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MORPHOLOGICAL ANALYSIS OF NEURODEGENERATION MODELS IN RAT RETINA

PhD thesis

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Introduction

The anatomy of the retina

The light reflected from objects travels through the refracting materials of the eye and the layers of the retina to the photoreceptors in the back of the eye where the light signal is transformed to chemical energy, which leads to neural transmission. This special function requires a finely structured retina.

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

1. Retinal pigment epithelium (supporting cells for the neural portion of the retina).
2. Photoreceptor layer (rods and cones).
3. External limiting membrane (a layer which imperfectly separates the inner segment portions of the photoreceptors from their cell bodies). This layer is built up from glial (Müller cell) processes
4. Outer nuclear layer (cell bodies of rods and cones).
5. Outer plexiform layer (rod and cone axons, horizontal cell dendrites, bipolar dendrites).
6. Inner nuclear layer (somatas of horizontal, bipolar and amacrine cells, main portion of Müller cells).
7. Inner plexiform layer (axons of bipolars and amacrines, dendrites of ganglion cells).
8. Ganglion cell layer (layer that contains nuclei of ganglion cells and gives rise to optic nerve fibers).
9. Nerve fiber layer (fibers from ganglion cells traversing the retina to leave the eyeball at the optic disk).
10. Inner limiting membrane: (Müller cell footplates).

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

Glutamate as retinal transmitter

The photoreceptors, the bipolar and the ganglion cells show glutamate-immunreactivity in the retina. By secreting glutamate, the photoreceptors signal information, collected from photons, for the second order neurons and ganglion cells, respectively. The glutamate transporters take up glutamate into the Müller cells with one K^+ cotransport and three Na^+, one OH^- or one HCO_3^- antiport.

Glutamate transporters keep the glutamate at low level in the synaptic cleft, thus avoiding glutamate-induced cell death. The Muller-cells transform the glutamate to glutamine and feed glutamine to surrounding neurons. The neurons then make glutamate from glutamine.

There are two types of glutamate receptors: ionotropic and metabotropic one. The ionotropic glutamate receptors allow Na^+ and Ca^{2+} to enter the cells upon ligand binding while the metabotropic glutamate receptors activate G-proteins.

Neuron degeneration

There are two possible ways of neuron degeneration, namely necrosis and apoptosis. These two kinds of degenerations have a characteristic series of processes marked by different morphological appearences. Necrosis is a kind of cell death caused by serious injury to the cell. The injury could be from mechanic intervention, hypoxia, toxic agents etc. Water and Ca^{2+} diffuse into the cell because of the increased membrane permeability, whereby the cell and the organelles become swollen and fragmented.

Apoptosis (programmed all death) may be caused by intrinsic or extrinsict factors or can be genetically determined. In the course apoptosis, the cells shrink and the chromatin in the nucleus condenses. The cell blebs into fragments but the cells membrane remains intact. Harmful materials of the cells are therefore contained, and this way the neighbouring cells do not acquire injury.

Role of glutamate in the neurotoxic processses

Glutamate is neurotoxic at a slightly higher than physiological level. The blood-brain barrier regulates the level of ingested extrinsic glutamate getting over to the brain and the retina, where it binds to the glutamate receptors, eventually inducing neuron degeneration. Since in the synapse there is an excitation control mechanism executed by glutamate transporters which are found in the presynaptic membrane and the surrounding glial cell membrane, the increased glutamate concentration has effects on both glutamate receptors and glutamate transporters, inducing neuronal degeneration.

Retina degeneration models

There are many types of retina degeneration models described in the literature. They can be grouped into two main categories: one is with a known genetic background, the other is 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.

Pathological activation of glutamate receptors is thought to play a key role in neuronal damage in many neurological diseases. In the eye, several pathological conditions such as ischemia and some types of glaucoma can be mimicked by experimentally elevating extracellular glutamate concentrations or applying its analogues. One such agent is monosodium L-glutamate (MSG) which can be administered in the form of subcutaneous

injection, and finally leads to the destruction of the entire inner retina. This treatment leads to depolarization of inner retinal cells and causes Ca^{2+} influx into the neurons.

In the same way, applying occlusion to the vessel supplying the eye or with bilateral carotid artery occlusion (BCCAO) we can induce retina degeneration. Electroretinographic and morphological studies also show that BCCAO induces severe retina damage.

AIMs OF THE STUDY

Our aim was to:

- induce retina degeneration applying different MSG treatments, and reveal its morphological features.
- find an optimal retina degeneration model that is reproducable and suitable for testing neuroprotective compounds.

We would also like to determine:

- the optimal glutamate concentration and dose, which gives measurable degeneration, but does not destroy the entire retina;
- the types of degenerating cells applying antibody against:
  - Ca^{2+}-binding proteins
  - glutamate transporters
  - tyrozin-hydroxylase (TH)
  - GABA-transporters.

Little change in the homeostasis of the retina could be fatal for the cells of the retina. One of the most important factor is the blood supply of the retina. Therefore, we performed:

- BCCAO for further investigation
- applied hypoxic conditions in a chamber and combined with unilateral carotid artery occlusion (UCCAO). These conditions were applied separetely or conjointly.

MATERIALS AND METHODS

MSG treatment: Experimental animals were derived from the local colony of Wistar rats. They were housed in individual cages, fed and watered *ad libitum*, under light/dark cycles of 12/12 h. The NIH and local animal care committee guidelines were carefully followed throughout the entire procedure. Newborn rats from both sexes (n=45) were injected s.c. with 2 mg/g b.w. MSG on postnatal days (PD) 1, 5, 9, 13, and 17. At 3 weeks of age, rats were killed with an overdose of anesthetic, the eyes were processed for normal light and electron microscopy.

BCCAO: Adult male Wistar rats (n=18) weighing 250-300 g were subjected to permanent bilateral carotid artery occlusion. Under pentobarbital anesthesia (35 mg/kg bodyweight), both

common carotid arteries were ligated with a 3-0 filament through a midline incision. Three weeks after the carotid occlusion, rats were sacrificed with an overdose of anesthetic, the eyes were processed for normal light microscopic technique.

UCCAO and hipoxic treatment: One week old Wistar rats (n=8) were subjected to permanent bilateral carotid artery occlusion. Under pentobarbital anesthesia (35 mg/kg bodyweight), left side common carotid arteries were ligated with a 3-0 filament through a midline incision. After the operation animals were put in hypoxic chamber one time for two hours. Two weeks after the carotid occlusion, rats were sacrificed with an overdose of anesthetic and the eyes were processed for normal light microscopic technique.

Immunchytochemical investigations: The eye-cup preparatums, 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 antibodies: anti-VGLUT1, -VGLUT2 (vesicular glutamate transporters), -TH (tyrosine-hydroxylase), -VGAT (vesicular GABA transporter), -calretinin, -calbindin, and -parvalbumin.

RESULTS

Repeated application of MSG causes progressively severe alterations in retinal morphology. One time MSG treatment did not cause remarkable alterations at light microscopic level. The thickness of the retina layers were the same as in the control retina, but we noticed signs of early degeneration processes (swollen cells, small holes in the tissue and picnotic nuclei).

Retinal tissue from animals treated with repeated (2x, 3x, 5x) MSG show severe degeneration compared to the controls. The distance of the outer limiting membrane and inner liminting membrane and the thickness of the inner retina layers significantly diminished. Much of the inner plexiform layer disappearsed and the inner nuclear- and ganglion cell layers are intermingled. As a consequence, the total thickness of the retina was significantly reduced. Only the photoreceptor layer seemsed unchanged.

Bilateral carotid occlusion resulted in severely reduced thickness of retinal layers as observed three weeks after ligation (compared to sham-operated control). The most marked reduction in thickness was found in the plexiform layers, and as a consequence, the distance between outer limiting membrane and inner limiting membrane was significantly less than in control preparations.

As a result of UCCAO and hypoxia treatment, the inner nuclear-, inner plexiform- and outer plexiform layers thickness decreased. Applying hypoxic environment caused a slightly increased thickness of the inner nuclear- and outer nuclear layers while the thickness of the inner plexiform- and outer plexiform layers significantly decreased.

In the MSG models the strength of immunolabelling and the number of labeled cells significantly decreased in the course of VGLUT-1, VGLUT-2, VGAT, calretinin, parvalbumin and TH immunostaining. The calbindin-immunolabeled horizontal cell number did not change, but the size of the cells grew.

DISCUSSION

Glutamate-induced toxicity is known to play a key role in several retinal pathologies. Glutamate released in retinal ischemia, initiates a cascade leading to retinal cell death. Increased glutamate level induces cell death in glaucoma patients too. Our results correspond to the finding that most glutamate receptors are found in the inner retina, and consequently the MSG treatment does not influence photoreceptors. Strictly the inner retina is degenerated. There was little difference between the animals treated with MSG at P1 (when new cells are still being generated) or P5 (when most intensive synaptogenesis occurs).

We did not expect an extensive damage in the outer plexiform layer, because just a slight change was seen at the light microscopic level. Interestingly, short time excytotoxic insult equally causes outer and inner plexiform layer degeneration. For finding, there could be an explanation; since we sacrificed the animals long time after the treatment, the retina would have enough time for a structural reorganization. One time MSG treatment at PD 1 does not cause much damage to the retina, therefore it is not a good model for studying neuroprotection on retina. At least 2x MSG treatment is necessery for the retina degeneration model causing enough damage to the retina for studying neuroprotection. Applying MSG, through possible increases in the intracellular Ca^{2+} level, cell death can be induced.

The Ca^{2+}-binding proteins may be able to buffer the increased Ca^{2+} level. Based on our experience we can declare that both the AII amacrine (parvalbumin-positive) and the ganglion cells (calretinin-positive) were damaged. Also, the horizontal cells (calbindin-positive) changed: the diamater of the their cell body increased. This is a symptom of glutamate excitotoxicity. The function of the dopaminergic cells depend on the absorbed light, and the light-driven synaptic inputs. Since the MSG treatment ruins these inputs, the dopaminergic (TH-immunolabeled) cells are also damaged.

The BCCAO-induced ischemia caused retina degeneration that may be a consequence of stimulated neurotransmitter exocytosis, glial disfunction, increase of the intracellular Ca^{2+} level, accumulating free radicals, increasing NO level and the appearance of the toxic mediator compounds exocytosed by imflammatory cells, for example, tumour necrosis factor or interleukin-1. These complete cascade processes lead to the degradation of the different groups of cells or the whole retina and depend on the strength and/or time of the events caused by retinal ischemia. In these processes both the outer nuclear and the outer plexiform layers were damaged, compared with MSG treatment, where almost the entire inner retina was degenerated.

The retina has a dual blood supply, reflecting its embryological origins. The photoreceptors, including their cell bodies in the outer nuclear layer, and the greater portion of the outer plexiform layer are nourished indirectly from the choriocapillaris, a richly anastomotic vascular layer that corresponds to the pia-arachnoid vessels in the rest of the brain. The inner retinal layers are nourished by branches of the central retinal artery, which arises directly from the ophthalmic artery in the region of the optic foramen. In our experiment, we ligated carotis communis artery on one side. The inner retina was much more sensitive to hypoxic conditions, but the entire retina was damaged to a certain extent.

In conclusion, all three retina degeneration models used in this study can be utilized to examine neuroprotection. Further experiments will be done to identify the cell types which suffer damage and the mechanisms of degeneration.

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