UNIVERSITY OF PÉCS

Biological Doctoral School
Comparative Neurobiology Program

Analysis of the effects of different neuroprotective compounds in retinal degeneration models in rats

PhD thesis

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Introduction

Anatomy of the retina

The retina is a multi-layered sensory tissue that lines the back of the eye. It contains millions of specific retinal cells that capture photons rays and translate light signals into electrical potential changes.

The vertebrate retina has ten distinct layers. From outermost to innermost, they include:

1. Retinal pigment epithelium (PE; supporting cells for the neural portion of the retina).
2. Photoreceptor layer (PL; composed of light and colour sensitive cells called rods and cones).
3. Outer limiting membrane (OLM; a layer which imperfectly separates the inner segment portions of the photoreceptors from their cell bodies). This layer is built up from adherens junctions between Müller cells and photoreceptor cell inner segments.
4. Outer nuclear layer (ONL; contains cell bodies of rods and cones).
5. Outer plexiform layer (OPL; the first layer, where connections between photoreceptors, and vertically running bipolar cells and horizontally oriented horizontal cells occur).
6. Inner nuclear layer (INL; somata of horizontal, bipolar and amacrine cells, main distribution of Müller cells).
7. Inner plexiform layer (IPL; in this layer bipolar cells synaps with different varieties of functionally specialized amacrine cells and dendrites of the various ganglion cells).
8. Ganglion cell layer (GCL; the layer of the retina containing primarily the cell bodies of ganglion cells, giving rise to optic nerve fibers, and some displaced amacrine cells).
9. Nerve fiber layer (NFL; fibers from ganglion cells traversing the retina to leave the eyeball at the optic disk).
10. Inner limiting membrane (ILM; inner surface of the retina bordering the vitreous body and thereby forming a barrier between neural retina and vitreous humor).

This structure provides the neuronal background of the first steps in processing contrast, colour and motion.

Retina degeneration models

There are many types of retina degeneration models described in the literature. They can be grouped into two main categories, (i) with a known genetic background or (ii) induced by metabolic or traumatic events. These induced retinal degenerations, among others, may be caused by aging, glaucoma, ischemic damage, autoimmune processes, diabetes, toxic agents and exposure to extremely strong light.

Over-activation of glutamate receptors is a central contributor to neuronal cell death in the brain and in the retina in numerous pathological conditions. Experimental elevation of glutamate concentrations can model several ophthalmic diseases. Direct increase of glutamate concentration can be reached by monosodium L-glutamate (MSG) administration, which finally leads to the destruction of the entire inner retina.

In the same way, bilateral common carotid artery occlusion (BCCAO) leads to moderate reduction in the cerebral blood flow in rats leading to subtle changes in biochemical and behavioral measures. It has been shown that BCCAO causes long-lasting white matter lesion, neuronal degeneration, microglial activation, astrocytosis, behavioral deficits and changes in several biochemical parameters. In the retina, it produces a characteristic pathologic appearance, paralleling the retinopathy of carotid artery occlusive disease in humans. Electroretinographical and morphological studies also show that BCCAO leads to ischemic damage of the retina.

The events finally leading to retinal cell death are very complex, providing an opportunity for a variety of protective pharmacological approaches. These involve reducing the detrimental effects of free radicals and increased Ca^{2+} levels, counteracting mitochondrial failure, prevention of apoptotic cascades, anti-inflammatory strategies and potentiating endogenous protective mechanisms.

Pituitary adenylate cyclase-activating polypeptide (PACAP)

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide first isolated from the hypothalamus, and later demonstrated in other areas of the central nervous system. PACAP exerts neurotrophic and neuroprotective effects in vitro and in animal models

in vivo, adding PACAP to the growing family of trophic factors in the nervous system. PACAP has been shown to reduce the damaged brain area in global and focal ischemia. The distribution of PACAP has also been described in various peripheral and sensory organs, including the eye. In the retina, PACAP immunoreactivity is present in the amacrine and horizontal cells, in the IPL, in the GCL and in the NFL. Recent studies have shown that PACAP attenuates glutamate toxicity in the retina *in vitro*.

Diazoxide (7-chloro-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide; DIAZ)

Mitochondrial dysfunction is involved in many key events of neuronal cell death in the retina. 7-chloro-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide (Diazoxide, DIAZ) is a mitochondrial ATP-sensitive K^+-channel-opener that has been implicated in cytoprotection in cardiac and cerebral ischemia, but relatively little is known about its putative protective effects in the retina. It has been shown that DIAZ enhances survival of retinal ganglionic cells, protects retinal neurons against excitotoxicity and inhibits the glutamate-induced mitochondrial depolarization in vitro. DIAZ has also been reported to block the hypoxia-induced horizontal cell depolarization and the reduction of the light-evoked hyperpolarization in vitro. *In vivo*, ischemic preconditioning can effectively be mimicked by DIAZ. However, the direct protective effect of DIAZ in different *in vivo* models of retinal degeneration has not yet been shown.

AIMs OF THE STUDY

The main goals of this study were to investigate:

- the optimal model for examining the neuroprotective effects of PACAP in MSG-induced retinal degeneration.

- whether PACAP could be effective in retinal ischemia induced by BCCAO.

- the effects of local administration of DIAZ in retinal degeneration induced by neonatal MSG treatment.

- the putative effects of DIAZ in BCCAO-induced ischemic damage of the retina.

We would also like to determine the effects of PACAP/ DIAZ treatments in some well-defined cell types after three times of MSG- or BCCAO-induced retinal degeneration in rats. Therefore, the expression of:

- vesicular glutamate transporter-1 (VGLUT-1),
- vesicular GABA transporter (VGAT),
- Ca^{2+}-binding proteins (calbindin, calretinin, parvalbumin),
- protein-kinase Cα (PKCα),
- glial fibrillary acidic protein (GFAP) was determined by immunhistochemistry.

These markers are found in neurochemically and morphologically identified retinal cell populations.

MATERIALS AND METHODS

MSG treatment schedule

Newborn rats (n=75) were injected subcutaneously (s.c.) with 2 mg/g body weight MSG dissolved in 100 µl physiological saline on postnatal days 1, 5 and 9 according to previous descriptions. Normal control animals (n=25) were given the same volume of physiological saline solution. Treatments were given immediately following each MSG injection on days 1, 5 and 9. PACAP (100 pmol in 5µl saline, n=30) or DIAZ (0.172 µg dissolved in 2 µl 0.01 M NaOH and phosphate buffered saline, PBS, n=20) was injected with a Hamilton syringe into the right vitreous body of animals. The left eyes received the same volumes of vehicle treatment (saline or PBS) and served as control MSG-treated eyes. Animals were sacrificed under anesthesia at the age of 21 days. The eyes were processed for histological observations as described below.

BCCAO operation

Adult male Wistar rats weighing 250-300 g were subjected to permanent BCCAO (n=69) under isoflurane anesthesia. The carotid region was exposed through a midline cervical incision. The common carotid arteries were ligated with a 3-0 mm suture. Sham/operated animals (n=16) underwent the same procedure except for ligation of the carotid arteries. PACAP (100 pmol in 5µl saline, n=29) or DIAZ (0.172 µg dissolved in 2 µl 0.01 M NaOH and phosphate buffered saline, PBS, n=24) was injected with a Hamilton

syringe into the right vitreous body of animals. The left eyes received the same volumes of vehicle treatment (saline or PBS) and served as control ischemic eyes. After two weeks of survival the animals were sacrificed under anesthesia and the eyes were processed as described below.

Histology

The eyes were immediately dissected in ice-cold phosphate buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. Tissues were embedded in Durcupan ACM resin, cut at 2 μm and stained with toluidine blue. Six tissue blocks from at least three animals were prepared and central retinal areas within 1 and 2 mm from the optic nerve were used for measurements (n=2-5 measurements from one tissue block). Sections where the GCL appeared thicker than a single cell row, were excluded from evaluation. The following parameters were measured: (i) cross-section of the retina from the outer limiting membrane to the inner limiting membrane; (ii) the width of the outer and inner nuclear and plexiform layers (ONL, OPL, INL, IPL), respectively; (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.

Immunocytochemical investigations

The eye-cup preparations, made from animal's eyes were cut in a cryostat and the slices were put on a gelatin-coated microscopic slide. We used the following primary monoclonal or polyclonal antibodies: anti-VGLUT-1 (vesicular glutamate transporter-1); VGAT (vesicular GABA transporter); Ca^{2+}-binding proteins: calretinin (CALR), calbindin (CALB), and parvalbumin (PARV); PKCα (protein-kinase Cα); GFAP (glial fibrillary acidic protein). After several washes in PBS, sections were incubated with the corresponding secondary antibodies (anti-rabbit Texas Red IgG, anti-mouse FITC IgG). For control experiments, primary antisera were omitted, and after protocol, specific cellular staining was not found.

RESULTS

MORPHOLOGY AND MORPHOMETRIC ANALYSIS

Effects of PACAP in MSG or BCCAO-induced retinal degeneration models

Repeated application of MSG causes severe alteration in retinal morphology. In control preparations all the layers characteristic for the mammalian retina are well visible. Under the pigment epithelium, several rows of photoreceptors are present, followed by the thin outer synaptic layer. The INL usually consists of 4-5 rows of cells, followed by a thick inner synaptic layer. Finally, cells in the GCL layer are frequent but not side by side. One time MSG treatment at postnatal day 1 does not cause a marked damage to the retina. The layers are similar in size to those of the control, although signs of degenerative processes, such as swollen cells and nervous profiles, small holes in the tissue, some pyknotic cell nuclei could be seen. The otherwise characteristic Müller glial cells identified on the basis of their dark rectangular nuclei are difficult to find in these preparations. When 3x MSG was applied the inner retinal layers were fused, and the number of cells in the GCL was significantly reduced. In the next series of experiments we used 3x MSG application with varying PACAP treatments. If only one time PACAP treatment was made at the first MSG application, it did not change the degenerative capacity of MSG. The retinal layers remain fused, although in some preparations more than two cell layers could be discerned in the inner retina. However, if animals received PACAP into the vitreous of the eye at the first 2 or all 3 times during MSG application, a substantial protective effect could be observed. The IPL remained well discernible, the INL was prominently present and retained 2-4 cell rows. The number of cells in the GCL was not significantly less than that of the control retina.

BCCAO resulted in severely reduced thickness of retinal layers as observed two weeks after ligation. All retinal layers bore the signs of degeneration with individual variations. The most marked reduction in thickness was found in the plexiform layers, and as a consequence, the distance between OLM-ILM was significantly less than in control preparations. The PL was also reduced. The outer segments were shorter than usual, their geometric arrangement was disturbed. Several empty cell body-shaped spaces were seen in the layer of photoreceptor cell nuclei. As a result of this anatomical situation the thickness of the ONL was markedly, though not significantly, less than in sham-operated animals. Similar empty spaces were also seen in the INL. This layer was significantly thinner than that of the control specimens. In the

IPL of several, but not all ischemic animals, dense dots of about 1 μm in diameter were seen, assumed to be degenerating bipolar cell terminals. Parallel with this process, OPL was narrower, leading to the fusion of the ONL and INL in several cases. Many cells in the GCL also suffered degeneration, shown by holes and necrotic cells in this layer. This fact is well reflected in the reduced number of cells in the GCL. Intraocular PACAP treatment following BCCAO 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 with that of the sham-operated animals and was significantly larger than that of the carotid occluded retinas. This was especially conspicuous in the OPL, which disappeared in several ischemic animals and was preserved in all PACAP-treated animals. This resulted in the clear separation of the nuclear layers in contrast to the bilateral carotid occluded rats, where the ONL and INL fused in most animals. Only the number of cells in the GCL seemed to be lower than in the sham-operated animals.

Effects of DIAZ in MSG- or BCCAO-induced retinal degeneration models

Retinal tissue from animals treated with MSG showed severe degeneration compared to control retinas, as described above. Much of the IPL disappeared and the INL and GCL were intermingled. As a consequence, the total thickness of the retina was significantly reduced, only the photoreceptor layer seemed unchanged. Local DIAZ treatment resulted in a retained retinal structure that was similar to that of the normal control retina. The number of cells in the GCL was not significantly less than in the untreated retina. The IPL remained visible, the INL and GCL were clearly separated at all places.

Carotid occlusion led to a severe reduction in thickness of retinal layers compared to sham-operated control rats, as described above, with all retinal layers displaying marks of serious degeneration. DIAZ proved to be retinoprotective also in this model: differences between sham-operated and DIAZ-treated retinas were statistically significant in almost all retinal layers, except for the ONL. Quantitative analysis demonstrated that DIAZ administration protected the cells in the GCL.

IMMUNOHISTOCHEMISTRY

In our normal control preparations, VGLUT-1 immunopositive structures were also present in the OPL and IPL. VGLUT-1 staining in the OPL and IPL of the rat retina shows the

terminals of photoreceptors and bipolar cells, respectively. Retinal tissue from animals treated with MSG or BCCAO showed severe degeneration compared to control or sham-operated retinas. Much of the IPL disappeared and the inner nuclear layer (INL) and ganglion cell layer (GCL) were intermingled. In BCCAO-treated retinas substantial reduction was found in the size of the terminals of photoreceptor cells in the OPL. Intraocular PACAP treatment following MSG application or ischemic injury led to a nearly intact appearance of VGLUT-1 immunoreactivity in retinal structures if animals received PACAP into the vitreous of the right eye. In this case, a substantial protective effect could be observed: the bipolar cell terminals in the IPL remained well discernible and the OPL was nearly intact. In the case of DIAZ treatment, the control/sham-operated retina preparation displayed VGLUT-1 immunopositive structures in the OPL and IPL. DIAZ also proved to be retinoprotective in these models.

We were able to verify the cellular localization of the VGAT in the OPL and IPL in control/sham retinas. Strong VGAT immunoreactivity could be detected in the IPL and weaker immunopositive structures were present in the OPL. After 3x MSG application, the strength of positive VGAT-immunoreactive structures was reduced. The entire inner retina, especially the IPL, was only faintly labeled. PACAP or DIAZ treatment significantly ameliorated the toxic effects of MSG. Under ischemic conditions, the strength of immunoreactivity was reduced, while PACAP or DIAZ diminished these harmful effects of ischemia.

In control/sham conditions the calcium binding proteins labeled as follows. In the case of PARV: AII amacrine cells; CALB: horizontal cells, CALR: ganglion and some amacrine cells. MSG treatment caused alterations in these labelings; PARV: the number of the stained cells were reduced; CALB: small alteration in the intensity of the immunoreactivity was observed; CALR: immunoreavtivity was weaker and the number of stained cells was decreased. PACAP or DIAZ treatment counteracted the MSG-induced changes in calcium binding protein immunolabeling. Conversely, the number of the labelled cells and the strength of stained neurons increased. In contrast, we observed that, in retinal ischemia caused by BCCAO the CALB-positive structures completely disappeared. Under this condition, PARV and CALR immonoreactivity patterns were the same as in MSG-treated retinas. After application of PACAP or DIAZ staining of retinas was similar to that of sham eyes, with no alterations in immunoreactivity patterns for the investigated calcium binding proteins.

It is known that rod bipolar cells show PKCα-immunoreactivity in the control/sham-operated retinas. The rod bipolar cell is characterized by a tight cluster of dendrites in the

OPL, oval cell body located in the INL, and a vertically directed axon, which expands into clusters of terminals in the IPL. Following MSG injections or ischemia, large and rapid reduction in PKCα intensity was observed. However, in the PACAP or DIAZ administered retinas the total amount of immunoreactivity was similar that of the control/sham-operated slides.

GFAP filaments are normally in the inner half of the Müller cells and their endfeet, as in our control/sham preparations, but following trauma to the retina such as excitotoxicity (MSG) or ischemia/hypoxia (BCCAO), GFAP is massively upregulated and found throughout the cell from the OLM to the ILM. The distribution pattern of GFAP in PACAP injected retinas was similar to that of the injured ones. In DIAZ-treated retinas, GFAP immunoreactivity was found at the inner margin of the retina, similarly to the control/sham situations.

DISCUSSION

The vertebrate retina uses glutamate as neurotransmitter in a so-called through-pathway (that is, the photoreceptor, bipolar and ganglion cells, connected with synapses in this order). The inner retinal cells, with the exception of ON bipolars, bear functional ionotropic glutamate receptors. Therefore, these cells are all potential targets of MSG toxicity. On the other hand, nearly the same set of neurons bear PACAP receptors. This explains why these cells could be able to survive the excitotoxic injury if PACAP is present in high concentration at the same time of the insult. The IPL remained slightly reduced after combined MSG and PACAP treatment in our experiments. We found that after the second PACAP injection, no further neuroprotection could be achieved with subsequent injection of PACAP. It is possible that repeated injection of PACAP leads to a primed state, which provides a long-lasting protection.

In the present study, we also showed that PACAP treatment effectively reduced retinal damage caused by BCCAO in adult male rats. The present morphological findings showed that PACAP protected all inner retinal layers, correlate with previous results which showed the distribution of PAC1 receptor in the retina. This pattern of receptor expression provides basis for the sites of action by intraocular PACAP administration in our present study. Apoptosis plays a major role in several retinal pathologies, including retinal ischemia. Anti-apoptotic agents have been shown to attenuate ischemic damage in the retina, including inhibition of caspases. PACAP is a very potent anti-apoptotic agent, which has been shown in

several cell types *in vitro*, and in the brain *in vivo* after cerebral ischemia. In the retina, we have recently shown that PACAP inhibits proapoptotic signaling pathways and stimulates anti-apoptotic signaling molecules in MSG-induced retinal damage *in vivo*. Oxidative stress is thought to play an important role in ischemic retinal cell death. Although the effects of PACAP in retinal oxidative stress has not been investigated yet, PACAP is able to ameliorate oxidative stress-induced apoptosis in neuronal and non-neuronal cells. Mitochondria are involved in many key events of neuronal cell death in the retina. We have recently shown that PACAP inhibits translocation of cytochrome- c and apoptosis inducing factor from the mitochondria and abolished the MSG-induced reduction of phospho-bad. This, along with other observations, indicates that PACAP attenuates the effects of stressors on mitochondria.

Another way of interfering with mitochondrial pathways is to alter ion permeability of the mitochondrial membrane. ATP-sensitive K^+ channels are located in different parts of the cell, including the inner mitochondrial membrane. The mitochondrial ATP-sensitive K^+ channels have been extensively studied in the heart and brain. The selective activation of these channels by means of pharmacological or physiological stimuli has been shown to be cytoprotective against ischemia or chemical stress. This type of neuroprotection represents a new mechanism of protection which is not dependent on blocking glutamatergic receptors or scavanging free radicals. DIAZ treatment also targets the mitochondria and induces a chain of intracellular protective mechanisms. DIAZ is a mitochondrial ATP-sensitive potassium channel opener that is mostly used as an antihypertensive and antihypoglycemic drug in human therapy and has been applied as a neuroprotective agent in animal studies. When DIAZ is used prior to the insult *in vitro*, it protects against neuronal cell death induced by oxidative stress or glutamate. *In vivo*, it has neuroprotective effects in various cerebral ischemic experimental conditions. BCCAO, which was also used in our studies, is mainly applied to induce cerebral hypoperfusion, where the protective effects of DIAZ have been described both using pre- and postischemic administration. Despite the numerous pieces of evidence showing the neuroprotective effects of DIAZ, little is known about its effects in the retina. The mitochondrial ATP-sensitive K^+ channels are also present in the retina, where the stimulatory effects of DIAZ have been reported. *In vitro*, the opening of these channels with different agents, including DIAZ, enhances survival of retinal ganglionic cells and protects retinal neurons against glutamate-induced excitotoxicity. DIAZ has also been shown to inhibit the glutamate-induced mitochondrial depolarization. Our present study provides *in vivo* evidence that local DIAZ administration attenuates both MSG- and ischemia-induced retinal degeneration. The mechanism could be multiple, including acute cytoprotective effects of the

drug as well as early and late preconditioning. If DIAZ is available for the cells at the time of the ischemia/hypoxia or other kind of depolarization, its protective mechanism can be mediated by reduction of the mitochondrial calcium load. Although the morphological appearance of the two retinal degeneration models is different, our present study shows that DIAZ is able to attenuate the degeneration induced by both MSG and BCCAO.

In summary, the present study showed that the severe degeneration of the retinal layers caused by neonatal MSG treatment or BCCAO was significantly attenuated by PACAP or DIAZ, also supported by immunocytochemical observations. PACAP and DIAZ may have further clinical implication in ophthalmic diseases induced by ischemia or excitotoxicity.

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PUBLICATIONS

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