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Post-ischemic environmental enrichment protects the retina from ischemic damage in adult rats

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ABSTRACT

The aim of this study was to elucidate whether post-ischemic enriched environment (EE) housing protects the retina from ischemic damage in adult rats, and the involvement of glutamate in retinal protection induced by EE housing. For this purpose, ischemia was induced by increasing intraocular pressure to 120 mm Hg for 40 min. After ischemia, animals were housed in a standard environment (SE) or EE and subjected to electroretinography and histological analysis. EE housing afforded significant functional protection in eyes exposed to ischemia/reperfusion injury. A marked reduction in retinal thickness and ganglion cell number, and an increase in Müller cell glial fibrillary acidic protein (GFAP) levels were observed in ischemic retinas from SE-housed animals, which were reversed by EE housing. A deficit in anterograde transport from the retina to the superior colliculus was observed in SE- but not in EE-housed animals. In SE-housed animals, ischemia induced a significant decrease in retinal glutamate uptake and glutamine synthetase activity, whereas EE housing reversed the effect of ischemia on these parameters. The intravitreal injection of supraphysiological levels of glutamate partially reproduced retinal alterations induced by ischemia/reperfusion, which were abrogated by EE housing. These results indicate that EE housing significantly protected retinal function and histology from ischemia/reperfusion injury in adult rats, likely through a glutamate-dependent mechanism.

Introduction

Ischemia is one of the key factors determining the pathophysiology of many retinal diseases, such as diabetic retinopathy, glaucoma, and age-related macular degeneration, among others. Retinal hypoxia and ischemia impair neuronal energy metabolism by launching a cascade of trigger reactions resulting in cell death. In addition, reperfusion with oxygenated blood after ischemia aggravates ischemic damage, an effect known as reperfusion injury. Several mechanisms such as excitotoxicity, oxidative stress, cell acidosis, and inflammation, acting in tandem are of considerable importance in retinal ischemia (reviewed by Osborne et al., 2004). Glutamate is the main excitatory neurotransmitter in the retina but it is toxic when present in excessive amounts. Thus, effective buffering of extracellular glutamate is important in preserving retinal structure and function. Glutamate excess is thought to cause neuronal cell death in the retina during ischemia/reperfusion (I/R) (Osborne et al., 2004). It was shown that electrophysiological and neuronal damage following ischemia resembles that caused by exposure to supraphysiological levels of glutamate (Fernandez et al., 2009a; Ikeda et al., 1992; Iversen, 1991) and that retinal ischemia induces a significant increase in glutamate release (Cazevieille and Osborne, 1997; Neal et al., 1994). At present, there is no effective treatment to protect the retina from I/R damage. Therefore, the development of resources to protect the retina against ischemia is a goal of vast clinical importance.

Besides pharmacological treatments, environmental conditions have been shown to modify the extent of ischemic damage in the central nervous system (Belayev et al., 2003; Briones et al., 2000; Ohlsson and Johansson, 1995). Enriched environment (EE) refers to conditions that facilitate or enhance sensory, cognitive, motor, and social stimulation relative to standard (laboratory) conditions. Several studies demonstrated that animals housed in EE after ischemic stroke obtain a better functional and structural outcome as compared with those housed in standard cages (Biernaskie and Corbett, 2001; Nygren and Wieloch, 2005; Ohlsson and Johansson, 1995; Sun et al., 2010). Moreover, it has been shown that EE causes several morphological and neurochemical changes in the central nervous system, like thicker cortex, increased dendritic spine density, increased expression of neurotrophic factors, and enhanced neurogenesis (Franklin et al., 2006; Nithianantharajah and Hannan, 2006).

Abbreviations: CTB, cholera toxin β-subunit; EE, enriched environment; I/R, ischemia/reperfusion; ERG, electroretinogram; OPs, oscillatory potentials; GFAP, glial fibrillary acidic protein; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OS, photoreceptor outer segments; RGCs, retinal ganglion cells; SC, superior colliculus; SE, standard environment.

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Retinal development is responsive to the experience provided by EE: the maturation of retinal acuity, which is a sensitive index of retinal circuitry development, is strongly accelerated by EE in rats (Landi et al., 2007). Prusky and co-workers have demonstrated that mice exposed to an EE from birth have an enhanced visual acuity when compared to restrained controls, suggesting that the performance of the visual system is greatly influenced by the complexity of the visual environment (Prusky et al., 2000). In addition, it was reported that EE provides protection against retinal degeneration induced by neonatal glutamate treatment in rats (Szabadfi et al., 2009). In contrast, the adult retina has long been considered less plastic than the brain cortex or hippocampus, the very sites of experience-dependent plasticity, and until now less is known on the protective effects of EE housing in the retina from adult rats. In that context, the aim of the present report was to analyze the beneficial effect of post-ischemic environmental enrichment on I/R damage in adult rats.

Materials and methods

Animals

All animal use procedures were in strict accordance with the NIH Guide for Care and Use of Laboratory Animals. The ethics committee of the University of Buenos Aires School of Medicine, (Institutional Committee for the Care and Use of Laboratory Animals, (CICUAL)) approved this study. Adult male *Wistar* rats (average weight, 250 ± 50 g) were housed in a standard animal room with food and water *ad libitum*, under controlled conditions of humidity and temperature (21 ± 2 °C). The room was lighted by fluorescent lights (200 lx) that were turned on and off automatically every 12 h (on from 8.00 AM to 8.00 PM). Animals from the control group (standard environment, SE) were housed in standard laboratory cages ($33.5 \times 45 \times 21.5$ cm) with two animals per cage. For enriched environment (EE) housing, six animals at a time were housed in big cages ($46.5 \times 78 \times 95$ cm), containing four floors and several food hoppers, water bottles, running wheels, tubes, ramps and differently shaped objects (balls, ropes, stones) repositioned once a day and fully substituted once a week. Particular care was taken not to repeat cage arrangement and object availability during the experiments. Animals were caged in the EE immediately after ischemia. Although food and water were offered *ad libitum*, location of the hoppers and bottles was changed daily in order to stimulate exploratory conduct. Cages were cleaned once a week at the same time and by the same protocol to that used for standard cage cleaning. The body weight was weekly monitored, and no significant differences in this parameter were observed between animals housed in SE and EE. Experimental groups and the experimental design used in this study are depicted in Fig. 1.

Retinal ischemia

Animals were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (2 mg/kg) administered intraperitoneally. After topical instillation of proparacaine, the anterior chamber of each eye was cannulated with a 30-gauge needle connected to a pressurized bottle filled with sterile normal saline solution. Retinal ischemia was induced in one eye by increasing intraocular pressure to 120 mm Hg for exactly 40 min, as previously described (Fernandez et al., 2009a). With this maneuver, complete ocular ischemia was produced, characterized by cessation of flow in retinal vessels, determined by funduscopic examination. During and after (before animals were returned to the animal house) the experiments, animals were kept normothermic with heated blankets. The contralateral eye was submitted to a sham procedure (*i.e.* eyes were cannulated without raising IOP); this procedure did not affect retinal function and histology as compared to intact eyes. A few animals in which cataracts developed due to lens injury, were not used any further in the experiments.

Electroretinography

Electroretinographic activity was assessed at 1, 2, and 3 weeks after ischemia, as well as at 7 days after an intravitreal injection of vehicle or glutamate, as previously described (Fernandez et al., 2009a). Briefly, after 6 h of dark adaptation, rats were anesthetized under dim red illumination. Phenylephrine hydrochloride and tropicamide were

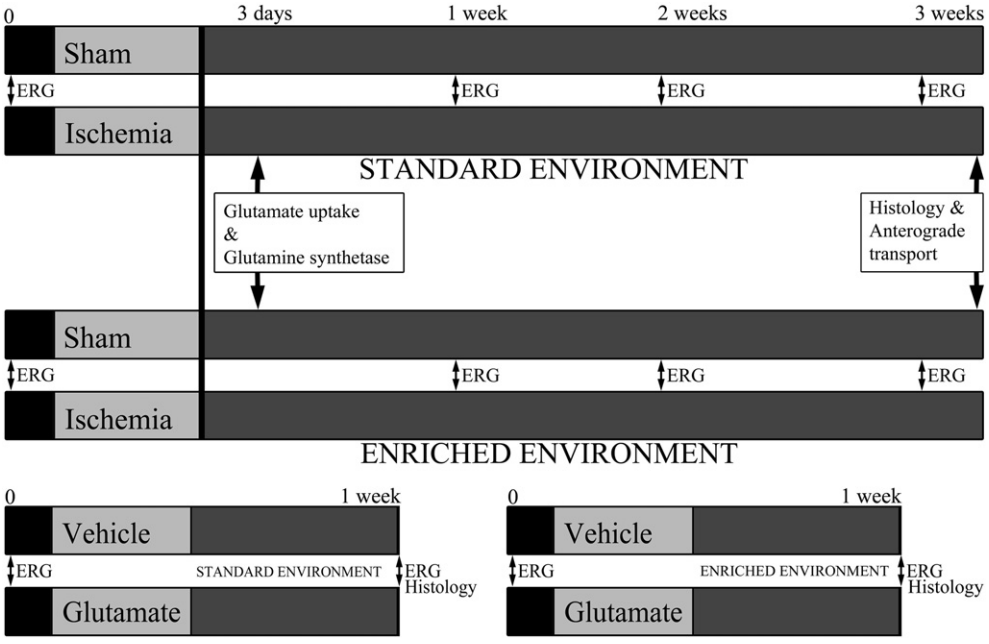

Fig. 1. Experimental groups. Animals were submitted to retinal ischemia, and caged in SE or EE immediately after ischemia. Electroretinograms were recorded at 1, 2, and 3 weeks after ischemia. Glutamate uptake and glutamine synthetase activity were assessed at 3 days post-ischemia, and histological and anterograde transport studies were performed at 3 weeks after ischemia. In another set of experiments, eyes were intravitreally injected with vehicle or glutamate, and animals were housed in SE or EE. In this case, functional and histological studies were performed at 1 week post-injection.

used to dilate the pupils, and the cornea was intermittently irrigated with balanced salt solution to maintain the baseline recording and to prevent keratopathy. Rats were placed facing the stimulus at a distance of 20 cm. All recordings were completed within 20 min and animals were kept warm during and after the procedure. A reference electrode was placed through the ear, a grounding electrode was attached to the tail, and a gold electrode was placed in contact with the central cornea. A 15 W red light was used to enable accurate electrode placement. This maneuver did not significantly affect dark adaptation and was switched off during the electrophysiological recordings. Scotopic electroretinograms (ERGs) were recorded from both eyes simultaneously and 10 responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a photic stimulator (light-emitting diodes) set at maximum brightness were amplified (9 cd s/m^2 without filter), filtered (1.5-Hz low-pass filter, 1000 high-pass filter, notch activated) and averaged (Akonic BIO-PC, Buenos Aires, Argentina). The a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Runs were repeated 3 times with 5 min-intervals to confirm consistency. Mean values from each eye were averaged, and the resultant mean value was used to compute the group means a- and b-wave amplitudes ± SE. Mean peak latencies and peak-to-peak amplitudes of the responses from each group of rats were compared.

Oscillatory potentials (OPs) were assessed as previously described (Moreno et al., 2005a). Briefly, the same photic stimulator with a 0.2 Hz frequency and filters of high (300 Hz) or low (100 Hz) frequency were used. The amplitudes of the OPs were estimated by measuring the heights from the baseline drawn between the troughs of successive wavelets to their peaks. The sum of three OPs was used for statistical analysis.

Histological evaluation

Three weeks after ischemia, rats were anesthetized and intracardially perfused with saline solution, followed by a fixative solution containing 4% formaldehyde in 0.1 mol/L PBS (pH 7.4). Then, the eyeballs and the brain were carefully removed and immersed for 24 h in the same fixative. After dehydration, eyes were embedded in paraffin wax and sectioned (5 μm) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured with a microscope (Eclipse E400, Nikon, Tokyo, Japan); 6-V halogen lamp, 20 W, equipped with a stabilized light source) and a camera (Coolpix s10; Nikon; Abingdon, VA, USA). Sections were stained with hematoxylin and eosin and were analyzed by masked observers. The average thickness (in μm) of the total retina, inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and photoreceptor outer segments (OS) were measured for each eye. Measurements (×400) were obtained at 1 mm dorsal and ventral from the optic disk. The number of cells in the ganglion cell layer (GCL) was counted along the whole retina section (×400). For each eye, results obtained from four separate sections were averaged, and the mean of 10 eyes was recorded as the representative value for each group.

Immunohistochemical studies

Antigen retrieval was performed by heating slices at 90 °C for 30 min in citrate buffer (pH 6.3). The following antibodies were used: a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody conjugated to Cy3 (1:1200; Sigma Chemical Co., St Louis, MO, USA), a goat anti-Brn3a antibody (1:500; Santa Cruz Biotechnology, Inc.), a goat anti-mouse IgM secondary antibody conjugated to Alexa 568 (1:500; Invitrogen, Molecular Probes) and a donkey anti-goat secondary antibody conjugated to Alexa 568 (1:500; Molecular Probes). Some sections were immersed in 0.1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) in 0.1 mol/L PBS for 10 min. Sections were preincubated with 5% normal horse serum for 1 h and then were incubated overnight at 4 °C with primary antibodies. After several washings, secondary antibodies were added, and sections were incubated for 2 h at room temperature. Regularly, some sections were treated without the primary antibodies to confirm specificity. Nuclei were stained and mounted with the fluorescent mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and observed under an epifluorescence microscope (BX50; Olympus, Tokyo, Japan) connected to a video camera (3CCD; Sony, Tokyo, Japan) attached to a computer running image analysis software (Image-Pro Plus; Media Cybernetics Inc., Bethesda, MD). Immunofluorescence studies were performed by analyzing comparative digital images from different samples obtained using identical exposure time, brightness, and contrast settings. The NIH ImageJ Software (National Institutes of Health, Bethesda, Maryland; http://imagej.nih.gov/ij/) was used to quantify GFAP intensity in identical rectangular areas of whole retinal sections (×200), as previously described (Haramati et al., 2011). For each eye, results obtained from four separate regions were averaged, and the mean of 5 eyes was recorded as the representative value for each group.

Morphometric analysis

All the obtained images were assembled and processed using Adobe Photoshop SC (Adobe Systems, San Jose, CA) to adjust the brightness and contrast. No other adjustments were made. For all morphometric image processing and analysis, digitalized captured TIFF images were transferred to ImageJ software version 1.42q (NIH, Bethesda, MD). All the nomenclature used herein follows that one of Paxinos and Watson (1997).

Terminal deoxynucleotidyltransferasedUTP nick end labeling (TUNEL) analysis

For DNA fragmentation of cells undergoing apoptosis, the ApopTag® Fluorecein *In Situ* Apoptosis Detection Kit (S7110, Chemicon, CA, USA) was used according to manufacturer's instructions. For each retinal section, the number of TUNEL(+) cells along the entire retina was calculated. For each eye, results obtained from four separate sections were averaged and the mean of five eyes was recorded as the representative value for each group.

Cholera toxin β-subunit transport studies

Rats were anesthetized, and a drop of proparacaine (0.5%) was topically administered for local anesthesia. Four microliters of a solution of 0.2% cholera toxin β-subunit (CTB) conjugated to Alexa 488 dye (Molecular Probes Inc., Eugene, OR) in 0.1 mol/L PBS (pH 7.4) were injected into the vitreous, using a 30-gauge Hamilton syringe (Hamilton, Reno, NV, USA). The injections were applied 1 mm from the limbus, and the needle was left in the eye for 1 min to prevent volume loss. Three days after injection, rats were anesthetized and intracardially perfused as previously described. Brains were carefully removed, postfixed overnight at 4 °C, and immersed in a graded series of sucrose solutions for cryoprotection; coronal sections (40 μm) were obtained using a freezing microtome. Nuclei were stained with the fluorescent dye DAPI, mounted with antifade medium, and viewed under an epifluorescence microscope as described.

Coronal sections (every other cut from the beginning to the end of the SC, approximately 40 sections) were used for the SC reconstruction using Matlab (The MathWorks Inc., Natick, MA). For each section, the retinorecipient SC was outlined using DAPI counterstaining, and the total retinotopic area was calculated. Digital images were converted to 8-bit grayscale, and the optic density of CTB staining was calculated. The total length was measured and divided into bins

(4 μm), from the medial to lateral region. The CTB density was obtained by dividing the total pixel area by CTB + pixels. Finally, a colorimetric thermal representation was applied (from 0% = blue to 100% = red). The number of sections and the thickness (2×) were used for a final reconstruction of the retinal projection to the SC.

Intravitreal injections

Animals were anesthetized as previously described. A drop of proparacaine was administered in each eye for local anesthesia. With a Hamilton syringe (Hamilton, Reno, NV, USA) and a 30-gauge needle, 4 μL of 0.3 M glutamate (estimated final concentration 20 mM, considering a vitreous volume of 60 μL (Sumioka et al., 2000) in sterile pyrogen-free saline was injected into one eye of anesthetized rats, while an equal volume of vehicle (saline solution) was injected in the fellow eye. Injections were applied at 1 mm of the limbus and the needle was left in the eye for 60 s; this small volume prevented the increase in intraocular pressure and volume loss. The dose of glutamate was selected on the basis of a previous report (Fernandez et al., 2009a). Animals were housed either in SE or EE immediately after they recovered from anesthesia.

L-[^{3}H]-glutamate uptake assessment

The influx of L-[^{3}H]-glutamate was assessed in a crude synaptosomal fraction of rat retinas, as previously described (Moreno et al., 2005b). Retinas were homogenized (1:9 w/v) in 0.32 M sucrose containing 1 mM $MgCl_2$, and centrifuged at 900 *g* for 10 min at 4 °C. Nuclei-free homogenates were further centrifuged at 30,000 *g* for 20 min. The pellet was immediately resuspended in buffer HEPES–Tris, containing 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (adjusted to pH 7.4 with Tris base), and aliquots (100–300 μg protein/100 μL) were incubated with 100 μL of L-[^{3}H]-glutamate (500,000–800,000 dpm/tube, specific activity: 17.25 Ci/mmol) and 10 μM glutamate. After 5 min, amino acid uptake was terminated by adding 4 mL of ice cold HEPES–Tris buffer. The mixture was immediately poured onto Whatman GF/B filters (Whatman Inc., Piscataway, NJ, USA) under vacuum. The filters were washed twice with 4 mL aliquots of ice-cold buffer and the radioactivity on the filters was counted in a liquid scintillation counter. Non-specific uptake of L-[^{3}H]-glutamate into synaptosomes was assessed by adding an excess of glutamate (10 mM).

Glutamine synthetase activity assay

Each retina was homogenized in 200 μL of 10 mM potassium phosphate, pH 7.2. Glutamine synthetase activity was assessed as described (Moreno et al., 2005b). Reaction mixtures contained 150 μL of retinal homogenates and 150 μL of a stock solution (100 mM imidazole-HCl buffer, 40 mM $MgCl_2$, 50 mM β-mercaptoethanol, 20 mM ATP, 100 mM glutamate, and 200 mM hydroxylamine, adjusted to pH 7.2). Tubes were incubated for 15 min at 37 °C. The reaction was stopped by adding 0.6 mL of ferric chloride reagent (0.37 M $FeCl_3$, 0.67 M HCl, and 0.20 M trichloroacetic acid). Samples were placed for 5 min on ice. Precipitated proteins were removed by centrifugation, and the absorbance of the supernatants was read at 535 nm against a reagent blank. Under these conditions, 1 μmol of γ-glutamylhydroxamic acid gave an absorbance of 0.340. Glutamine synthetase specific activity was expressed as μmoles of γ-glutamylhydroxamate per hour per milligram of protein.

Protein level assessment

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis of results was made by a two-way analysis of variance (ANOVA) followed by a Tukey's test, as stated.

Results

Fig. 2 depicts the effect of EE housing for 1, 2, or 3 weeks after 40-min ischemia on the retinal dysfunction induced by I/R. The average amplitudes of ERG a- and b-waves and OPs in non-ischemic eyes or 1, 2, and 3 weeks after 40-min ischemia from animals housed in SE or EE, as well as representative scotopic ERG traces from rats submitted to these treatments are shown in Fig. 2. In SE-housed animals, ischemia for 40 min and reperfusion for 1, 2, or 3 weeks induced a significant decrease in ERG a- and b-wave amplitudes, while their latencies remained unchanged (data not shown). The ERG a-wave amplitude was completely restored by EE at 2 and 3 weeks of housing, and the b-wave amplitude was partly recovered at 1 and 2 weeks after ischemia, and completely recovered at 3 weeks post-ischemia. A similar profile was observed for OPs, as shown in Fig. 2. No significant differences were observed between non-ischemic eyes from animals kept in SE and from those housed in EE for 3 weeks. The effect of EE housing on retinal histological alterations induced by 40-min ischemia was examined. Fig. 3 shows a representative photomicrograph of non-ischemic retinas from animals housed in SE (C + SE, detail in A) or EE (C + EE, detail in B). In the retina from animal housed in SE for 3 weeks after 40-min ischemia (I + SE, detail in C), typical histopathological features of ischemic damage were observed, showing marked reduction in the total retinal thickness and frequent formation of folds in the ONL with loss of photoreceptors. In animals housed in EE for 3 weeks after ischemia, the retinal structure was notably preserved (I + EE, detail in D). Apoptotic cell death was evaluated by the TUNEL assay. A significant increase of TUNEL(+) cell number in the GCL was observed in ischemic retinas from animals housed in SE, whereas EE housing significantly decreased the number of apoptotic cells (Fig. 3). No TUNEL(+) cells were observed in non-ischemic retinas from animal housed in SE or EE or in other retinal layers from ischemic retinas in SE or EE housed animals. In animals housed in SE, I/R provoked a significant decrease in total retina, IPL, OPL, ONL, and OS thickness, and ganglion cell layer (GCL) cell number, whereas EE housing for 3 weeks significantly preserved these parameters (Table 1). In order to further analyze the morphological protection induced by EE housing, retinal Brn3a (a specific marker of retinal ganglion cells (RGCs), and GFAP levels were analyzed, as shown in Fig. 4. In SE-housed animals, ischemia induced a significant decrease in Brn3a(+) cell number, whereas EE housing reversed the effect of ischemia on this parameter. In non-ischemic retinas from animals housed in SE or EE, astrocytes localized in the nerve fiber layer and GCL were weakly GFAP-immunopositive, whereas 3 weeks after 40-min ischemia, a significant increase in retinal GFAP levels in astrocytes and spread to glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells was observed in animals housed in SE. EE housing decreased the levels of GFAP in Müller cells. Brn3a and GFAP immunoreactivity was similar in non-ischemic eyes from animals housed in SE and EE.

The active anterograde transport of RGC projections to the SC was analyzed using CTB (Fig. 5). At the optic chiasm, most optic nerve axons from each eye meet, and cross the midline and project to the contralateral SC. In the SC innervated by a non-ischemic eye, CTB labeled the entire retinotopic projections to the stratum zonale (SZ) and the stratum griseum superficiale (SGS). A clear reduction in CTB-staining was observed in the central and temporal regions of the contralateral SC to an ischemic eye from animals housed in SE; in the central region, areas with virtually none CTB-staining were observed, whereas in animals housed in EE, the CTB anterograde

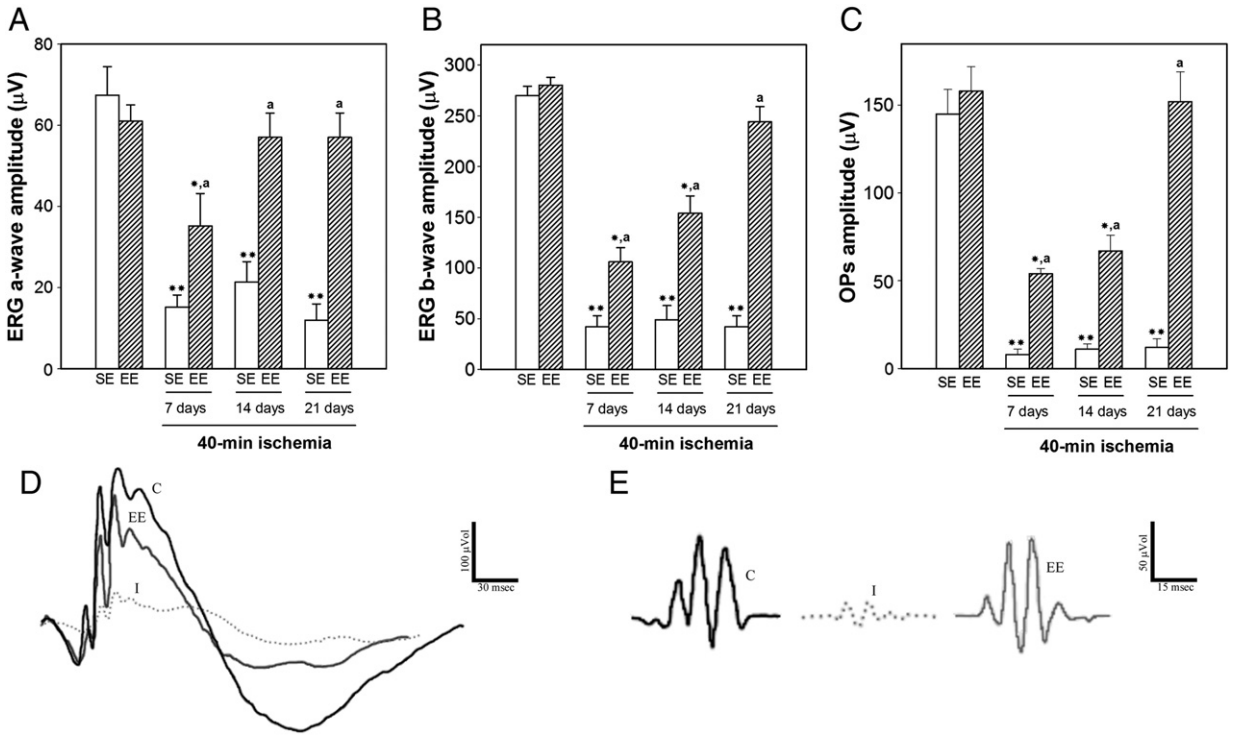

Fig. 2. Effect of EE housing on the retinal dysfunction induced by I/R. Upper panel: average amplitudes of scotopic ERG a-wave, b-wave, and OPs. A significant decrease in the amplitudes of ERG a-wave, b-wave, and OPs was observed in animals submitted to 40-min ischemia and housed in SE, whereas EE housing significantly reversed the effect of I/R on these parameters. Data are mean ± SE (n: 10 eyes/group). * $p<0.05$, and ** $p<0.01$ *vs.* non-ischemic eyes in SE, a: $p<0.01$ *vs.* ischemic eyes in SE, by Tukey's test. Lower panel: Representative scotopic ERG and OP traces of control and ischemic eyes from animals housed in SE or EE for 3 weeks. C + SE: control in SE, I + SE: ischemia + SE, I + EE: ischemia + EE.

transport was notably preserved. CTB-staining in the contralateral SC to a non-ischemic eye was similar in animals housed in SE and EE.

In order to analyze the influence of EE housing on retinal glutamate recycling, retinal glutamate influx and glutamine synthetase activity were assessed at 3 days after ischemia in animals housed in SE or EE. As shown in Fig. 6, these parameters significantly decreased in ischemic eyes from animals housed in SE as compared with non-ischemic eyes. EE housing, which did not affect these parameters in non-ischemic eyes, significantly reversed the effect of ischemia.

The effect of an intravitreal injection of glutamate on scotopic ERG from animals housed in SE or EE is shown in the upper panel of Fig. 7. The injection of glutamate induced a significant decrease in the ERG a-wave, b-wave, and OP amplitude (but not their latencies) in animals housed in SE, whereas EE housing for 7 days after glutamate injection significantly abrogated the effect of glutamate. The effect of an intravitreal injection of glutamate on retinal histology is shown in the lower panel of Fig. 7 and Table 1. Seven days after injection, glutamate induced clear alterations in the retinal structure from animals housed in SE (G + SE), as compared with vehicle-injected eyes (C + SE), whereas EE housing (G + EE) preserved the retinal structure from glutamate-induced damage. As shown in Table 1, glutamate injection induced a significant reduction in the total retina and IPL thickness, and GCL cell number in animals housed in SE, as compared with vehicle injected eyes, whereas EE housing reversed the effect of glutamate. Glutamate induced a significant decrease in Brn3a(+) cell number and an increase in GFAP Müller cell levels, whereas EE housing reversed these alterations (Fig. 8).

Discussion

For the first time, the foregoing results indicate that a non-invasive, and biologically meaningful stimulation of the sensory pathway, such as that induced by EE housing, reversed retinal functional and histological damage induced by I/R in adult rats. Other manipulations such as ischemic pre- and post-conditioning are able to induce a highly robust protection against retinal ischemic damage (Fernandez et al., 2009b; Roth et al., 1998). However, despite their beneficial effects, due significance should be given to the fact that in these cases, the eye, a very delicate and crucial structure is being manipulated by inducing brief ischemic events. As shown herein, EE housing, which does not need any direct manipulation of the eye, was able to significantly restore retinal function and histology after a deleterious ischemic event. Moreover, in spite of the high effectiveness of ischemic pre-conditioning for retinal protection, its utilization as a clinical strategy is mostly limited because the onset of retinal ischemia is largely unpredictable, in contrast to the onset of reperfusion which could be more predictable. In that context, the beneficial effects of post-ischemic environmental enrichment could be clinically translatable to patients who have experienced retinal ischemia.

In transient retinal ischemia, the nature of neurodegeneration occurs in a delayed fashion (Fernandez et al., 2009a), suggesting the possibility that early intervention may be able to mitigate neuronal cell death. Compared to non-ischemic values, the ERG a-wave, b-wave, and OP amplitudes were strongly reduced at 1, 2, and 3 weeks after 40-min ischemia. Despite limitations of our method for ERG assessment due to using a single intensity recordings (lack of intensity response plot, risk of artifact and error), EE housing, which showed no effect *per se* on retinal function, significantly reversed the effect of ischemia on the ERG.

The functional protection induced by EE seems to be time of exposure-dependent, since the recovery was partial at 1 or 2 weeks and complete at 3 weeks of EE housing. In addition, a clear preservation of retinal structure was observed in ischemic retinas from animals housed in EE for 3 weeks after ischemia. The a-wave of the flash ERG is classically thought to represent photoreceptor activity, whereas the b-wave reflects bipolar and Müller cell function. As for the OPs, their origin has not been definitively determined, but OPs are thought to originate from feedback neural pathways in the inner retina, especially around the IPL and mainly from amacrine cells,

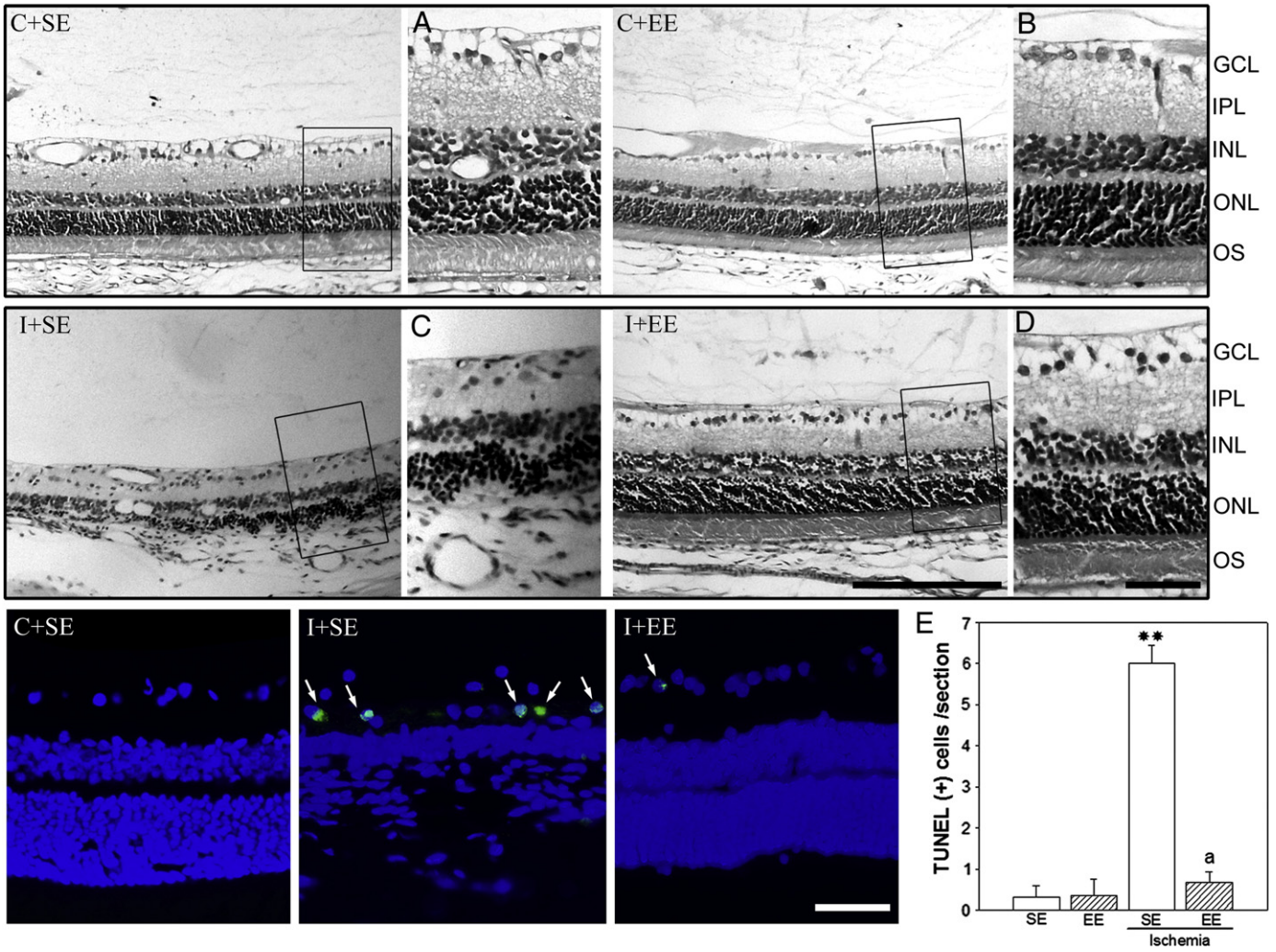

Fig. 3. Effect of EE housing on retinal structure. Representative photomicrographs showing histological appearance of non-ischemic retinas from animals housed in SE (C + SE, detail in A) or EE (C + EE, detail in B), and ischemic retinas from animals housed in SE (I + SE, detail in C), or EE (I + EE, detail in D). Severe retinal damage is shown in the retina from eyes submitted to ischemia in SE, whereas in animals housed in EE for 3 weeks after ischemia, the retinal structure was notably preserved. Scale bar = 200 μm and 50 μm (for details). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer, OS, outer segment of photoreceptors. Lower panel: TUNEL assessment after 3 weeks of retinal ischemia. The number of TUNEL(+) cells in the GCL was significantly higher in ischemic retinas from animals housed in SE than in non-ischemic eyes (in SE or EE), whereas EE significantly decreased this parameter in ischemic eyes. Scale bar = 50 μm. Data are mean ± SEM (n = 5 eyes per group), **p<0.01 *versus* control eyes from animals caged in SE, a: p<0.01, *versus* ischemic eyes from animals housed in SE, by Tukey's test.

although RGCs and bipolar cells may contribute to some parts of the OPs (Wachtmeister, 1998). Therefore, it was not surprising that EE housing which significantly reversed the ischemia-induced decrease in ERG a-wave, b-wave, and OP amplitudes, also reversed the effect of ischemia on OPL, ONL, and OS thickness. The structural preservation induced by EE was confirmed by the analysis of Brn3a and GFAP levels. Brn3a is a POU domain transcription factor that is specifically expressed in RGC nuclei (Quina et al., 2005). In SE-housed animals, ischemia provoked a significant loss of RGCs, whereas exposure to EE significantly preserved Brn3a(+) cell number. GFAP is an intermediate filament that is normally expressed in astrocytes but not in retinal Müller cells (Sarthy, 2007). However, in a variety of retinal

Table 1
Histological analysis of retinas from control and eyes exposed to ischemia or glutamate injection light from animals housed in SE or EE.

	Total retina	IPL	INL	OPL	ONL	OS	GCL cell count
Control + SE	165.6 ± 7.9	37.0 ± 3.4	23.5 ± 0.8	9.0 ± 0.6	42.1 ± 0.7	29.6 ± 1.5	460.2 ± 9.2
Control + EE	173.1 ± 6.0	36.4 ± 1.5	23.9 ± 1.9	8.5 ± 0.8	42.4 ± 2.7	31.8 ± 1.7	467.4 ± 17.7
Ischemia + SE	85.4 ± 17.5**	24.5 ± 4.1*	18.0 ± 2.9	3.0 ± 1.0**	12.3 ± 5.1**	7.7 ± 3.0**	265.4 ± 8.5**
Ischemia + EE	160.6 ± 8.9[a]	32.3 ± 1.9	23.5 ± 1.3	9.4 ± 0.5[a]	42.0 ± 2.0 [a]	28.4 ± 2.0[a]	498.5 ± 11.5[a]
Glutamate + SE	126.1 ± 4.5**	19.4 ± 1.5**	18.9 ± 1.0	10.5 ± 0.6	40.8 ± 1.9	28.1 ± 1.7	266.3 ± 6.0**
Glutamate + EE	159.5 ± 6.0[b]	28.8 ± 0.4*,[c]	23.3 ± 1.2	10.8 ± 0.6	45.7 ± 1.6	32.9 ± 1.7	497.0 ± 23.1[b]

Total retinal and retinal layer thicknesses (in μm) in control eyes and eyes exposed to 40-min ischemia or glutamate injection from animals housed in SE or EE. In SE-housed animals, ischemia provoked a significant decrease in total retina, IPL, OPL, ONL, and OS thickness, and GCL cell number, while glutamate injection induced a significant decrease total retina and IPL thickness and GCL cell count. EE housing significantly reversed the effect of ischemia and glutamate. Data are mean ± SEM (n = 10 eyes per group). *p<0.05, **p<0.01 *vs.* control eyes (in SE), [a]: p<0.01 *vs.* ischemia in SE-housed animals, [b]: p<0.01 and [c]: p<0.05 *vs.* glutamate in SE-housed animals, by Tukey's test. OS, photoreceptor outer and inner segment; OPL, outer plexiform layer; ONL, outer nuclear layer; IPL, inner plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

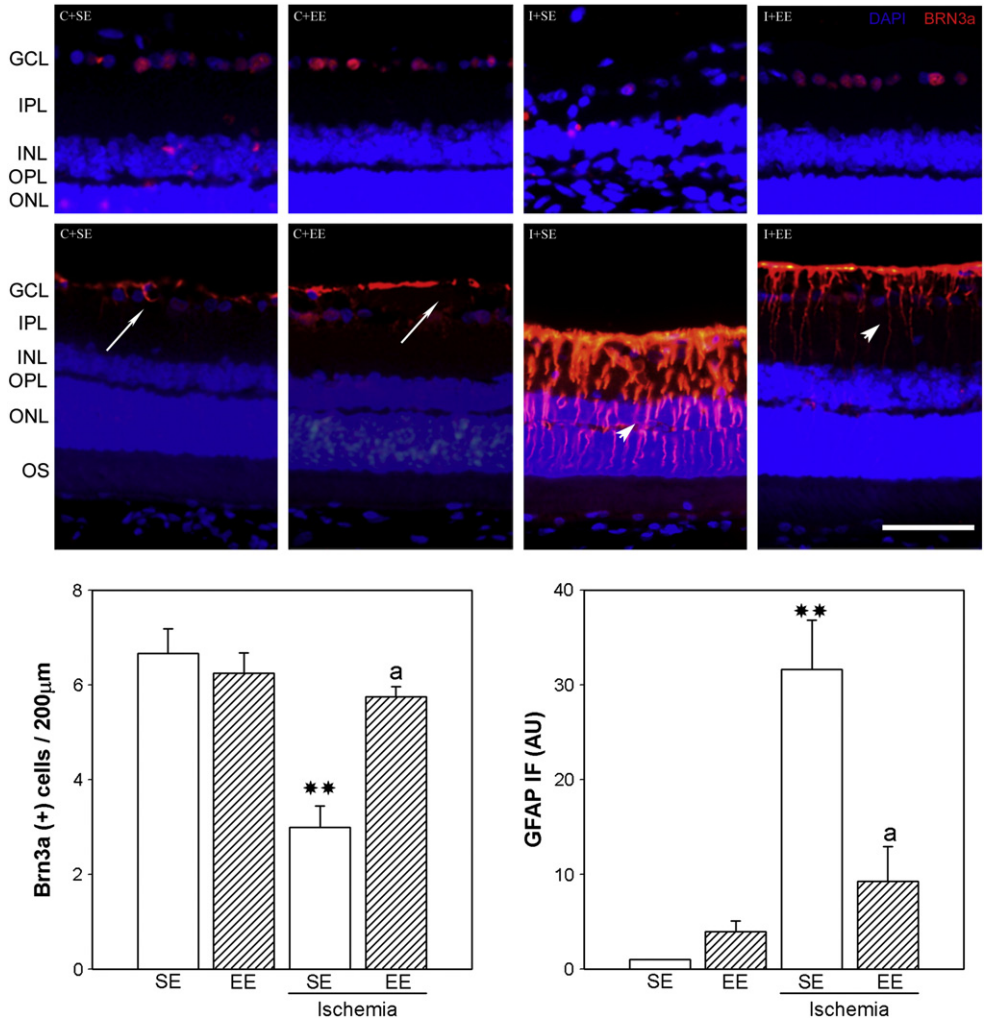

Fig. 4. Immunohistochemical detection of Brn3a and GFAP in control eyes and eyes submitted to ischemia from animals housed in SE or EE for 3 weeks after ischemia. Upper panel: The presence of Brn3a was confined to the GCL in all experimental groups. A decrease in Brn3a(+) cell number was observed in ischemic eyes from SE-housed animals (I+SE) as compared with non-ischemic eyes (in SE or EE), whereas EE housing (I+EE) preserved this parameter in ischemic eyes. In control eyes from animals housed in SE or EE, GFAP(+) immunoreactivity was observed in astrocytes (arrow), whereas in ischemic retinas from animals housed in SE, an intense GFAP(+) immunoreactivity was observed in Müller cell bodies and their processes (arrow head). EE housing reduced GFAP immunoreactivity in ischemic retinas, showing only few GFAP(+) Müller cells. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar = 50 µm. Lower panel, left: The number of Brn3a positive cells was significantly lower in ischemic eyes from animals housed in SE than in non-ischemic eyes (in SE or EE), whereas EE housing reversed the decrease in Brn3a(+) cell number induced by ischemia. Lower panel, right: The intensity of GFAP immunoreactivity in Müller cells was significantly increased in ischemic eyes from animals housed in SE, whereas in ischemic eyes from animals housed in EE, a reversion of the increase in Müller cell GFAP immunoreactivity induced by ischemia was observed. Data are mean ± SEM (n = 5 eyes per group), **$p<0.01$ *versus* control eyes from animals in SE, a: $p<0.01$, *versus* ischemic eyes from animals in SE, by Tukey's test.

injuries, including retinal ischemia (Barnett and Osborne, 1995; Fernandez et al., 2009a), GFAP expression in Müller cells became evident. Therefore, GFAP expression in Müller cells is widely used as a molecular indicator for retinal stress. In agreement, 40-min ischemia provoked a significant alteration in Müller cells (as shown by an increase in GFAP immunoreactivity), whereas EE reduced the reactive expression of GFAP in Müller cells. CTB tracing studies were performed in order to visualize surviving RGCs which remain connected with the SC (its primary projection site) after ischemia. In animals housed in SE, ischemia provoked a "misconnection" between the retina and the SC, whereas exposure to EE restored the deficit in the anterograde transport from the retina to the SC.

In animals housed in SE, a significant increase in the number of apoptotic cells was observed in the GCL from eyes submitted to ischemia, whereas EE housing decreased the number of apoptotic cells in ischemic retinas. At present, we cannot formally exclude the possibility that, in addition to a decrease in the number of apoptotic cells, EE housing also increased cell proliferation. However, since a panretinal protection (as shown by ERG and histological analysis), as well as a clear preservation of the active anterograde transport of RGC projections to the SC was observed in animals housed in EE, different cellular type proliferation, and RCG axon replacement seem unlikely.

The precise mechanisms responsible for the retinal protection against I/R damage induced by EE remain to be established. High levels of glutamate can be toxic to retinal cells. The retinal glutamate/glutamine cycle efficiently prevents excessive accumulation of glutamate in the interstitium which, if unchecked, would induce excitotoxic neurodegeneration. This pathway involves the synaptic release of glutamate from neurons, rapid and efficient glutamate uptake from the synaptic space by glia (astrocytes and Müller cells), conversion of glutamate to glutamine by glutamine synthetase (exclusively expressed in Müller cells) (Grossman et al., 1994), followed by release of glutamine to the interstitium and uptake by the neurons for conversion to glutamate (Poitry et al., 2000; Thoreson and Witkovsky, 1999). In this way, the neurotransmitter pool is replenished and glutamate neurotoxicity is prevented. Based on the well-established link between excitotoxicity and retinal ischemic damage, we hypothesized that the protective effect of EE housing

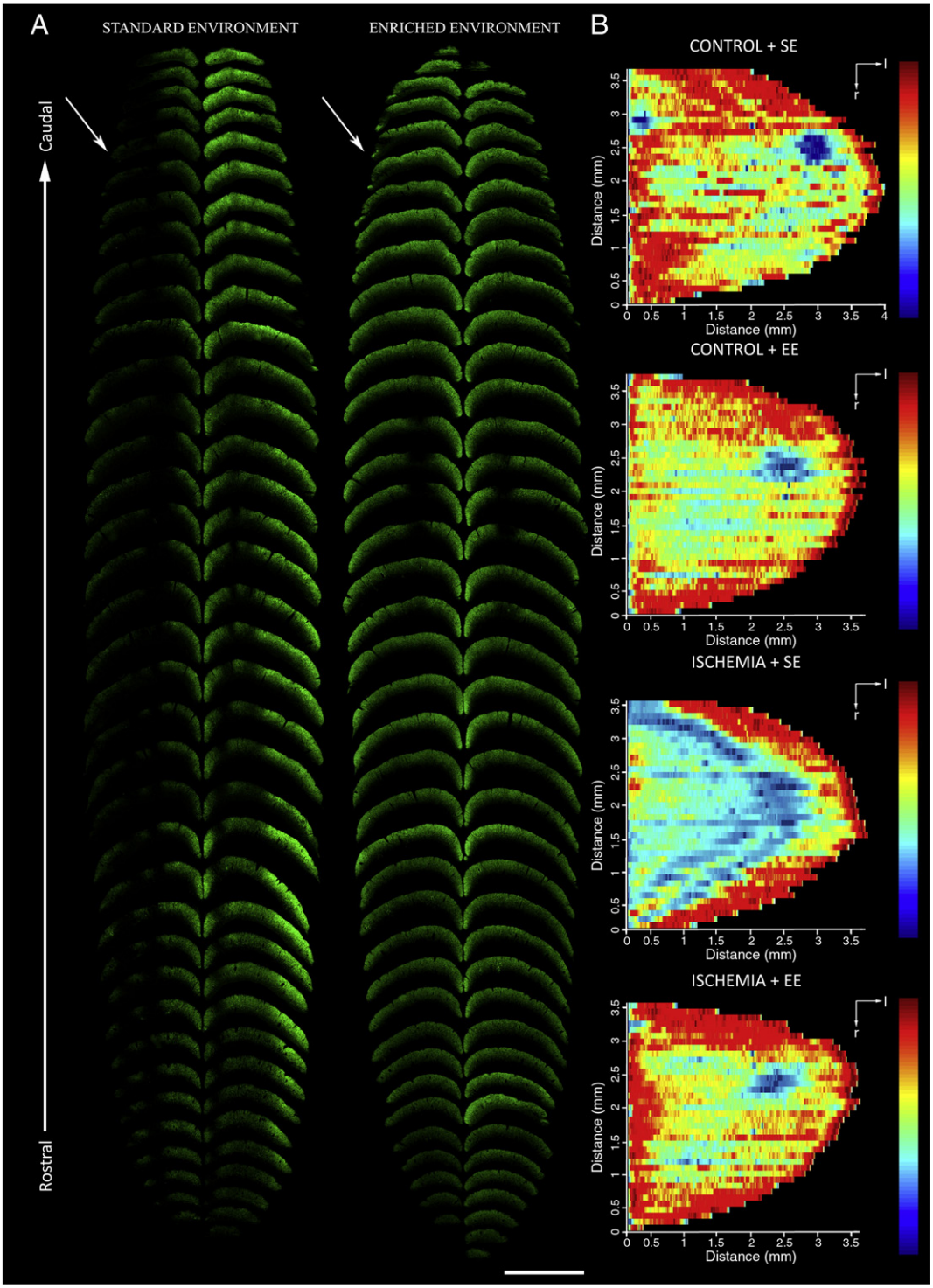

Fig. 5. CTB tracing studies. Retinal terminal field in the SC from animals submitted to ischemia in one eye and a sham procedure in the contralateral eye and housed in SE or EE for 3 weeks after ischemia. Panel A: Photomicrographs showing the CTB-staining pattern in the superficial layers of the SC from an animal housed in SE (left) or EE (right). Representative dorsal views of retinotopic SC map reconstruction corresponding to control eyes from animals housed in SE or EE or to ischemic eyes from animals housed in SE or EE are shown. In the contralateral SC to the eye submitted to a sham procedure, no signs of alterations were observed for SE- or EE-housed animals. In the contralateral SC to an ischemic eye (arrow) from an animal housed in SE, a clear reduction in the density of retinal terminals and zones of none CTB-staining was found, whereas EE housing preserved retinal terminal field in the contralateral SC to an ischemic eye. Shown are image representative of five animals per group. Scale bar = 2 mm.

could be a glutamate-dependent process. To test this hypothesis, retinal glutamate uptake and glutamine synthetase activity, two key processes involved in the regulation of glutamate synaptic concentrations, were assessed in ischemic retinas from animals housed in SE or EE for 3 days after ischemia. In agreement with previous results (Fernandez et al., 2009a), retinal glutamate uptake and glutamine synthetase activity significantly decreased in ischemic eyes from animals housed in SE, whereas EE housing significantly avoided the decrease in these parameters. Notably, EE housing *per se* (in non-ischemic retinas) did not change glutamate uptake and glutamine synthetase activity, which is in agreement with the fact that this maneuver *per se* did not affect retinal function and histology. These

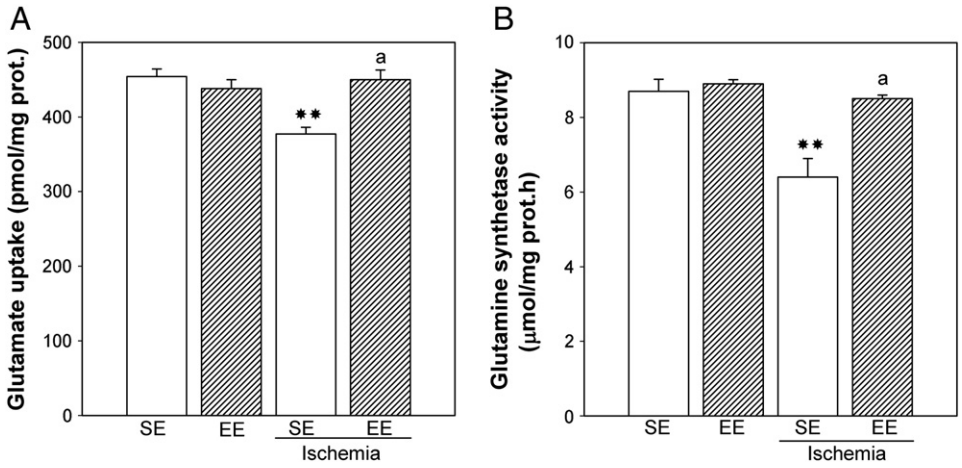

Fig. 6. Effect of SE or EE housing on retinal glutamate uptake and glutamine synthetase activity. Three days after ischemia, a significant decrease of glutamate uptake (panel A) and glutamine synthetase activity (panel B) was observed in ischemic retinas from animals housed in SE, whereas EE, which showed no effect *per se* in non-ischemic eyes, significantly reversed the effect of ischemia on these parameters. Data are mean ± SEM (n = 10–12 animals per group). ** $p<0.01$ *vs.* non-ischemic retinas in SE; a: $p<0.01$ *vs.* ischemic retinas in SE, by Tukey's test.

results support that ischemic damage could be mediated by an increase in retinal synaptic glutamate concentrations and that the recovery induced by EE housing could involve a normalization of glutamatergic transmission. Two necessary (albeit not sufficient) conditions are needed to confirm this hypothesis: i) retinal damage induced by high levels of glutamate should be similar to that provoked by I/R, and ii) EE housing should protect retinal function and histology from glutamate toxicity. Although at the dose used herein,

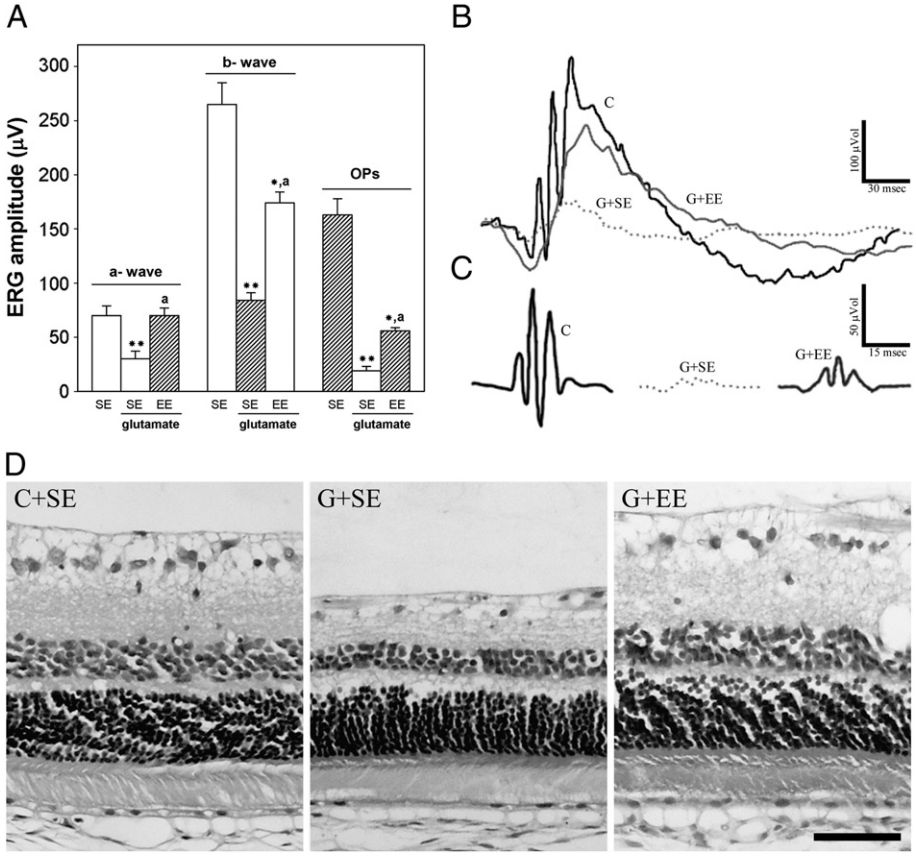

Fig. 7. Effect of an intravitreal injection of glutamate on retinal function and histology. Eyes were intravitreally injected with vehicle or glutamate and animals were housed in SE or EE for 7 days after injections. In SE-housed animals, glutamate induced a significant decrease in the ERG a- and b-wave, and OP amplitudes, whereas EE housing significantly reversed the effect of glutamate. Panel A: Average amplitudes of scotopic ERG a-wave, b-wave, and OPs; Panel B: Representative ERG traces, Panel C: Representative OP traces. Shown are means ± SEM (n = 10 animals/group). * $p<0.05$ and ** $p<0.01$ *vs.* control eyes in SE-housed animals; a: $p<0.01$ *vs.* glutamate-injected eyes from animal housed in SE, by Tukey's test. Lower panel: Representative micrographs of hematoxylin and eosin stained sections of retinas 7 days after injection of vehicle (C + SE) or glutamate (G + SE) in animals housed in SE, and eyes injected with glutamate from animals housed in EE (G + EE). Glutamate induced a decrease in total retinal and the inner plexiform layer thickness and a decrease in GCL cell number, whereas EE housing preserved retinal morphology from glutamate damage. Scale bar = 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer, OS, outer segment of photoreceptors.

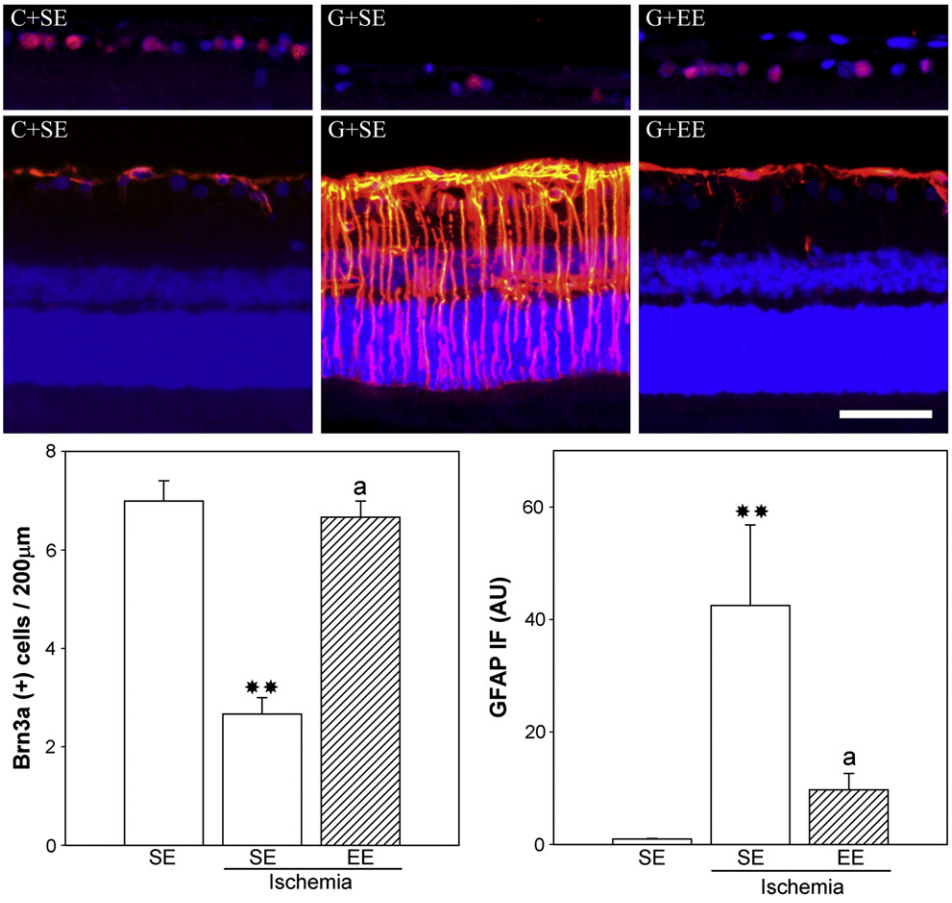

Fig. 8. Effect of glutamate injection on retinal Brn3a and GFAP levels. Upper panel: Immunohistochemistry for Brn3a after the following treatments: vehicle (C + SE), and glutamate in SE (G + SE) or in EE (G + EE). Glutamate decreased Brn3a(+) cell number in SE but not in EE. Lower panel: Immunohistochemistry for GFAP in the same experimental groups. In glutamate-injected eyes from SE-housed animals, GFAP-immunoreactivity was observed in astrocytes and Müller cells, throughout the plexiform layers. EE housing reduced GFAP immunoreactivity, showing only few positive Müller cells. Scale bar = 50 µm. Lower panel, left: The number of Brn3a positive cells was significantly lower in glutamate-injected eyes from animals housed in SE than in vehicle-injected eyes, whereas EE housing reversed the decrease in Brn3a(+) cell number induced by glutamate. Lower panel, right: The intensity of GFAP immunoreactivity in Müller cells was significantly increased in glutamate-injected eyes from animals housed in SE, whereas in glutamate-injected eyes from animals housed in EE, a reversion of the increase in Müller cell GFAP immunoreactivity induced by glutamate was observed. Data are mean ± SEM (n = 5 eyes per group), **$p<0.01$ *versus* vehicle-injected eyes from animals in SE, a: $p<0.01$, *versus* glutamate-injected eyes from animals housed in SE, by Tukey's test.

glutamate injection caused greater functional damage and lesser histological injury than I/R, both conditions were partly met. Supraphysiological concentrations of glutamate induced functional (ERG a- and b-wave, and OP amplitudes), and histological damage similar to that provoked by ischemia, and EE housing protected the retina from the deleterious effects of high concentrations of glutamate. In agreement, it was previously shown that at early postnatal and pre-weaning age, EE is able to ameliorate the severe retinal degeneration induced by neonatal glutamate treatment (Szabadfi et al., 2009). In addition, several reports show that the neural plasticity seen after EE housing is mediated by glutamate signaling (Andin et al., 2007; Li et al., 2007).

A remarkable feature of neurons is their ability to modify their function and organization in response to changes in their inputs. This ability for neuronal plasticity is particularly robust and widespread during development, but can extend into adulthood under certain circumstances and to a more limited extent. Compared to the developing visual system, where neuronal plasticity has been well characterized at multiple levels (Berardi et al., 2000; Morishita and Hensch, 2008), little is known about retinal plasticity in the adult. Since the illumination level was held constant for all conditions, it seems unlikely that this parameter could account for differences across experimental groups. However, the increased number and novelty of objects in the visual field may have increased the occurrence of edges or other high contrast visual features, which would increase retinal information processing. The EE is visually complex in comparison with typical laboratory home cages. Thus, it is tempting to speculate that the number and diversity of stimuli in the visual environment (contrast, spatial frequency, and the widened area of the accessible environment) invoked mechanisms of plasticity within the adult retina, involving the main retinal neurotransmitter (*i.e.* glutamate).

Conclusions

The present results support that post-ischemic EE housing significantly protected the adult retina from ischemic damage. This phenomenon seems to be a post-developmental response which demonstrates a remarkable retinal plasticity. The mechanism underlying the ability of this adaptation/flexibility could involve the regulation of the principal retinal neurotransmitter clearance that encompasses many aspects of retinal pathophysiology. Moreover, since EE housing was able to ameliorate the retinal toxic effects of glutamate, EE housing could have promise for application in retinal diseases which involves glutamate-mediated retinal cell death. In this vein, these results may provide new insight into the development of therapeutic approaches to treat retinal degeneration.

Disclosures

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References

Andin, J., Hallbeck, M., Mohammed, A.H., Marcusson, J., 2007. Influence of environmental enrichment on steady-state mRNA levels for EAAC1, AMPA1 and NMDA2A receptor subunits in rat hippocampus. Brain Res. 1174, 18–27.

Barnett, N.L., Osborne, N.N., 1995. Prolonged bilateral carotid artery occlusion induces electrophysiological and immunohistochemical changes to the rat retina without causing histological damage. Exp. Eye Res. 61, 83–90.

Belayev, A., Saul, I., Liu, Y., Zhao, W., Ginsberg, M.D., Valdes, M.A., Busto, R., Belayev, L., 2003. Enriched environment delays the onset of hippocampal damage after global cerebral ischemia in rats. Brain Res. 964, 121–127.

Berardi, N., Pizzorusso, T., Maffei, L., 2000. Critical periods during sensory development. Curr. Opin. Neurobiol. 10, 138–145.

Biernaskie, J., Corbett, D., 2001. Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury. J. Neurosci. 21, 5272–5280.

Briones, T.L., Therrien, B., Metzger, B., 2000. Effects of environment on enhancing functional plasticity following cerebral ischemia. Biol. Res. Nurs. 1, 299–309.

Cazevieille, C., Osborne, N.N., 1997. Retinal neurones containing kainate receptors are influenced by exogenous kainate and ischaemia while neurones lacking these receptors are not: melatonin counteracts the effects of ischaemia and kainate. Brain Res. 755, 91–100.

Fernandez, D.C., Chianelli, M.S., Rosenstein, R.E., 2009a. Involvement of glutamate in retinal protection against ischemia/reperfusion damage induced by post-conditioning. J. Neurochem. 111, 488–498.

Fernandez, D.C., Bordone, M.P., Chianelli, M.S., Rosenstein, R.E., 2009b. Retinal neuroprotection against ischemia-reperfusion damage induced by postconditioning. Invest. Ophthalmol. Vis. Sci. 50, 3922–3930.

Franklin, T.B., Murphy, J.A., Myers, T.L., Clarke, D.B., Currie, R.W., 2006. Enriched environment during adolescence changes brain-derived neurotrophic factor and TrkB levels in the rat visual system but does not offer neuroprotection to retinal ganglion cells following axotomy. Brain Res. 1095, 1–11.

Grossman, R., Fox, L.E., Gorovits, R., Ben-Dror, I., Reisfeld, S., Vardimon, L., 1994. Molecular basis for differential expression of glutamine synthetase in retina glia and neurons. Brain Res. Mol. Brain Res. 21, 312–320.

Haramati, S., Navon, I., Issler, O., Ezra-Nevo, E., Gil, S., Zwang, R., Hornstein, E., Chen, A., 2011. microRNA as repressors of stress-induced anxiety: the case of amygdalar miR-34. J. Neurosci. 31, 14191–14203.

Ikeda, H., Hankins, M.W., Asai, T., Dawes, E.A., 1992. Electro-physiological properties of neurones following mild and acute retinal ischaemia. Exp. Eye Res. 55, 435–442.

Iversen, L.L., 1991. Pharmacological approaches to the treatment of ischaemic neuronal damage. Eye 5, 193–197.

Landi, S., Sale, A., Berardi, N., Viegi, A., Maffei, L., Cenni, M.C., 2007. Retinal functional development is sensitive to environmental enrichment: a role for BDNF. FASEB J. 21, 130–139.

Li, C., Niu, W., Jiang, C.H., Hu, Y., 2007. Effects of enriched environment on gene expression and signal pathways in cortex of hippocampal CA1 specific NMDAR1 knockout mice. Brain Res. Bull. 71, 568–577.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Moreno, M.C., Marcos, H.J., Oscar Croxatto, J., Sande, P.H., Campanelli, J., Jaliffa, C.O., Benozzi, J., Rosenstein, R.E., 2005a. A new experimental model of glaucoma in rats through intracameral injections of hyaluronic acid. Exp. Eye Res. 81, 71–80.

Moreno, M.C., Sande, P., Marcos, H.A., de Zavalía, N., Keller Sarmiento, M.I., Rosenstein, R.E., 2005b. Effect of glaucoma on the retinal glutamate/glutamine cycle activity. FASEB J. 19, 1161–1162.

Morishita, H., Hensch, T.K., 2008. Critical period revisited: impact on vision. Curr. Opin. Neurobiol. 18, 101–107.

Neal, M.J., Cunningham, J.R., Hutson, P.H., Hogg, J., 1994. Effects of ischaemia on neurotransmitter release from the isolated retina. J. Neurochem. 62, 1025–1033.

Nithianantharajah, J., Hannan, A.J., 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat. Rev. Neurosci. 7, 697–709.

Nygren, J., Wieloch, T., 2005. Enriched environment enhances recovery of motor function after focal ischemia in mice, and downregulates the transcription factor NGFI-A. J. Cereb. Blood Flow Metab. 25, 1625–1633.

Ohlsson, A.L., Johansson, B.B., 1995. Environment influences functional outcome of cerebral infarction in rats. Stroke 26, 644–649.

Osborne, N.N., Casson, R.J., Wood, J.P., Chidlow, G., Graham, M., Melena, J., 2004. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Prog. Retin. Eye Res. 23, 91–147.

Paxinos, G., Watson, C., 1997. The Rat Brain in Stereotaxic Coordinates. Elsevier, Amsterdam.

Poitry, S., Poitry-Yamate, C., Ueberfeld, J., MacLeish, P.R., Tsacopoulos, M., 2000. Mechanisms of glutamate metabolic signaling in retinal glial (Müller) cells. J. Neurosci. 20, 1809–1821.

Prusky, G.T., Reidel, C., Douglas, R.M., 2000. Environmental enrichment from birth enhances visual acuity but not place learning in mice. Behav. Brain Res. 114, 11–15.

Quina, L.A., Pak, W., Lanier, J., Banwait, P., Gratwick, K., Liu, Y., Velasquez, T., O'Leary, D.D., Goulding, M., Turner, E.E., 2005. Brn3a-expressing retinal ganglion cells project specifically to thalamocortical and collicular visual pathways. J. Neurosci. 25, 11595–11604.

Roth, S., Li, B., Rosenbaum, P.S., Gupta, H., Goldstein, I.M., Maxwell, K.M., Gidday, J.M., 1998. Preconditioning provides complete protection against retinal ischemic injury in rats. Invest. Ophthalmol. Vis. Sci. 39, 777–785.

Sarthy, V., 2007. Focus on molecules: glial fibrillary acidic protein (GFAP). Exp. Eye Res. 84, 381–382.

Sumioka, K., Shirai, Y., Sakai, N., Hashimoto, T., Tanaka, C., Yamamoto, M., Takahashi, M., Ono, Y., Saito, N., 2000. Induction of a 55-kDa PKN cleavage product by ischemia/reperfusion model in the rat retina. Invest. Ophthalmol. Vis. Sci. 41, 29–35.

Sun, H., Zhang, J., Zhang, L., Liu, H., Zhu, H., Yang, Y., 2010. Environmental enrichment influences BDNF and NR1 levels in the hippocampus and restores cognitive impairment in chronic cerebral hypoperfused rats. Curr. Neurovasc. Res. 7, 268–280.

Szabadfi, K., Atlasz, T., Horváth, G., Kiss, P., Hamza, L., Farkas, J., Tamás, A., Lubics, A., Gábriel, R., Reglodi, D., 2009. Early postnatal enriched environment decreases retinal degeneration induced by monosodium glutamate treatment in rats. Brain Res. 1259, 107–112.

Thoreson, W.B., Witkovsky, P., 1999. Glutamate receptors and circuits in the vertebrate retina. Prog. Retin. Eye Res. 18, 765–810.

Wachtmeister, L., 1998. Oscillatory potentials in the retina: what do they reveal. Prog. Retin. Eye Res. 17, 485–521.