonal transport block. Poststroke, stressed transgenic mouse RGCs were identifiable by their lacZ expression. RGCs within the retinal region affected by ON stroke accumulated lacZ at different levels by three days post-insult. Affected RGCs are strongly Bex-ir positive at this stage in all experimental animals, unlike Brn3b expression which declines soon after rAION induction [18,19], suggesting that soon after axonal stroke, Bex-ir accumulates in the cytoplasm of axonally blocked RGCs. Thus, similar to the axon blockade-induced edema produced by rAION at the junction of the ON [29], affected RGCs also respond with an accumulation of cytoplasmic components upstream of the ON block. Bex-ir signal is present within in the nucleus of affected cells, suggesting that RGC-synthesized Bex 1 and 2 proteins may shuttle between nuclear, cytoplasmic and axonal compartments and may be utilized for both nuclear and cytoplasmic functions.

Late postaxonal stroke, there is loss of RGCs and their axons. Retinal Bex1 expression three weeks after rAION reveals that stroke-induced RGC soma and axonal loss occurs regionally, with a decreased density of axons and cell bodies in the area affected by ON damage. This regional loss is readily seen in flat mount preparations, and can be correlated both by quantitative transmission electron microscopy (TEM) and retinal flat mount stereology (data not shown). The patterns of RGC loss in rats and Thy-1-CFP mice following rAION are similar (data not shown). The effectiveness of Bex-ir visualization of RGC structures pre-and poststroke suggests that retinal Bex-ir localization may be useful in evaluating neuroprotective effectiveness of candidate drugs in CNS axonal stroke by enabling the direct and rapid visualization of the area of affected neurons. Use of Bex-ir, coupled with transgenic technology, may provide the basis for more effective analysis of the impact of axonal ischemia than previously available.

**ACKNOWLEDGEMENTS**

This project was funded by grants from the NEI (R01 EY015304-01) to SLB, NIH-NIDCD (DC-03112) to FLM,
and by an unrestricted departmental grant from Research to Prevent Blindness (RPB). The authors would like to thank Dr. A. Puche (Department of Anatomy and Neurobiology, University of Maryland-Baltimore, Baltimore, MD) for helpful comments and assistance in confocal utilization, and for the mounting medium. SLB thanks Dr. C. M. Bernstein for helpful comments and critical reading in the preparation of this manuscript.

REFERENCES


