UNIVERSITY OF PÉCS

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Two factors in fight for sight: a neuroprotective agent and environmental enrichment

PhD thesis

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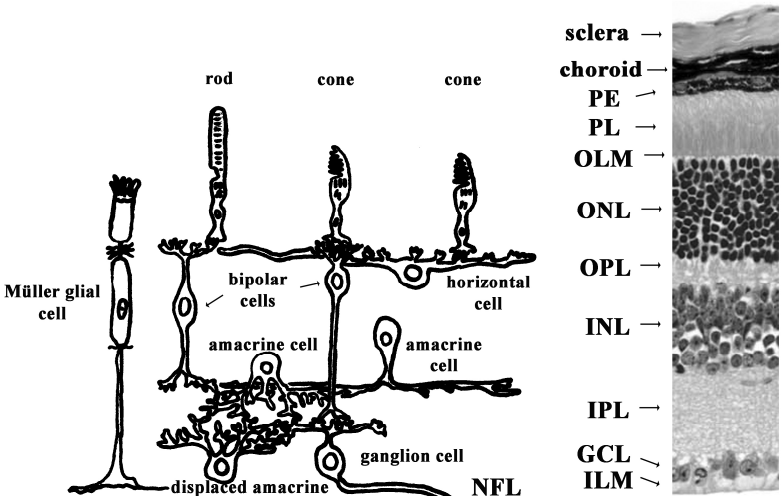
INTRODUCTION

The retina, like many other central nervous system structures, contains a huge diversity of neuronal types. Mammalian retinas contain approximately 55 distinct cell types, each with a different function. The basic idea of retina was originated by Ramón y Cajal's (1894) classical descriptions of the retina as a "true nervous centre", as clarified by his silver-stain preparations.

„The retina is the complex sample of the neural tissue, a microprocessor located in the eye."
Richard H. Masland, 1987

1. The structure of the mammalian retina

The vertebrate retina is the thin sheet of neural tissue at the back of the eye. The retina is oriented that the photoreceptors lie at the back, so to reach the photoreceptors light must pass through overlying layers of the retina (inverse eye). The retina, like the other parts of the CNS is a neurosensory centre. It has a highly organized laminar structure that is similar in all tetrapod vertebrate species, including rats. The distinct ten layers with six major retinal cell types are represented below:



Cross section of vertebrate retina showing different layers with the six major retinal cells types and their synaptic connections.
PE: pigment epithelium, PL: photoreceptor layer, OLM: outer limiting membrane, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, ILM: inner limiting membrane, NFL: nerve fiber layer.

2. Degeneration models of the retina

Choosing the right animal species for testing drug-induced retinal toxicity depends on several factors including similarity of the ocular structures to those of humans, availability, cost, simplicity for experimentation, and prior knowledge about retinal structure and function. Rats have a well-developed retinal vascular system, such as humans, with the arteries and veins emerging radially from the optic disc. The retinal structure is similar to that of humans, composed of three layers of nuclei (outer and inner nuclear layers and the ganglion cell layer), interposed by two synaptic layers, the outer and inner plexiform layers (OPL and IPL). The animals are relatively inexpensive and are raised for laboratory use. Drugs are frequently tested for retinal toxicity in animal models in order to address applied and basic research questions. Animal studies can be expanded to provide valuable information on cellular and molecular mechanisms, their importance for retinal function, and their susceptibility to specific compounds. This information serves to increase our understanding of retinal function in health and disease, and for future developments of drugs for ophthalmic use.

Hypoperfusion and ischemia are common conditions in the elderly, leading to decreased oxygenation of all tissues, including the highly sensitive retinal neurons and thus, leading to their death and consequent blindness. Ischemia is a serious condition leading to death of retinal neurons and blindness. Ocular ischemia plays an important role in the pathophysiology of various ocular diseases such as diabetic retinopathy, retinal vascular occlusion, anterior optic neuropathy, and possibly glaucoma. It may ultimately lead to neuronal death by inducing apoptosis or necrosis. Our ischemic model, the bilateral common carotid artery occlusion in the retina produces a characteristic pathologic appearance, paralleling the retinopathy of carotid artery occlusive disease in humans.

Pathological increase of glutamate levels plays a key role in neuronal damage in many diseases. In the eye, several pathological conditions can be mimicked by experimentally elevating extracellular glutamate concentrations. The damaging effects of monosodium glutamate (MSG) on the retina have long been known. In accordance with others, we have also demonstrated that three times systemic treatment of neonatal rats with MSG leads to the destruction of the entire inner retina.

3. Urocortin 2 (Ucn 2)

Since the isolation of corticotropin-releasing factor (CRF), three mammalian CRF-like paralogs (urocortin 1, 2, 3) have been identified. Members of the CRF peptide family themselves are potent activators of adenylate cyclase and cAMP production by G-protein coupled CRF receptors (CRF-R1, CRF-R2). CRF peptides are potent neuroprotectants, as shown, for example in cortical and hippocampal neurons. This action is mediated entirely by CRF-R1. Ucn 2 is a CRF paralog that preferentially activates CRF-R2s (endogenous ligand). Ucn 2 is the selective agonist of CRF-R2 and has a low affinity to the CRF-R1, but activates this latter receptor in very high concentration.

The members of the CRF superfamily and their receptors have been shown in the retinas of various species (ep.: goldfish, turtle, chicken, rat). Little is known regarding the potential retinoprotective effects of Ucns despite the known presence of CRF family peptides and their receptors (predominantly CRF-R2α) in the mammalian retina. Immortalised Y79 retinoblastoma cells express functional CRF-R1 and CRF-R2α *in vitro*.

Little is known about the potential protective effects of CRF receptor activation mediated by Ucn 2 against retinal injuries and cortico-visual degeneration.

4. Environmental enrichment

Donald O. Hebb, the creator of the "scientific optimal neurophysiological milieu" concept considered that environmental clues can influence neural development. His rearing of rats in an enriched environment altered neural development and he proved that sensory – neural connections were shaped by experience. Rosenzweig with his colleagues started research by comparing isolated rats in normal cages, and those placed in ones with toys, ladders, tunnels, running wheels in groups. They created the concept of enriched environment in 1978. Data emphasize the need to consider early life events as etiological factors for delayed neuropsychiatric disturbances and neurodegenerative diseases. Environmental enrichment increases the levels of neurotrophic factors in different brain areas and induces the development of synaptic structure and plasticity.

The complex stimulating environment facilitates structural reorganization of the brain, particularly in the visual cortex. The retina, in contrast to the cortex and hippocampus, has a low degree of plasticity. Therefore, it was assumed that the retinal development is

independent of the sensory inputs. However, several research groups have also shown that in rats raised from birth in stimulating environment, visual acuity increased by about 18%, thereby enhancing the complexity of the visual system and the plasticity of the visual cortex. Brain derived neurotrophic factor (BDNF) plays an important role in retinal development and is also one of the key molecules in cell differentiation, mediating the stimulating effect of the environment.

AIMS OF THE STUDY

The aims of the present study were to investigate, in our retina degeneration models (BCCAO and 3xMSG), whether:

1. the potential neuroprotective agent Ucn 2 by intravitreal administration is retinoprotective in BCCAO-induced ischemic and 3xMSG-induced excitotoxic models
    a. with standard histological (morphological and morphometric) analyses.
    b. with immunhistochemical method by cell- (calbindin, parvalbumin, calretinin, PKC α, TH, GFAP), and tissue-specific (VGLUT1, VGAT) antibodies.

2. the potential retinoprotective effects of different environmental conditions in ischemic and excitotoxic retinal degenerations: expanded cage and complex enriched environment.
    a. with standard histological (morphological and morphometric) analyses.
    To determine the degree of retinoprotection by comparison analyses between the animals, housing in standard and expanded cage and in enriched environment.

3. Comparison analyses of the effects of Ucn 2 and environmental conditions in different types of retinal degeneration models in the light of hypothetic retina protection.

MATERIALS AND METHODS

All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-20/2006).

1. Bilateral common carotid artery occlusion (BCCAO)

Experimental subjects (Wistar rats) underwent permanent BCCAO. Under isoflurane anesthesia, the carotid region was exposed through a midline cervical incision, and both common carotid arteries were permanently ligated with a 3–0 filament.

2. Monosodium-glutamate (MSG) treatment

The pups in each groups (Ucn 2 treated, standard housing, expanded cage and environmental enrichment) were injected subcutaneously with 2 mg/g bodyweight MSG dissolved in 100 µl physiological saline on postnatal days 1, 5 and 9. The control litters received the same volume of physiological saline.

3. Intravitreal injection of Ucn 2

Immediately following the operation and also the MSG treatments, 2 nmol Ucn 2 was injected into the vitreous body of the right eye in a 3 µl or 5 µl volume of phosphate-buffered saline (PBS). The same volume of PBS vehicle was injected into the left eye, serving as a control BCCAO and 3xMSG eye.

4. Enriched environment paradigm

Animals of both sexes were cross-fostered immediately after birth, to minimize litter differences. Pups were placed in one of the following three cages immediately after birth or BCCAO operation. Normal control rats were placed in a regular (control) cage with 43×30×20 cm dimensions. A second group of rats was placed in a larger cage (expanded field), the floor of which was 88×50 cm with 44 cm high walls. A third group of pups was

placed in a large cage with the same parameters as for group 2 (88×50×44 cm) supplemented with a complex environmental enrichment. Rats were continuously exposed to intensive multisensory stimulation. The cage contained different toys, objects, running tunnels and rotating rods with various shapes, materials and colors. Half of the objects were changed daily, while the other half was left unchanged.

5. Morphological and morphometric analyses

Two weeks after the BCCAO and three weeks after 3xMSG treatment, rats were sacrificed with an overdose of anesthetic, the eyes were immediately dissected in ice-cold PBS and fixed in 4% paraformaldehyde dissolved in 0.1M phosphate buffer. Tissues were embedded in Durcupan ACM resin, cut at 1-2 µm and stained with toluidine blue. The sections were mounted in Depex medium and examined in Nikon Eclipse 80i microscope. The following parameters were measured in a blinded fashion: (i) cross-section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM); (ii) the width of the outer and inner nuclear and plexiform layers (ONL, OPL, INL, IPL); and (iii) the number of cells/100 µm section length in the ganglion cell layer (GCL). Results are presented as mean ± S.E.M. Statistical comparisons were made using the ANOVA test followed by Tukey-B's post hoc analysis.

6. Immunohistochemical preparation

For cryostat sectioning, retinas were embedded in tissue freezing medium, cut in a cryostatat 10-12 µm to obtain radial sections. Sections were stored at -20 °C until use. During the immunohistochemical preparation the retinal sections were incubated with the primary monoclonal or polyclonal antibody overnight at room temperature. We used the following cell-specific antibodies: calbindin, calretinin, parvalbumin, protein-kinase Cα, glial fibrillar acidic protein and also tissue-specific antibodies: vesicular glutamate transporter 1 and vesicular GABA transporter. On the second day sections were incubated for 2 h in the dark with the corresponding secondary (red - Alexa Fluor "568" or green – Alexa Fluor "488" fluorescence) antibodies. For control experiments, primary antisera were omitted, resulting in no specific staining.

RESULTS

1. BCCAO+Ucn 2

BCCAO led to a severe reduction in the thickness of the retinal layers two weeks after ligation as compared to control rats. Each retinal layer showed width reductions suggestive of serious degeneration. The observed degree of retinal degeneration corresponds to our previous results with the BCCAO model. Intravitreal injection of Ucn 2 led to a marked amelioration of the retinal layers, suggesting retinoprotection in the BCCAO retinal ischemia model. In contrast to the vehicle-treated BCCAO retinas, the OPL remained visible in Ucn 2-treated eyes, with the INL and GCL clearly separated. Ucn 2 attenuated ischemic degeneration in the retina *in vivo*. The layers significantly differed between vehicle-treated and Ucn 2-treated BCCAO retinas. Quantitative morphometric analysis also demonstrated that Ucn 2 administration protected cells in the GCL from degeneration, with the number of cells in this layer significantly more than those from BCCAO-operated retinas. The present results show that acute intravitreal Ucn 2 administration attenuates the marked degeneration of retinal layers that otherwise is seen two weeks following permanent BCCAO in rats. The *in vivo* findings demonstrate that protective actions of Ucn 2 extend to sparing retina from ischemic injury. Immunohistochemical studies (by using cell- and tissue specific markers) confirmed the already proven protective effects of Ucn 2 in ischemic degeneration *in vivo*.

2. 3xMSG+3xUcn 2

Retinal tissue from animals treated with 3xMSG showed severe degeneration compared to normal retinas. Much of the IPL disappeared and the INL and GCL were intermingled and the retinal cells were swollen in ONL. Intravitreal Ucn 2 treatment resulted in a retained retinal structure reminded that of the normal retina. The IPL remained visible; the INL and GCL were clearly separated at all places. There was no statistical difference in the cell number of GCL between the 3xMSG and 3xMSG + 3xUcn 2 treated retinas. The application of the 3xUcn 2 treatment following MSG slightly, but significantly ameliorated the MSG-induced retinal degeneration. Immunohistochemical studies (by using cell- and tissue specific markers) confirmed the already proven (by morphological and morphometric analyses) protective effects of Ucn 2 in 3xMSG degenerated retinas *in vivo*.

3. Environmental enrichment – BCCAO

Normal retinas showed all characteristic layers of the mammalian retina. No apparent morphological differences could be observed in preparations from saline-treated rats in any of the groups.

BCCAO resulted in severely reduced thickness of retinal layers as observed two weeks after ligation in the case of pups housed in standard cage, consistently with our previous descriptions. All retinal layers were bearing the marks of degeneration with individual variation. Several empty cell body-shaped spaces were seen in the ONL.

Amelioration could be observed in the BCCAO retinas of rats kept in enlarged cages. The retinal layers could be clearly separated, but we could also observe picnotic cells and empty cell body shapes.

Environmental enrichment following bilateral carotid occlusion led to a nearly intact appearance of the retinal layers. This is well reflected in the morphometric measurements. The thickness of the major retinal layers was almost identical to that of the sham-operated animals and was significantly larger than that of the BCCAO ones kept in standard and expanded cage. The entire thickness of the retina was approximately 17% more than in animals kept in expanded cage, and only 25% less than in the case of control animals.

4. Environmental enrichment – 3xMSG

All the layers characteristic for the mammalian retina were well visible in normal preparations without MSG treatment. No apparent morphological differences could be observed in preparations from saline-treated rats in any of the groups.

Retinal tissue from animals treated with MSG showed severe degeneration compared to the normal controls, consistent with our previous descriptions. Much of the IPL disappeared and the INL and GCL were intermingled. In addition, swollen cell bodies were observed in the ONL.

A slight amelioration could be observed in the retinas of rats kept in large cages. The retinal layers could be clearly separated, the thickness of ONL, OPL, INL, and IPL, and so the entire thickness of the retina were significantly increased compared to standard housed MSG-treated retinas. The number of cells in the GCL, however, was not changed when compared to preparations from MSG-treated animals kept in standard cages.

Pups kept in enriched environment had a markedly lower degree of retinal degeneration following MSG treatment and the appearance of the retinal structure was well preserved. All layers of the retina were significantly thicker than in the retinas of MSG treated rats kept in expanded cages. The entire thickness of the retina was only approximately 15% less than in normal animals. The number of cells in the GCL was markedly higher than standard MSG-treated or those kept in expanded cages without enrichment.

DISCUSSION

Neuroprotection is a paradigm that aims to reduce or even prevent neuronal damage by pharmaceutical intervention or molecular genetic techniques. Neuroprotection can be quantified by morphological, morphometric and immunhistochemical techniques.

In the present study we showed that both Ucn 2 treatment and housing in enriched environment effectively reduced retinal damage caused by BCCAO or 3xMSG treatment.

In the rat retina permanent BCCAO, depending on the duration of ischemia, caused progressive morphological changes from the inner retinal layers (GCL, INL) to the outer retinal layers.

Repeated subcutaneous application of MSG causes progressively more severe alterations in retinal morphology. Pathological activation of glutamate receptors is thought to play a key role in neuronal damage in many neurological diseases. The vertebrate retina uses glutamate as neurotransmitter in the so-called through-pathway. Inner retinal cells, with the exception of ON bipolars, bear functional ionotropic glutamate receptors. Therefore, these cells are all potential targets of MSG toxicity.

Evidence suggests that Ucn 2 provides slightly higher protection against ischemic degeneration in the retina. The present study also showed that expanded cage provided some degree of neuroprotection, while a complex environmental enrichment led to a manifest protection against retinal degeneration induced by neonatal MSG treatment in rats. Compared to Ucn 2, the degree of the retinal protection was better in the case of the retinal layers. The number of cells in the GCL was also markedly higher.

The effects of enriched environment are multifocal, possibly including altered levels of different neurotrophic factors. This effect is likely to be mediated by BDNF coupled to high activity of tyrosine kinase receptor type 2 (TrkB). BDNF and TrkB were shown to be expressed in the retina, superior colliculus and visual cortex. There are no data about the Ucn 2 receptors in the rat retina. We have no information about the direct or indirect influence of Ucn 2 on neurotrophic factor expression. However, other members of the CRF superfamily have been reported to elevate the level of BDNF. It can be assumed that Ucn 2, a member of the CRF superfamily, plays a role in elevating BDNF expression via in both CRF-R1 and the CRF-R2.

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PUBLICATIONS

1. Publications releated to the thesis

Atlasz T., Babai N., Reglődi D., Kiss P., Tamás A., **Szabadfi K.**, Tóth G., Hegyi O., Gábriel R. (2007): Pituitary adenylate cyclase activating polypeptide is protective in bilateral carotid occlusion-induced retinal lesion in rats. *Gen. Comp. Endocrinol.,* 153; 108-114. (2007. IF: 2.562)

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