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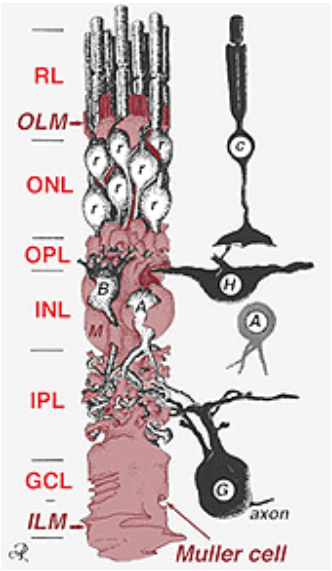

Stefanie M. Hauck

Dissertation

zur Erlangung des Doktorgrades

an der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

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vorgelegt von Stefanie M. Hauck

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drawing of retinal Mueller glia

by A. Reichenbach

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe.

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SUMMARY

A major cause of blindness in the Western world is degeneration of photoreceptors as a result of point mutations in genes coding for either phototransduction-related proteins or other proteins important for retinal function. Despite the diversity of mutated genes and proteins involved in this heterogeneous group of progressive retinal dystrophies with homologous phenotypes, the final event leading to blindness is apoptosis of photoreceptors. This has led to intensive studies of the effects of neuroprotective agents on the survival of photoreceptors in animal models of retinitis pigmentosa. One such effective molecule discovered to date to exert substantial rescue of retinal photoreceptors is glial cell line-derived neurotrophic factor (GDNF). However, the molecular mechanism of action underlying GDNF-mediated neuroprotection remains unresolved. This dissertation and the herein described studies were carried out with the goal of elucidating neuroprotective mechanisms using the porcine retina as a model. This species was selected due to its morphological and anatomical similarities to human retina. In order to clarify possible cellular mechanisms involved in neuroprotection, the initial studies involved analysis of GDNF action in porcine retina. It soon became evident that the GDNF-receptive cell in retina was not the photoreceptor itself but rather retinal Mueller glial cells (RMG), which are the major retinal glial cells. Thus, primary RMG cell cultures prepared from porcine retina were established and characterised to analyse this cell type without extraneous effects from the retinal environment. Proteomic profiling revealed profound changes in expression of RMG-specific marker proteins as an effect of *in vitro* conditions. Thus, the *in vitro* experiments for studying GDNF-induced signalling were performed with primary RMG cultures in an early state (two weeks *in vitro*) in order to study cells resembling the *in vivo* phenotype. GDNF was found to induce the ERK, SAPK and PKB/AKT pathways, as well as upregulating basic fibroblast growth factor (bFGF). Application of bFGF to primary porcine photoreceptors *in vitro* promoted a concentration-dependent rescue. Therefore a model of RMG-mediated indirect survival promoting mechanism induced by GDNF could be proposed. The finding that RMG are mediators of photoreceptor survival prompted further screenings for RMG-specific, secreted molecules promoting photoreceptor survival. A large-scale primary photoreceptor survival assay (96well format) was developed, in which RMG-conditioned medium (RMG-CM) was tested for survival activity. Conditioned medium was observed as having specific photoreceptor survival-promoting activity stemming from previously unidentified protein/s. Reducing the complexity of RMG-CM by anionic chromatography revealed that the activity does not bind

to anionic resins. Mass spectrometric identifications of the mono-Q flow-through identified 23 different proteins from the active fraction, among them three potential new candidates for neuroprotective activity in the context of photoreceptor survival: connective tissue growth factor (CTGF), insulin-like growth factor binding protein 5 (IGFBP5) and insulin-like growth factor binding protein 7 (IGFBP7). Expression cloning and re-testing of these candidates for their ability to promote photoreceptor survival revealed that CTGF and IGFBP5 were effective in protecting photoreceptors when applied in combination with the RMG-conditioned media. Taken together, these results indicate that such survival-promoting activity is multi-factorial.

RMG are likely to support photoreceptors by either cell to cell-mediated paracrine signalling or by secreting factors into the intercellular space between retina and retinal pigment epithelium, which consists of a complex matrix of proteins and polysaccharides. This matrix, designated as interphotoreceptor matrix (IPM), directly borders three cell types: photoreceptors, RMG and the retinal pigment epithelium and predisposes the IPM to function as repository of neuroprotective molecules possibly secreted from adjacent cells to protect and support photoreceptors. In order to identify such novel neuroprotective substances, the composition of IPM was investigated in this thesis by comparative proteomics. Over 140 different proteins were identified, the majority of which had never been previously detected in the IPM. Among these, 13 candidates were found, which in other tissue systems have been already reported to have a functional role in neuroprotection.

INTRODUCTION

1 Retina

1.1 The structure of the mammalian retina

The retina is a specialized sensory organ capable of transforming light into electric signals that are transmitted via the optic nerve to the visual centers of the brain. The retina derives during embryogenesis from the neuroectoderm, a part of the ectoderm that gives rise to formation of the central nervous system (CNS). The mature mammalian retina consists of two distinct tissues: the neural retina composed of neurons and glial cells, and the retinal pigmented epithelium (RPE), a single epithelial cell layer (Figure 1). The cells of the neural retina derive from multipotent progenitor cells and their differentiation follows a precise chronological order that is found in many species (Cepko, 1993). The mature neural retina shows a highly organized structure composed of three cellular layers: the outer nuclear layer (ONL) composed of photoreceptors, the inner nuclear layer (INL) containing neurons (horizontal, bipolar, amacrine and interplexiform cells) and retinal Mueller glial cells (RMG), and the ganglion cell layer (GCL) that contains in addition to ganglion cells also displaced amacrine cells and astrocytes. Two synaptic layers separate these nuclear layers: the outer plexiform layer (OPL) and the inner plexiform layer (IPL). The axons of the ganglion cells converge to the exit of the optic nerve, forming the nerve fiber layer.

1.2 The retinal pigmented epithelium (RPE)

The RPE consists of a single layer of cuboid shaped epithelial cells, situated between the photoreceptors of the neural retina and the choroid, where it controls the flow of nutrients from the choroidal vascular system to the retina. The RPE cells are highly polarized, the basal cell membranes being in contact with Bruch's membrane and highly folded to increase the surface area allowing the exchange of metabolites, for example retinol, from circulation (Bok, 1999). The apical membranes of RPE cells terminate in numerous long microvilli that intercalate with the photoreceptor outer segments.

The RPE cells are indispensable for the development and maintenance of the neural retina (Raymond and Jackson, 1995). They participate in the formation of the blood-retina

barrier and control the transport of ions and metabolites that circle through the retina. RPE cells phagocytose continuously the shed discs of photoreceptor outer segments, and recycle the visual pigments (Young, 1978; Bok, 1985; Clark, 1986).

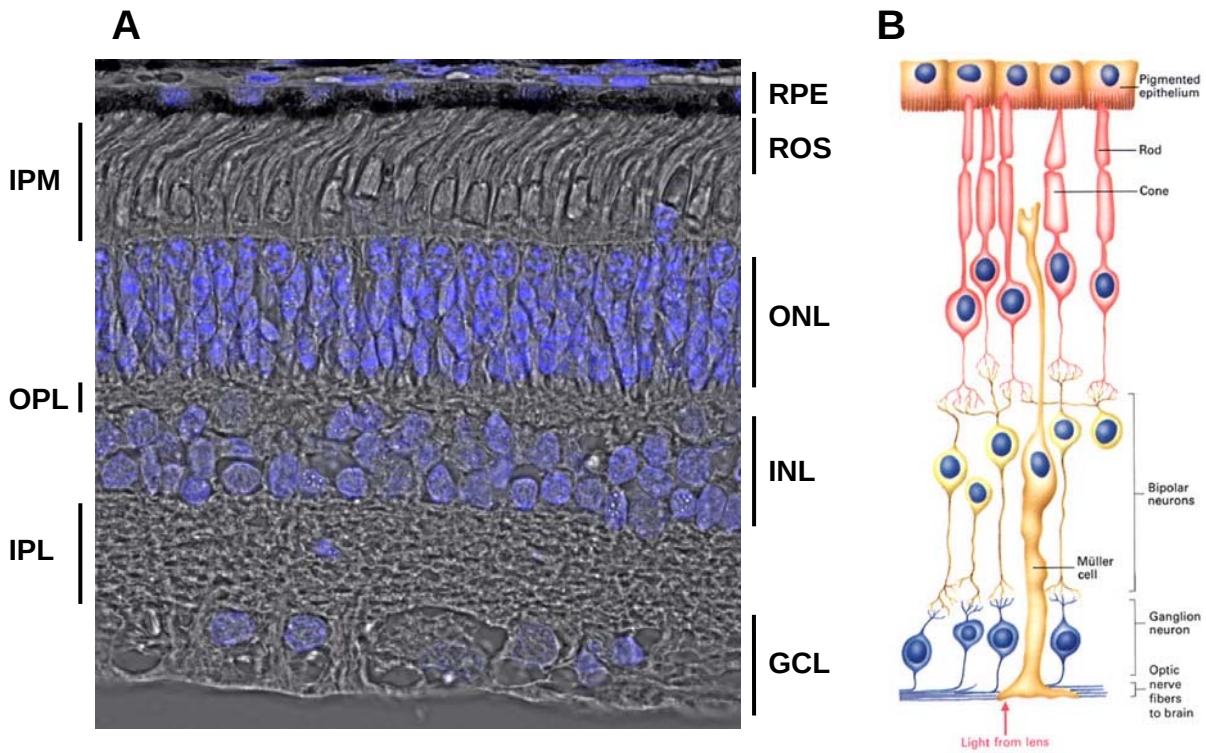

Figure 1: Cellular structure of the retina

A: Porcine eye was fixed and embedded in paraffin, sectioned (5 µm) and nuclei were stained with DAPI. Nomarski image and blue fluorescence image were overlaid to visualize retinal structures. RPE: retinal pigment epithelium, ROS: rod outer segments, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer, IPM: interphotoreceptor matrix, OPL: outer plexiform layer, IPL: inner plexiform layer.
B: Schematic of retinal cellular structure

2 Retinal neurons

Transformation of the light signal into electric signal is performed by photoreceptor cells of the retina; further transmission and processing of the signal within the retina is conducted by different functional classes of neurons. Bipolar and ganglion cells transmit the signals along a "vertical" direct pathway, whereas horizontal, amacrine and interplexiform cells modulate the signals.

2.1 Photoreceptors

Photoreceptors are polarized neurons that capture light and transform this energy into a chemical message through a process called phototransduction. Their cell bodies are

localized in the outer nuclear layer and are in tight contact with the RPE and retinal Müller glial cells (RMG), forming synapses with cells of the inner nuclear layer (bipolar and horizontal cells). Photoreceptors are the most abundant cell type in the retina and are divided into two types, rods and cones. Rods are responsible for scotopic or nocturnal vision whereas cones recept photopic or diurnal color vision.

The unique morphology of photoreceptors distinguishes them from other neurons of the CNS. They have a very short axon and a specialized dendrite constituting the inner and outer segment joined by a connecting cilium (Figure 4B). The inner segment and the cell body contain the majority of cell organelles involved in metabolic activities. The rod outer segment is composed of a stack of flattened discs, surrounded by the plasma membrane. The visual pigment rhodopsin is densely packed in the disc membranes but is also found to a lesser degree in the surrounding plasma membrane. The cone outer segment differs from the rod in that increased surface area is achieved by repeated enfolding of the plasma membrane. The cone outer segment is usually shorter than that of the rod and tapers in the distal direction.

The adult human retina contains about 96 million photoreceptors, of which approximately 5% are cones, the remainder are rod photoreceptors (Curcio et al., 1990). In mouse, only 1% of the photoreceptors are cones (Jeon et al., 1998). Physiologically, humans are trichromats and their cones are separated into three types: dependent on the expression of different opsins, the cells are sensitive to short- (S), middle- (M), or long- (L)-wavelength light (Nathans et al., 1986). Mice are dichromats expressing an M opsin and an ultraviolet (UV)-wavelength sensitive opsin (Jacobs et al., 1991).

2.2 Bipolar cells

Bipolar cells are stimulated by photoreceptors and transmit their signals to the ganglion cells. Glutamate, the photoreceptor neurotransmitter is constantly released in the dark (Trifonov, 1968) rendering the photoreceptor depolarized. Upon light stimulation the photoreceptor responds with a hyperpolarization, and inhibition of transmitter release. The postsynaptic bipolar cells respond with either hyperpolarization or depolarization of their membranes. The hyperpolarizing type of bipolar cell is called an OFF-center cell while the depolarizing bipolar cell is called an ON-center cell (Kolb, 2001). Rod photoreceptors transfer the signal to ON bipolar cells, cone photoreceptors signal to either ON or OFF bipolar cells (see Figure 2). The cone-derived parallel sets of visual channels for ON (detecting light areas on dark backgrounds) and OFF (detecting dark areas on light backgrounds; e.g. black letters on white paper) qualities of an image are a fundamental for detection of contrast in images.

Signals from ON and OFF bipolar cells stimulated by cones are transmitted to ganglion cells, signals from ON bipolar cells stimulated by rods are transmitted to amacrine cells.

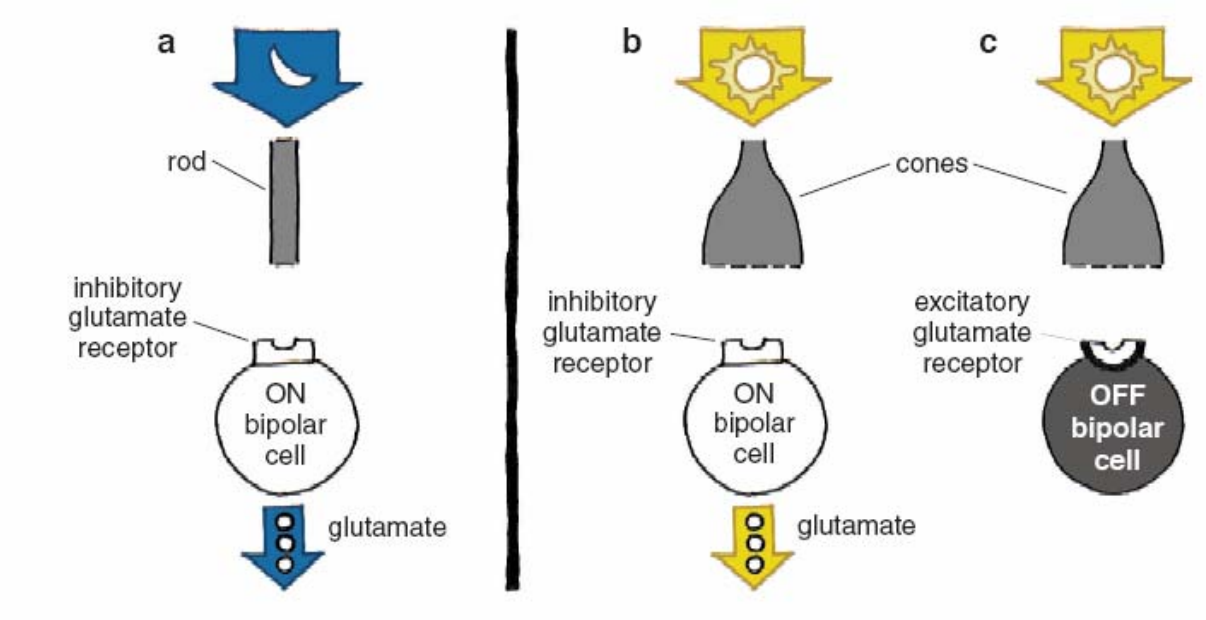

Figure 2: ON and OFF pathways along bipolar cells

Photoreceptors transmit information to bipolar cells using the molecule glutamate, but different bipolar cells respond differently to the presence of the molecule; some fire in response, whereas others cease firing, depending on the kind of glutamate receptor on their surface. ON bipolar cells have a depolarizing receptive field *(a, b)*; OFF cells have a hyperpolarizing receptive field *(c)*. Contrary to what one might expect, photoreceptors *stop* releasing glutamate when stimulated by light, in turn causing ON bipolar cells to release glutamate.
(from http://www.americanscientist.org/articles/03articles/kolb.html)

2.3 Horizontal cells

The horizontal cells modulate the electric signal coming from photoreceptors laterally and provide a first level of treatment of visual information. They are situated in the inner nuclear layer neighboring the outer plexiform layer where they form synapses with photoreceptors. Morphologically two types of horizontal cells are described for primates: type I horizontal cells that form dendritic connections with cones and axonal connections with rods, and type II horizontal cells that are uniquely connected to cones. Each horizontal cell receives input from many cones, which results in a large receptive field. This receptive field is even enlarged by close contact between different horizontal cells through gap junctions.

Horizontal cells sharpen the contrasts of the visual image by the so-called "center surround" organisation: if stimulated close to the center of the large horizontal cell, it transfers an ON signal, but if stimulated in the periphery, it sends an OFF signal. Without this first level of signal processing, the images would remain coarse-grained and blurry (Kolb, 2001).

2.4 Amacrine cells

The amacrine cells play an important role in transmitting information from rod photoreceptors to ganglion cells. The amacrine cells collect messages from many rod-connected bipolar cells, allowing the perception of very dim light and modulate visual information at the level of the inner plexiform layer, where they establish synapses with bipolar, ganglion, interplexiform or other amacrine cells. Two major types of amacrine cells exist: small-field AII cells, which interconnect approximately 30 bipolar cells and wide-field A17 cells, which may collect signals from thousands of rod-bipolar cells. A17 amacrine cells usually emit GABA as neurotransmitter and additionally release at least one other neuroactive substance. Those secondary substances are neuromodulators, e.g. substance p, somatostatin, vasointestinal peptide, serotonin, dopamine, acetylcholine, adenosine or nitric oxide, and they rather act more slowly and at longer distances than neurotransmitters. Neuromodulating substances are believed to influence retinal circuitry under changing light conditions and change retinal activity in response to different day-times as given by the circadian-clock (Kolb, 2001).

2.5 Ganglion cells

The ganglion cells receive their information directly from cone-bipolar cells or from amacrine cells (rod-derived signals) and transmit action potentials along their axons to higher brain centers for the treatment of visual information. The converging ganglion cell axons constitute the optic nerve. In human retinas, two basic types of ganglion cells exist that receive signals from cone-bipolar cells: ON-center ganglion cells are activated when a spot of light falls in the center of their receptive field and are inactivated when light falls on the cell's periphery (see Figure 3, left panel). OFF-center ganglion cells react the opposite way (see Figure 3, left panel). In the human fovea, which contains only cone photoreceptors, a different type of ganglion cells exists: the midget ganglion cell, connected in a one-to-one ratio with midget bipolar cells (see Figure 3, right panel). The one-to-one signal transmission produces a point-to-point image from the fovea transferred to the brain (Kolb, 2001).

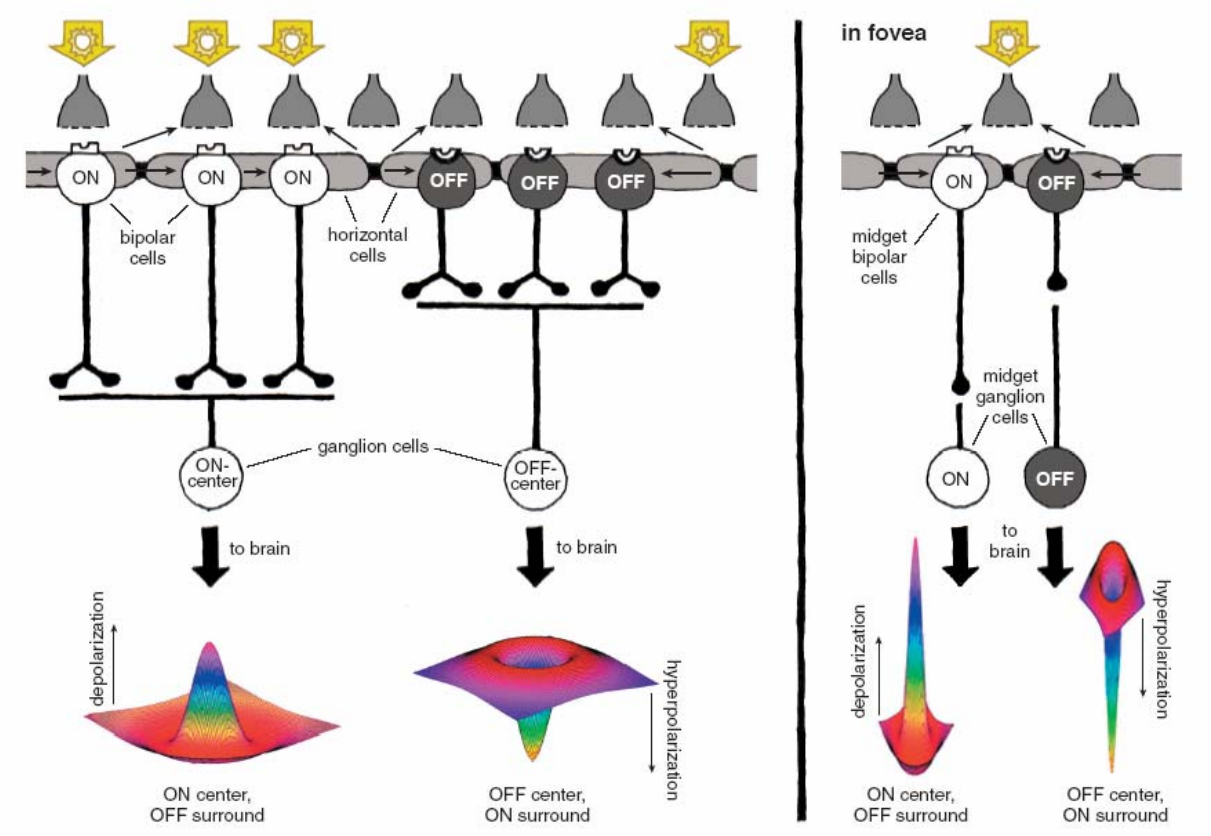

Figure 3: Processing of cone-derived signals along ON and OFF pathways

Human retinas have two types of ganglion cells: ON-center and OFF-center cells. ON center ganglion cells are activated when a spot of light falls in the center of their receptive fields, whereas OFF-center ganglion cells fire in response to light falling on their fields' periphery leaving their center dark. Horizontal cells convey antagonistic surround signals to bipolar cells and thence to ganglion cells. Ganglion cells have receptive fields with a Mexican-hat shape, reflecting their integration of opposing information about centers and surrounds. This kind of processing helps sharpen the boundaries of images. In the fovea *(right)*, ganglion cells have much narrower receptive fields; in fact, each carries information from a single cone. A cone feeds information to two of these midget ganglion cells; at all times each foveal cone transmits either an ON or an OFF signal to the brain. This signal also carries a colour message regarding the type of cone (red or green) it comes from.
(from http://www.americanscientist.org/articles/03articles/kolb.html)

2.6 Interplexiform cells

The interplexiform cells represent a rare cell type in the retina and modulate information in the inner nuclear layer. Interplexiform cells extend their neurites in the outer and inner plexiform layers or establish synaptic contacts with other neurons in the inner nuclear layer. In primates two types have been described based on their neurotransmitter: GABA-positive interplexiform cells and tyrosine-hydroxylase-positive interplexiform cells.

3 Retinal glial cells

Three different types of glial cells are found in mammalian retina: retinal Mueller glia, astrocytes and microglia.

3.1 Retinal Mueller glial cells (RMG)

The RMG are the principal glial cells of the retina. They are elongated cells that span radially throughout the entire neural retina and make close contact to every retinal cell type (Figure 4 A). The terminations of the RMG form the inner and outer limiting membrane, while their cell bodies are situated in the inner nuclear layer. The apical portions of the RMG envelop completely the photoreceptor cell bodies within the outer nuclear layer, and secondary extensions envelop dendritic or axonal processes of other retinal neurons (see Figure 4). RMG derive from the same progenitor cells as neurons during retinal development and they play a primordial role in the orientation, migration and movement of neurons (Rakic, 1981). In the mature retina RMG act as support cells but also possess crucial metabolic functions, important for the survival of retinal neurons. In detail RMG are involved in a wide range of functions:

(1) regulation of the extracellular homeostasis, pH and water content of the extracellular space, as well as homeostasis of K^+ ions: active retinal neurons release K^+ ions, and RMG siphon K^+ from the extracellular space and redistribute it into different sinks, such as the vitreous body, blood vessels, and the subretinal space (Karwoski et al., 1989; Newman et al., 1984; Reichenbach et al., 1992);

(2) delivery of trophic substances to neurons and removal of metabolic endproducts (Poitry, et al., 2000); and

(3) metabolism of glucose to lactose, which is preferentially taken up by photoreceptors as a source of energy for their oxidative metabolism (Poitry-Yamate et al., 1995). In addition, RMG contribute to neuronal information processes through the fast reuptake of released neurotransmitters, by providing neurons with precursors of neurotransmitters and by reabsorbing neuronally released glutamate via the glutamate-aspartate transporter (GLAST) (Derouiche and Rauen, 1995; Otori et al., 1994); the latter is expressed exclusively by RMG and astrocytes. Glutamate reuptake is crucial for the rapid termination of light-evoked activity in retinal ganglion cells (Matsui et al., 1999), as well as maintaining low extracellular glutamate levels for prevention of glutamate excitotoxicity and disturbances in neuronal processing. The main degradation pathways of glutamate in RMG are the synthesis of glutamine by glutamine synthetase (Linser and Moscona, 1979) and the synthesis of

glutathione from glutamate, cysteine, and glycine; glutathione acts as an intraretinal antioxidant (Huster et al., 1998; Makar et al., 1994). Moreover, supported by increasing amount of evidence (Bringmann and Reichenbach, 2001), RMG also support photoreceptor and ganglion cell survival by yet unknown mechanisms (Garcia et al., 2002). RMG have been shown to play a crucial role in trophic factor-induced signalling within the retina (Wahlin et al., 2000) and are implicated in mediating photoreceptor rescue effects of several neurotrophic factors (Frasson et al., 1999a, Wahlin et al., 2001).

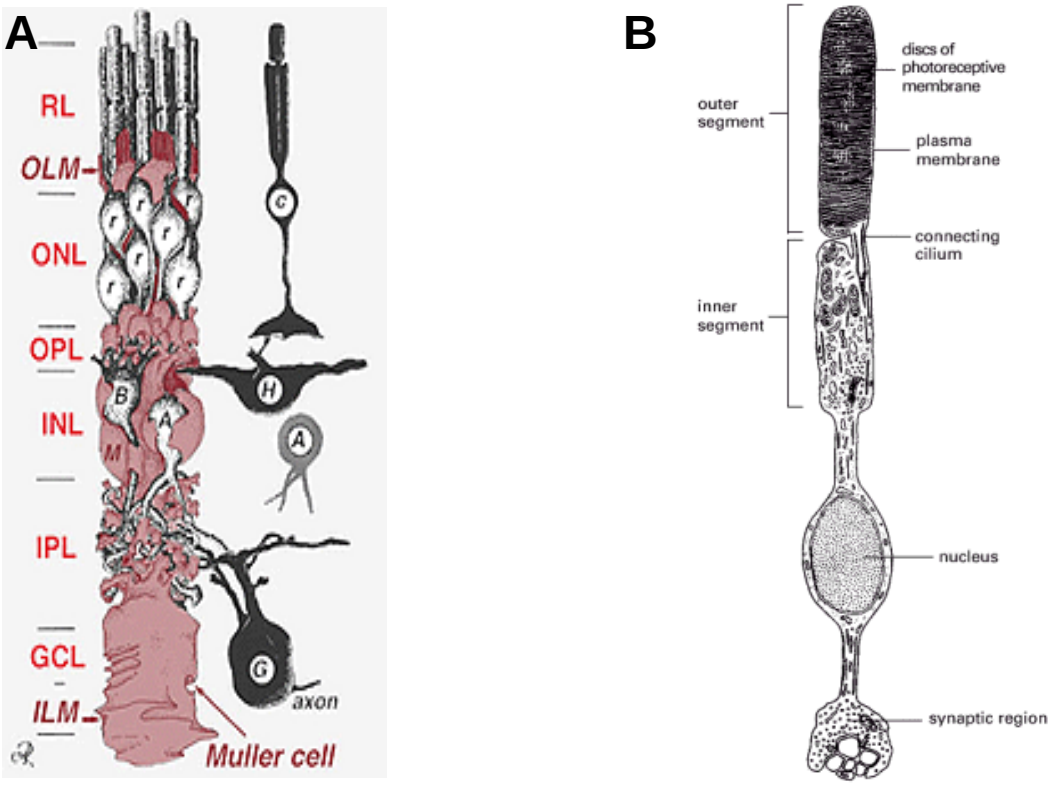

Figure 4: (A) Retinal Mueller glial cell (RMG) and (B) rod photoreceptor

A: Retinal Mueller glial cells span the entire thickness of the retina and ensheath every neuron. They form the outer limiting membrane (OLM) and the inner limiting membrane (ILM). RL: receptor layer, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer, IPM: interphotoreceptor matrix, OPL: outer plexiform layer, IPL: inner plexiform layer (drawing by A. Reichenbach).
B: Rod photoreceptor cell is highly compartimentalised in an outer segment, where light is recepted and phototransduction occurs, an inner segment containing most organelles, and the cell body with the nucleous and the synaptic region, where the photoreceptor cells form synapses with bipolar cells (picture from “Molecular Biology of the Cell”, New York, 2002)

3.2 Astrocytes

Astrocytes are not intrinsic to the retina, but during development they migrate along the optic nerve and insert into the retina (Watanabe and Raff, 1988). The positioning of astrocytes in the retina depends on the co-migration of vascular endothelium cell precursors (Chan-Ling and Stone, 1991); this explains their absence in non-vascularized retinas. In the mature retina, astrocytes are found in the ganglion cell layer and optic fiber layer where also numerous blood vessels are situated (Bussow, 1980). Astrocytes play a role in the construction of the blood-retina barrier and can modulate the growth of endothelial cells (Jiang et al., 1993).

3.3 Microglia

The third glial cell type in the retina is originating from mesoderm and not from ectoderm like RMG and astrocytes. These cells enter the retina along with mesenchymal precursors of retinal blood vessels in development (Chan-Ling, 1994). Microglial cells are found in every retinal layer and can be stimulated into macrophagic function upon trauma to the retina (http://webvision.med.utah.edu/).

4 Interphotoreceptor matrix

The interphotoreceptor matrix (IPM) is a carbohydrate-rich complex occupying the extracellular compartment between the outer neural retina and the apical surface of the retinal pigment epithelium (RPE) in the vertebrate eye (Figure 1) (Rohlich, 1970). As such, it surrounds the elongate, light sensitive photoreceptors extending from the outer retinal surface. Several structural and functional activities, with a fundamental importance to vision have been proposed to occur within the IPM, including visual pigment chromophore exchange, retinal adhesion, metabolite trafficking, photoreceptor alignment and cell-cell interactions, thought to be involved in photoreceptor membrane turnover (reviewed in Hageman and Johnson, 1991).

IPM consists of two different structural compartments, which are classified by their solubility in phosphate buffered saline (PBS): saline rinses of the outer retinal surface removes “soluble” IPM components, such as interphotoreceptor retinoid-binding protein (IRBP), a variety of enzymes, mucins and immunoglobins (Hageman and Johnson, 1991). Resistant to saline rinses are the distinct matrix domains surrounding rod and cone photoreceptors which are accordingly called “insoluble” IPM. Nevertheless, these IPM

components can be released as a relatively intact, sheet-like unit by hypotonic treatment of the retina (Hollyfield et al., 1990a; Hollyfield et al., 1990b; Johnson and Hageman, 1991). "Insoluble" IPM consists of a scaffold of hyaluronan (HA) to which the secreted glycoprotein SPACR and chondroitin sulfate proteoglycan SPACRCAN bind via HA-binding motifs in their polypeptides (Hollyfield, 1999). Other molecules containing specific HA-binding motifs have been identified in the plasma membranes of adjacent cells, e.g. CD44 expressed in the apical region of retinal Mueller glial cells (RMG) (Chaitin, et al., 1994) and RHAMM in the apical region of RPE (Chaitin, et al., 1999) (see Figure 5).

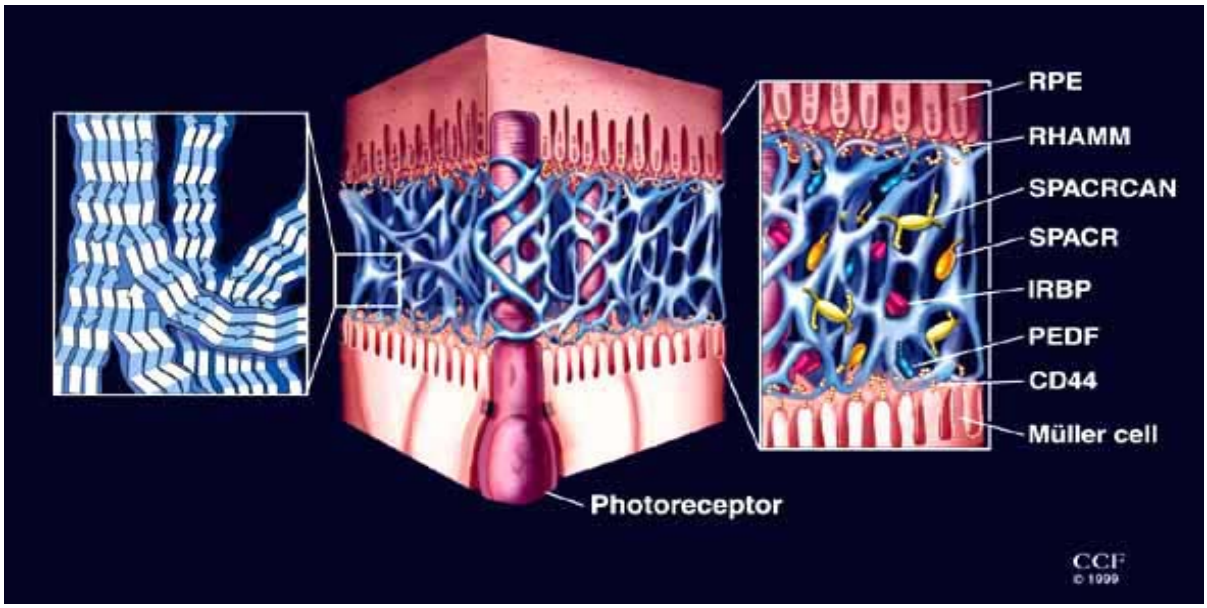

Figure 5: The structure of Interphotoreceptor Matrix

The left panel depicts the possible antiparallel alignment of linear hyaluronan molecules forming the basic matrix scaffold structure. The center panel depicts the continuous three-dimensional scaffold complex (not to scale) in the extracellular compartment, adapted from electron microscope images of the IPM (http://www.glycoforum.gr.jp/science/hyaluronan/). The right panel depicts the interaction of the scaffold (not to scale) with hyaluronan-binding motifs on cells that border the IPM: CD44 is present in the apical microvilli of Müller cells, and RHAMM has been identified on apical RPE processes) and secreted molecules within the IPM (SPACR, SPACRCAN, pigment epithelium derived factor PEDF and interphotoreceptor matrix-binding protein IRBP). Since IRBP can be removed from the IPM with saline rinses, it is not considered part of this insoluble IPM complex. (Drawing by David Schumick, Medical Illustrator, the Cleveland Clinic Foundation.)

Additionally to structural proteins, some ubiquitous growth, survival and neurotrophic factors have been identified in IPM of normal and pathological retinas: basic fibroblast growth factor (bFGF) (Hageman and Johnson, 1991; Gao and Hollyfield, 1992; Gao and Hollyfield, 1995; Hanneken and Baird, 1992), insulin-like growth factor 1 (IGF-I) and

insulin-like growth factor binding-protein (IGF-BP) (Waldbillig et al., 1991). Pigment-epithelium derived factor (PEDF), a member of the serine protease inhibitor family (Steele et al., 1993) is presumably secreted by RPE cells into IPM (Becerra et al., 2004; Tombran-Tink et al., 1995). PEDF carries a RHAMM-type motif in its primary sequence through which it binds HA, being thus strongly attached to the insoluble IPM scaffold (Hollyfield, 1999).

5 Phototransduction

Light enters the mammalian eye through the pupil and after transversion of the vitreous body enters the retina. The photons pass all retinal layers and are finally absorbed at the outer segments of rod and cone photoreceptors where the electromagnetic wave is transformed into an electrochemical signal (phototransduction) (reviewed in Molday, 1998). The phototransduction cascade (Figure 6) in rods is initiated by rhodopsin, a seven-pass transmembrane protein covalently linked to an 11-cis retinal chromophore. Photoexitation converts 11-cis retinal to its all-trans isomer, creating Meta II rhodopsin, which catalyses the activation of the G-protein transducin. This in turn leads to the activation of phosphodiesterase (PDE), which hydrolyses cGMP to 5´-GMP. One rhodopsin molecule can activate multiple PDE molecules, amplifying the signal. The decrease in intracellular cGMP causes the cGMP-gated cation channels in the outer segment membrane to close. Without the balanced influx of Ca^{2+} the cell becomes hyperpolarized. This leads to inhibition of release of neurotransmitter glutamate from the synaptic region of the photoreceptors and subsequently to the generation of an electric signal in the downstream neuron of the activated photoreceptor (bipolar cell) which is finally sent to the visual cortex.

The resting phase is restored when Meta II rhodopsin is inactivated by rhodopsin kinase and binds to arrestin (S-antigen). Transducin and PDE are inactivated and disassociate due to the hydrolysis of the bound GTP by intrinsic GTPase activity of the transducin α subunit. The low level of intracellular Ca^{2+} caused by the closure of the cGMP-gated channels activates guanylate cyclase, which synthesizes cGMP. As intracellular levels of cGMP increase, the cGMP-gated Na^{+} and Ca^{2+} channels reopen and a depolarized dark state is re-established. As Ca^{2+} levels raise again, guanylate cyclase activity is inhibited and cGMP synthesis returns to basal levels.

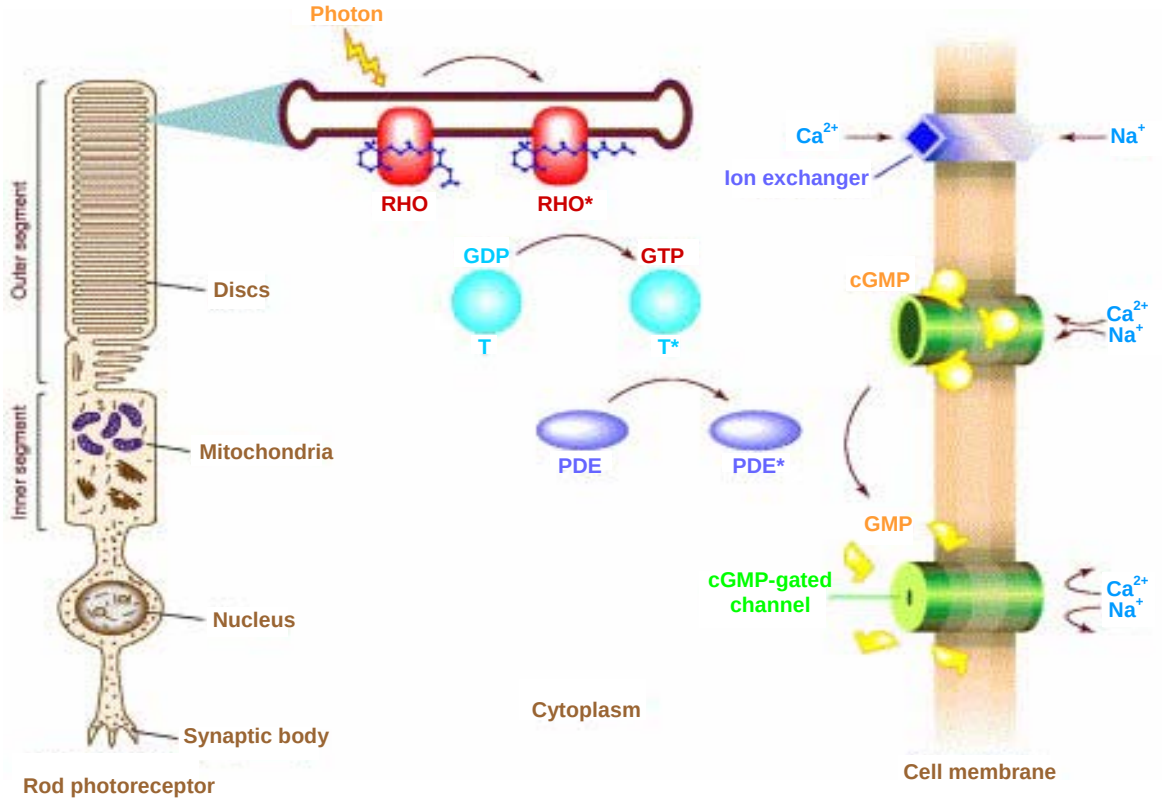

Figure 6: Phototransduction cascade

Light induced phototransduction takes place in the membrane discs of rod outer segments. The absorbtion of a photon induces the formation of activated rhodopsin, which then activates transducin. Activated transducin activates phosphodiesterase, which then catalyses the hydrolysis of cyclicGMP to GMP. This leads to a closure of cGMP-gated cation channels in the outer segment membrane and to a hyperpolarisation of the photoreceptor cell (from Farrar et al., 2002).

6 Retinal degeneration

In some eye diseases the retina becomes damaged or compromised and degenerative changes eventually lead to death of the retinal neurons and, as a consequence to loss of vision. Major retinal pathologies include glaucoma, where elevated pressure in the eye leads to death of retinal ganglion cells; diabetic retinopathy, where blood vessels in the retina become leaky with serious consequences; age related macular degeneration and retinitis pigmentosa.

6.1 Inherited degenerative diseases

6.1.1 Retinitis pigmentosa:

The disease named retinitis pigmentosa (RP) describes a heterogeneous group of inherited progressive retinal dystrophies, primarily affecting the photoreceptors. Main symptoms include progressive loss of visual functions with night blindness, narrowing of the visual field, reduced central vision and increasing sensitivity to glare. It is the most common inherited retinal dystrophy, affecting approximately 1 in 3,500 people or around 2 million patients worldwide (Hims et al., 2003). RP can be inherited in autosomal dominant, autosomal recessive, X-linked and rare mitochondrial and digenic forms. Mutations in many different genes have been shown to cause retinal degenerations and to date 39 loci have been found for non-syndromic RP (retinal degeneration in the absence of other defects). For 30 of these loci the disease-causing gene has been identified, for 9 loci the responsible gene-mutation remains unknown. Further 33 genes or loci have been implicated in various syndromic forms of RP and in total, 158 genes or loci have been shown to underlie all the different forms of human retinal dystrophies (RetNet; http://www.sph.uth.tmc.edu/RetNet/).
The knowledge of 30 different genes causing non-syndromic RP when mutated, enabled a functional grouping of the protein products of these genes (summarized in Table 1; adapted from Hims et al., 2003).

Table 1 (according to Hims et al., 2003): adRP: autosomal dominant RP; arRP: autosomal recessive RP; adCSNB: autosomal dominant congenital stationary night blindness; arCSNB: autosomal recessive congenital stationary night blindness; LCA: Leber congenital amaurosis

Gene:	Protein:	Associated dystrophies:
1. phototransduction cascade		
RHO	Rhodopsin	adRP, arRP and adCSNB
PDE6A	Phosphodesterase α subunit	arRP
PDE6B	Phosphodesterase β subunit	arRP
SAG	Arrestin	arRP, arCSNB
CNGA1	Rod cGMP-gated channel α subunit	arRP
CNGB1	Rod cGMP-gated channel β subunit	arRP
2. visual cycle		
RPE65	Retinal pigment epithelium-specific 65kd protein	arRP, LCA
RLBP	Cellular retinaldehyde-binding protein	arRP, rRPalbescens
ABCA4	ATP-binding cassette transporter	arRP, CRD, AMD
LRAT	Lecithin retinol acyltransferase	arRP
RGR	RPE-retinal G protein-coupled receptor	arRP, dominant choroidal sclerosis
3. structural proteins		
RDS	Peripherin/RDS	adRP, LCA, adCRD
ROM1	Retinal outer segment protein 1	adRP
RHO	Rhodopsin	adRP, arRP and adCSNB
4. transcription factors		
CRX	Cone-rod otx-like homeobox transcription factor	adRP
NRL	Neural retinal leucine zipper	adRP
NR2E3	Nuclear receptor subfamily 2 group E3	adRP
5. splicing factors		
PRPF8	Pre-mRNA processing factor 8	adRP
PRPF3	Pre-mRNA processing factor 3	adRP
PRPF31	Pre-mRNA processing factor 31	adRP
6. intracellular transport		
FSCN2	Retinal fascin	adRP
RP1	RP1 protein	adRP
RPGR	Retinitis pigmentosa GTPase regulator	X-linked RP
RP2	RP2 protein	X-linked RP
TULP1	Tubby-like protein	arRP
7. cell-cell adhesion/signalling		
MERTK	C-mer proto-oncogene receptor tyrosine kinase	arRP
CRB1	Crumbs homolog 1	arRP, LCA
USH2A	Usherin	arRP, Ushers syndrome
8. miscellaneous		
IMPDH1	Inosine monophosphate dehydrogenase 1	adRP
RP9	RP9 protein	adRP
CERKL	Ceramide kinase	arRP

Mutations for non-syndromic RP are found in all major proteins involved in phototransduction cascade, namely rhodopsin, phosphodiesterase, arrestin and cGMP-gated channel (see Table 1). For rhodopsin alone, over 100 different disease-causing mutations have been reported. Additionally to RP, mutations in components of the phototransduction cascade have also been implicated in other retinal dystrophies: retina-specific guanylate cyclase (GUCY2D) in cone-rod dystrophy or Leber´s amaurosis; guanyate cyclase-activating protein 1A (GUCA1A) in cone dystrophy; rhodopsin kinase (RHOK) and rod transducin a subunit (GNAT1) in recessive CSNB; cone cyclic nucleotide-gated channel (CNGA3) in achromatopsia (RetNet; http://www.sph.uth.tmc.edu/RetNet/sum-dis.htm).

The visual cycle, reviewed by Thompson and Gal (2003), is the name given to a series of biochemical steps that recycle the chromophore component of rhodopsin. 11-cis retinal is converted by photoexcitation into all-trans retinal, which is then released from rhodopsin, bound to phosphatidylethanolamine and transported from the interior of the rod-discs into photoreceptor cytoplasm by the ATP-binding cassette transporter (ABCA4). Within the cytoplasm the chromophore is converted into all-trans retinol and released from the cell for transportation to the RPE (bound to transporting protein IRBP). Inside the RPE, all-trans retinol is bound to cytosolic retinoid-binding protein (CRBP) and is esterified by lecithin retinol transferase (LRAT) to all-trans retinol ester, followed by isomerisation into 11-cis-retinol, a process believed to involve the RPE-specific protein RPE65. 11-cis retinol is then bound by cellular retinaldehyde-binding protein (CRALBP) and converted into 11-cis retinal by catalysis of 11-cis dehydrogenase 5 (RDH5). The 11-cis retinal is again transported via the IPM (and bound to IRBP) from the RPE to the photoreceptor and subsequently bound to rhodopsin, thus completing the cycle. Mutations causing recessive RP have been found in four genes encoding components of the visual cycle, namely RPE65, CRALBP, LRAT and ABCA4 (see Table 1).

Several structural proteins have been implicated in RP when mutated: the photoreceptor-specific proteins peripherin/RDS and ROM1 are thought to be vital for the maintenance of the flattened shape of the discs in the photoreceptor outer segments. Additionally, rhodopsin, which accounts for 70% of outer segment protein content (Hamm and Bownds, 1986), has been suggested to have an important role in the morphology of the rod outer segments (van Soest et al., 1999)

The transcription factors encoded by CRX, NRL and NR2E3 genes, namely cone-rod homeobox protein, neural retina-specific leucine zipper protein and photoreceptor-specific nuclear receptor, are all thought to be specifically involved in the control of many

photoreceptor-specific genes (Furukawa et al., 1997; Kobayashi et al., 1999; Kumar et al., 1996). This explains why mutations described for those transcription factors cause different retinal degeneration phenotypes (see Table 1).

Most astonishingly, mutations in three different pre-mRNA splicing factors, which are ubiquitously expressed in all cells of the body and not only in retinal cells, have been identified to cause autosomal dominant RP in patients without other pathologic phenotype. The implicated genes PRPF8, PRPF31 and HPRP3 all encode small nuclear ribonucleoprotein (snRNP) proteins that are essential components of the U4/U6:U5 tri-snRNP which is a major component of the spliceosome (McKie et al., 2001; Vithana et al., 2001; Chakarova et al., 2002). All three splicing factors are expressed ubiquitously and it is unresolved why their mutation leads to a dominant retina-specific disease. It has been speculated that the continuous need of large amounts of proteins of the phototransduction cascade renders the photoreceptors particularly vulnerable to defects in translation machinery. Or else, a photoreceptor-specific transcript is specifically affected by these mutations (Hims et al., 2003).

Efficient transportation of proteins within photoreceptor cells from the site of synthesis in the cell body and inner segment to the outer segment has to pass the connecting cilium and would be expected to be essential to photoreceptor function. Accordingly, several genes involved in this transport have been linked to RP: tubby related protein 1 (gene: TULP1) (Hagstrom et al., 2001) and the product of the RPGR gene (X-linked retinitis pigmentosa GTPase regulator) (Roepman et al., 2000). Furthermore, three genes found to cause RP appear to have a role in the formation of the cell cytoskeleton and may thus be also involved in intracellular transport mechanisms. The RP1 gene shows homology to doublecortin, which is believed to regulate microtubule dynamics and stability during neuronal development (Bowne et al., 1999). The RP2 gene is mutated in some forms of X-linked RP and shares homology to human cofactor C, which is involved in β-tubulin folding (Schwahn et al., 1998; Schwahn et al., 2001). The retina-specific gene FSCN2 (retinal fascin), which has been associated with dominant RP, is believed to play a role in actin bundling (Tubb et al., 2000).

Proteins involved in intercellular signalling have been shown to be mutated in some cases of RP: the C-mer proto-oncogene tyrosine kinase (MERTK) is thought to have a role in cell-cell signalling between the RPE and photoreceptor cells (Feng et al., 2002) and is essential for proper function of PR-outer segment phagocytosis by the RPE (D'Cruz et al., 2000a); the transmembrane protein crumbs (gene: CRB1) has been shown to be essential for

correct photoreceptor morphogenesis (Izaddoost et al., 2002); the usherin gene USH2A product is thought to be a cell-cell adhesion molecule (Rivolta et al., 2000).

6.1.2 Age-related macular degeneration (AMD)

AMD is one of the most common causes of vision loss in developed countries. The most characteristic clinical finding in the retinae of patients with AMD are so called drusen, an extracellular deposit of protein, lipid and debris that accumulate underneath the retinal pigment epithelium (RPE). In AMD the area of the retina which is responsible for acute bright light vision, the macula which contains predominantly cone photoreceptors is affected. Loss of vision from AMD occurs either as a result of choroidal neovascularisation with exudation and haemorrhage (the "wet" form) or by slow atrophy of the RPE and adjacent photoreceptors (the "dry" form). Wet AMD is twice as common as the dry type and causes rapid vision loss (Constable, 2004). Prevalence of AMD in population is high with a distinct trend of increase: about 10-20% of people over the age of 65 suffer from an early-state maculopathy or overt macular degeneration. In US, about 1.75 million people suffer from AMD, this number will increase to about 3 million in 2020 (Friedman et al., 2004). While the pathogenesis of AMD is likely to result from several interacting components such as mutations in potential causative or susceptibility genes, metabolic stress on the RPE and adjacent tissues and exogenous factors (Silvestri, 1997; Hayward et al., 2003; Tomany et al., 2004), other forms of macular disease are inherited, like Stargardt´s or Best´s disease, Sorsby´s fundus dystrophy or cone dystrophies (Kellner et al., 2004).

6.2 Degeneration pathways in retinal diseases

Apoptosis of photoreceptors (and the RPE) is the final cell death pathway in RP and in AMD (Chang et al., 1993; Portera-Cailliau et al., 1994). While the apoptotic end stage is well-documented in animal models and in human autopsy eyes, there is a large gap between the profound knowledge of gene mutations (see, for example, RetNet at http://www.sph.uth.tmc.edu/Retnet/) on the one hand, and events on the cellular and molecular levels leading to cell death including signalling and death pathways on the other hand. It remains unresolved, how cells, carrying a gene mutation, can survive for extended periods before undergoing apoptosis. Which are the intrinsic factors enabling prolonged cell survival under those pathological conditions? And which factors eventually trigger the cell death? In the case of RP, mutations in different genes and different mutations in the same gene can result in similar phenotypes; however, different phenotypes may also be caused by

the same mutation. Maculopathy and "physiological" ageing of the retina remain without severe visual disturbances in many people, while in others disease with loss of central vision is developing from those stages. Molecular components of drusen can differ in AMD-eyes and healthy age-matched controls (Crabb et al., 2002; Dentchev et al., 2003) thus offering potential clues to the pathogenesis.

6.3 Animal models of retinal degeneration

The inherent complexity of both RP and AMD requires the elucidation of common neuroprotective pathways and common death mechanisms in order to develop possible therapies. An important prerequisite for studying retinal degeneration is the availability of appropriate animal models. For research in RP there is by now a multitude of animal models available reflecting the human disease, but animal models are rare for AMD. Only recently a mouse model was described which showed several morphological features typical for AMD including apoptotic cell death (Ambati et al., 2003).

In general two different approaches to access animal models exist: screening for naturally occurring models and genetical engineering of artificial mutations leading to retinal degeneration and mimicking mutations found in patients.

6.3.1 Naturally-occurring animal models:

The models most commonly studied are the naturally occuring rd1 mouse (retinal degeneration 1), the rds mouse (retinal degeneration slow) and the Royal College of Surgeons (RCS) rat. The rd1 mouse carries a mutation on the gene encoding the β-subunit of the cyclic GMP-phosphodiesterase, an essential enzyme of the phototransduction cascade (Bowes et al., 1990), leading to very rapid loss of rods, followed by loss of cones (Carter-Dawson et al., 1978). The rds mouse lacks the gene encoding peripherin 2, which is required for normal development of photoreceptor outer segment discs (Travis et al., 1991). Finally, the RCS rat presents a mutation in the receptor tyrosine kinase gene Mertk which is responsible for ineffective phagocytosis of outer segments by the RPE and ultimately causes the photoreceptors to die (D'Cruz et al., 2000b).

Several canine models of inherited retinal degeneration have been identified, namely the Irish setter, carrying a nonsense mutation in PDEβ-subunit (Aguirre et al., 1978); the Cardigan Welsh corgi, carrying a truncated PDEα-subunit and the Swedish Briard/Briard Beagle dog, carrying a 4-bp deletion in RPE65 gene leading to early onset retinal degeneration including Leber´s congenital amaurosis (a group of autosomal recessive retinal

dystrophies characterized by visual impairment identified at or within a few months from birth) (Narfstrom et al., 1989).

A line of chicken (Rhode Island Red Chicken) was identified with very rapid retinal degeneration. The cause is a deletion/rearrangement of the guanylate cyclase-1 gene, resulting in a null allele and very low cGMP levels (Ulshafer et al., 1984; Semple-Rowland et al., 1998).

6.3.2 Transgenic animal models

Due to rapidly advancing transgenic and gene targeting technologies, the number of laboratory-generated vertebrate animals in which specific genes are targeted is increasing. Transgenic technology is based on the incorporation into the genome of a transgene construct which often contains a mutation linked to disease. The mutant transgene is expressed in specific cell types (photoreceptors) or tissues (retina) of the animal, and transmitted to progeny. Thus, the consequence of a mutation on the development, differentiation and metabolism of a cell can be studied. Knockout technology is based on targeted replacement of a gene by a construct that disrupts (knocks out) expression of the gene. As a consequence, the gene product cannot be produced, leading in many cases to specific phenotypes. Aditionally, a gene can be replaced by a construct with flanking loxP sites. In a second step, the bacteriophage enzyme cre recombinase is expressed in the desired cell type by standard transgenic technology; when expressed, cre recognizes loxP sites and excises DNA flanked by loxP. Thus, a gene is knocked out only in tissues or cells expressing cre. Conditional or tissue-specific knockouts are particularly desirable if a universal knockout is lethal during embryonic development. Currently, these techniques are applied mostly in rodents; however, other species (pig, Xenopus) are increasingly used in research.

Gene knockout mouse models comprise rhodopsin ($Rho^{-/-}$) (Humphries et al., 1997), rhodopsin kinase ($RK^{-/-}$) (Chen et al., 1999a), cone-specific cyclic nucleotide gated channel ($CNG3^{-/-}$) (Biel et al., 1999), arrestin ($arrestin^{-/-}$) (Chen et al., 1999b), RPE65 ($Rpe65^{-/-}$) (Redmond et al., 1998), retinal-specific ATP-binding cassette transporter ($ABCR^{-/-}$) (Weng et al., 1999) and interphototreceptor retinoid-binding protein ($IRBP^{-/-}$) (Liou et al., 1998).

Transgenic mouse models comprise P23H, P347S, V20G, P27L, Q334ter rhodopsin mutants (Olsson et al., 1992; Chang et al., 1993; Huang et al., 1993; Naash et al., 1993; Portera-Cailliau et al., 1994; Roof et al., 1994; Goto et al., 1995) and P216L peripherin mutant (Kedzierski et al., 1997)

Two widely used transgenic rat models are S334ter (Liu et al., 1999) and P23H (Lewin et al., 1998b) rhodopsin mutants.

Rat and mouse eyes are very small and not ideally suited for subsequent manipulations of the retina, such as injections of viral constructs for somatic gene therapy. Moreover, rodent eyes do not have a macula, and are therefore not suitable for macular degeneration models. The pig eye is more similar to the human eye in terms of the size and ratio of rod/cone photoreceptors (Hendrickson and Hicks, 2002). Therefore, efforts have been made to engineer a pig transgenic model for RP. The transgenic animals express a mutated rhodopsin gene (*Pro*347Leu) and develop early rod cell loss with slower cone cell degeneration (Li et al., 1998; Petters et al., 1997; Tso et al., 1997).

6.3.3 Animal models of light-induced retinal degeneration

The protracted cell death in most animal models (and humans) renders the study of apoptotic mechanisms rather difficult due to non-synchronic apoptosis. Only few animal models have a short period of accumulated cell death that might provide a fair amount of material to study and a time frame for experimental manipulations. However, in those cases disease-induced cell death coincides with histogenetic, developmental cell death, such as in the rd1 mouse, a condition that could mask the mechanisms of degenerative apoptosis. It has been observed that bright light damages vision in laboratory animals (Noell et al., 1966). Consequently several setups for controlled light-induced retinal degeneration have been developed (reviewed in Reme et al., 1998; Wenzel et al., 2005). These models have several advantages: (1) light exposure triggers apoptosis of photoreceptors in a synchronized way, (2) apoptosis is rapid and (3), although it is not an exact model for mutation-induced retinal degeneration, light appears to be a disease-triggering cofactor in most inherited forms of RP.

7 Therapeutic strategies in retinal degenerative diseases

Several strategies to preserve visual functions are developed in animal models. They comprise transplantation of stem cells, RPE or retina, delivery of a large number of neuroprotective cytokines, gene therapy including replacement of missing proteins or removal of harmful molecules, implantation of subretinal or epiretinal chips or cortically based electrodes (Eckmiller, 1997; Zrenner, 2001; Alteheld et al., 2004). At present, neuroprotection and certain gene-therapies appear to be among the most promising approaches. Because apoptosis is the final pathway of cell removal, prevention of apoptosis is conceptually feasible for all types of mutations and for AMD. This is advantageous to gene therapy, where genetic

screening for each patient needs to be performed before a therapy strategy can be developed. Either the inhibition of apoptosis as the downstream final event in retinal degeneration or prolongation of photoreceptor survival by neuroprotective molecules appears to be a reasonable approach. However, successful prevention of apoptosis will require, at least in part, the understanding of neuroprotective signalling and apoptotic death mechanisms in the retina.

7.1 Gene therapy

Different gene-mediated therapy strategies have been developed for the treatment of inherited retinal degenerations. In mutations leading to loss of function (mostly autosomal recessive or X-linked retinal degenerations), the principle of gene therapy is to correct the genetic defect by the introduction of a wild-type version of the mutated gene into the cells in which normal functioning of this gene is required. Gene therapy has been performed using several different vectors and has resulted in the slowing down of the photoreceptor degeneration process in the rd1 mouse (Bennett et al., 1996; Jomary et al., 1997; Kumar-Singh and Farber, 1998; Takahashi et al., 1999), in the *rds* mouse (Ali et al., 2000) and in the RCS rat (Vollrath et al., 2001). The most conclusive result was observed in the $RPE65^{-/-}$ dog. By injecting a recombinant AAV carrying the missing wild-type RPE65 cDNA into the subretinal space, recovery of visual function was obtained as assessed by psychophysical and behavioral tests (Acland et al., 2001).

In mutations leading to a toxic gain of function (autosomal dominant retinal degenerations), the principle of gene therapy is to inhibit the expression of the gene responsible for deleterious effects. Gene silencing can be obtained by the use of single-stranded antisense oligonucleotides or the use of ribozymes (Farrar et al., 2002). The potential effects of ribozyme therapy was evaluated in the P23H transgenic rat (LaVail et al., 2000; Lewin et al., 1998a). They demonstrated that *in vivo* expression of either a hammerhead or hairpin ribozyme specific to the mutant transcript significantly delays the degeneration of the photoreceptors in transgenic rats for at least 8 months.

Additionally, several antiapoptotic gene therapies have been developed. In mutant *drosophila*, it was demonstrated that targeted transfer of antiapoptotic gene p35 blocked apoptotic mechanisms in retinal cells and preserved visual function (Davidson and Steller, 1998). Bcl-2 is known to control mitochondrial membrane permeability (Sharpe et al., 2004) and integrity in general (Donovan and Cotter, 2004). Several experiments using expression of Bcl-2 in photoreceptors in a variety of models have been performed with the aim to stabilize

mitochondria and thus prevent photoreceptor apoptosis. The results are controversial: no protection resulted from transgenic Bcl-2 overexpression in the rd1 mouse in one study (Joseph and Li, 1996), while protection was achieved in another study (Chen et al., 1996) or with Bcl-2 expressed from a viral vector (Bennett et al., 1998). Furthermore, while Bcl-2 overexpression was without effect in mice expressing a K296E mutation in rhodopsin (Joseph and Li, 1996), but animals with S334ter rhodopsin mutation were protected (Chen et al., 1996) and in the rds mouse, Bcl-2 overexpression had a very robust effect (Nir et al., 2000).

7.2 Transplantation

Numerous attempts have been made to transplant tissue into animal models of retinal degeneration. Two different types of tissue have been transplanted: RPE transplantation attempts to obtain beneficial effects upon the adjacent photoreceptors, and retinal neuronal transplantation, on the other hand, aims at replacing the degenerate tissue altogether.

In the RCS rat, RPE fails to phagocytose shed outer segments, and the photoreceptor cells subsequently die. Using this model, studies showed that fresh RPE cell transplants considerably delayed the loss of photoreceptors (Li and Turner, 1988; Sheedlo et al., 1989; Gouras and Lopez, 1989), restored normal metabolism (Lavail et al., 1992) and improved visual function (Jiang and Hamasaki 1994; Whiteley et al., 1996). It has been proposed that additionally to direct rescue from the transplant, there is indirect rescue by trophic factor release in these studies, because rescue effects are also observed in untransplanted areas of the eye (Sahel et al., 2001).

Neuronal transplantation aims at replacing lost photoreceptor cells. Transplantation of either embryonic dissociated cells or retinal sheets into the subretinal space of rodent models of retinal degeneration demonstrate that transplants survive and differentiate and that neuronal fibers originating from the transplant develop synapses with the remaining host retina which are at least sufficient to mediate a simple light-dark preference (Kwan, et al., 1999; Aramant and Seiler, 1995). Still, numerous obstacles remain before RPE or retinal transplantations may be used in clinical therapy (see reviews by Berson and Jakobiec, 1999; Lund et al., 2003).

Beyond the interesting prospect of replacing the lost cells, retinal transplants were shown to exert trophic effects on the remaining host retina. Transplantation of rod-rich photoreceptor sheets into the subretinal space of rd1 mice, which at this age contain few remaining rods but numerous surviving cones, induced a significant increase in host cone survival (Mohand-Said et al., 1997; Mohand-Said et al., 2000).

7.3 Pharmacological treatment

7.3.1 Anti-apoptotic treatment

Calcium overload has been a prime candidate for the induction of neuronal apoptosis and in retinal light damage it seems to be involved in the early phase of apoptotic cell death of photoreceptors (Donovan et al., 2001). Accordingly, application of the calcium channel blocker D-diltiazem prevented light damage as revealed by the absence of TUNEL positive cells in the ONL (Donovan and Cotter, 2002). Reports analyzing effects of calcium antagonists on photoreceptor dystrophy in animal models of RP are conflicting: D-diltiazem was reported to be protective in the rd1 mouse in one report (Frasson et al., 1999b), while no effect was reported in two other studies using the rd1 mouse (Pawlyk et al., 2002; Takano et al., 2004) or in a corresponding dog model (Pearce-Kelling et al., 2001). In the study by Takano et al., however, the calcium antagonists nilvadipine and nicardipine slowed the course of degeneration (Takano et al., 2004). Further evidence for a deleterious effect of calcium was derived from rd1 mice additionally lacking L-type voltage gated calcium channels. In these mice, retinal degeneration was slowed as compared to rd1 mice expressing the calcium channel (Read et al., 2002). Calcium antagonists have also been tested in two other models: D-diltiazem had no protective effect in the P23H transgenic rat (Bush et al., 2000). On the other hand, nilvadipine and not D-diltiazem caused a slowing of retinal degeneration and some preservation of function in the RCS rat (Yamazaki et al., 2002; Sato et al., 2003).

Exposure of photoreceptors to blue light *in vitro* results in the generation of reactive oxygen species by a mechanism involving mitochondrial electron transport (Yang et al., 2003). In several studies using prolonged exposure to broad band green or white light, antioxidants like dimethylthiourea (DMTU) and phenyl-*N*-tert-butylnitrone (PBN) were shown to be protective against light damage (Organisciak et al., 1999; Ranchon et al., 1999; Specht et al., 1999 ; Ranchon et al., 2001). The anti-oxidant had to be applied before the onset of light to be protective; indicating that reactive oxygen species generation in fact is an early event (Organisciak et al., 2000). Likewise injection of recombinant thioredoxin (TRX) or transgenic expression of TRX, an endogenous redox-regulating protein, conferred protection by reducing oxidative stress (Tanito et al., 2002a; Tanito et al., 2002b). DMTU and PBN have consequently been tested for a protective effect against the deleterious effect of light in two models of inherited retinal degeneration. While DMTU could protect photoreceptors of P23H and S334ter transgenic rats (Organisciak et al., 2003), PBN only protected P23H transgenic rats. PBN had no effect on either P23H or S334ter caused degeneration in the absence of

additional light insults (Ranchon et al., 2003). These results indicate that anti-oxidative treatments may have beneficial effects on certain forms of retinal degeneration by reducing the disease-accelerating effects of light.

The involvement of caspases in retinal apoptosis is still unresolved due to conflicting results. In the rd1 mouse, Doonan and colleagues did not observe caspase-3 activation or a protection of the rd1 retina by caspase inhibitors (Doonan et al., 2003), other studies reported an activation of caspase-3 (Jomary et al., 2001; Kim et al., 2002) and indicate a role of this protease in the rd1 degeneration by showing that inhibition of caspase-3 delayed cell death (Yoshizawa et al., 2002; Sharma and Rohrer, 2004) and that ablation of caspase-3 led to a transient mild preservation of retinal morphology (Zeiss et al., 2004). Similarly, the involvement of caspase-8 appears unclear in the rd1 model: Activation was observed by Jomary et al. (Jomary et al., 2001), but not by Doonan (Doonan et al., 2003). Caspase-3 inhibitors provided protection in two other models of inherited retinal degeneration: the S334ter rat (Liu et al., 1999) and the tubby mouse (Bode and Wolfrum, 2003). Furthermore, activation of caspases-1 and -2 was demonstrated in the RCS rat and caspase-1 inhibition transiently reduced the number of apoptotic photoreceptors (Katai et al., 1999).

Exposure to acute bright white light induces the expression of caspase-1 (Grimm et al., 2000) and its protein levels increase in the photoreceptor layer following exposure to intense green light (Wu et al., 2003). Both findings suggest an involvement of at least caspase-1 in light induced photoreceptor apoptosis. However, no activation (cleavage) of caspases-1, -3, -7, -8 or -9 was observed after acute light damage (Donovan et al., 2001) (Donovan and Cotter, 2002).

7.3.2 Neuroprotective cytokines and growth factors

Several compounds have been tested for their neuroprotective potential in retinal degeneration based on evidence derived from other systems, in particular the CNS. Attempts for neuroprotection in the retina involve a broad spectrum of cytokines (Chaum, 2003).

7.3.2.1 LEDGF

Lens epithelium derived growth factor (LEDGF) promotes survival of cells against stress by inducing the expression of heat shock proteins, antioxidant proteins, and detoxication enzymes (Shinohara et al., 2002). Injection of LEDGF into the vitreous of rats prior to 48 h of light exposure resulted in protection of retinal function and preservation of retinal morphology. The number of surviving photoreceptors was twice as high as compared

to the vehicle injected eye (Machida et al., 2001). Similar effects on morphology were observed when LEDGF was applied to retinas derived from rd1 mice in organ culture, where it caused an increased survival of photoreceptors (Ahuja et al., 2001). Likewise, LEDGF prolonged the survival of functional photoreceptors in the RCS rat (Machida et al., 2001). On the other hand, no protection resulted from LEDGF application in the P23H rat (Machida et al., 2001).

7.3.2.2 b-FGF

Basic fibroblast growth factor (bFGF) has been extensively studied as neuroprotective cytokine in the retina. It seems to play a major role in the endogenous defence against stress of retinal cells. Endogenous b-FGF is released from retinal cells as an answer to different stress-stimuli and subsequently protects retinal cells from apoptosis.

b-FGF has been studied for its neuroprotective capacity in several models of inherited retinal degeneration: Attempts to decrease retinal degeneration in several mouse models by intravitreal injections of the recombinant protein failed (LaVail et al., 1998). However, longer lasting expression of b-FGF from a virally delivered transgene in rats transgenic for rhodopsin carrying the S334ter mutation led to conservation of retinal morphology, but not function (Lau et al., 2000). Similarly, viral transfer of b-FGF to the retina of the RCS rat protected retinal morphology (Akimoto et al., 1999). In the same model, intravitreal implantation of encapsulated cells expressing b-FGF slowed the course of degeneration (Uteza et al., 1999) (Neuner-Jehle et al., 2000). Two other members of the FGF family (FGF-5 and FGF-18) have been shown to rescue morphology but not the function in transgenic rats carrying the rhodopsin mutations P23H and S334ter (Green et al., 2001). Even though species differences may underlie the variation in rescue, it appears that the route of application and the duration of the presence of high FGF levels might define the success of treatment.

Intravitreal injections of recombinant b-FGF was neuroprotective in two light damage setups using constant exposure to white light (LaVail et al., 1998; Cao et al., 2001). The neuroprotective effect could be enhanced by a combination of b-FGF and PEDF (Cao et al., 2001). In contrast, no protection against constant light exposure for 1 week was achieved when b-FGF was expressed locally from a transgene delivered by a AAV vector (Lau and Flannery, 2003). In a very similar setup, transgenic expression of b-FGF protected photoreceptor morphology, but not the function (Spencer et al., 2001). Pre-conditioning with light increases the expression of b-FGF and protects against damaging doses of light (Liu et al., 1998; Li et al., 2003). Light pre-treatment also enhanced photoreceptor survival in RCS rats, but failed to do so in P23H rats. In both cases b-FGF was up regulated. Light pre-

treatment may have failed to rescue the P23H-induced degeneration due to the intrinsic susceptibility of P23H photoreceptors to light damage (Nir et al., 1999; Nir et al., 2001). Ischemic pre-conditioning of the retina by elevation of the intraocular pressure likewise resulted in an increased resistance against light damage and was accompanied by increased expression of b-FGF (Casson et al., 2003). Similarly, induction of ganglion cell death yielded the same effects (Casson et al., 2004).

7.3.2.3 PEDF

Pigment epithelium derived factor (PEDF) is considered a potent neuroprotective factor against, for example, glutamate excitotoxicity and oxidative damage (Tombran-Tink and Barnstable, 2003) for neurons from CNS. Consequently, it was also tested for neuroprotective activity at the site of its origin, the eye. Intraocular injection of PEDF slowed degeneration in two mouse models of inherited degeneration, rd1 and rds (Cayouette et al., 1999). SIV-mediated PEDF gene transfer preserved morphology and function in the RCS rat (Miyazaki et al., 2003). Injection of recombinant PEDF prior to light exposure doubled the number of surviving photoreceptors and rescued retinal function following constant light exposure for up to 10 days (Cao et al., 2001).

7.3.2.4 CNTF

Ciliary neurotrophic factor (CNTF) is a very well-studied molecule with regard to retinal degeneration. Like b-FGF it seems to be part of an intrinsic retinal response to stress. CNTF has been applied to a large variety of inherited retinal degenerations: intraocular injections failed to display a protective effect in several models (LaVail et al., 1998) but expression from a virally delivered transgene slowed photoreceptor loss in the rd2 mouse (Cayouette et al., 1999; Liang et al., 2001; Schlichtenbrede et al., 2003), the P216L transgenic mouse (Bok et al., 2002), the rhodopsin S334ter and P23H transgenic rats (Liang et al., 2001), the RCS rat (Huang et al., 2004) and the rhodopsin knock-out mouse (Liang et al., 2001). Interestingly, retinal function turned out to be less (or not) protected than morphology. CNTF, released by engineered cells, slowed retinal degeneration in the rcd1 dog model (Tao et al., 2002) and, after intravitreal injection, in an autosomal dominant feline model of rod-cone dystrophy (Chong et al., 1999). Protective effects were also reported for the rd1 mouse on retinas in organ culture (Caffe et al., 2001). CNTF expression was found to be upregulated by pre-conditioning with light (Liu, C., et al., 1998) or by injury to ganglion cells (Casson et al., 2004). Recombinant CNTF injected intravitreally protected against exposure to white light for 1–2 weeks (LaVail et al., 1998).

Interestingly, the receptors for CNTF (CNTFα) are mainly expressed in inner retina on neurons and glial cells and on RPE, but do not appear to be expressed by photoreceptors (Chaum, 2003). This leads to the hypothesis that the survival signal promoted by CNTF, is transduced indirectly by other retinal cells, e.g. retinal Mueller glial cells (RMG).

7.3.2.5 NGF

Reports about neuroprotective effects of nerve growth factor (NGF) in retinal degeneration have been contradicting. Intravitreal injection of NGF in an autosomal dominant feline model of rod-cone dystrophy slowed degeneration (Chong et al., 1999).

In light-induced retinal degeneration NGF may have deleterious effects by reducing the endogenous b-FGF response. This activity seems to involve activation of $p75^{NTR}$ receptors on retinal Müller glial cells, as blocking or knock-out of this receptor increases retinal resistance in acute bright light damage (Harada et al., 2000). No effect of $p75^{NTR}$ receptor knock-out on light damage susceptibility, however, was found with a different light damage setup using constant exposure for 2–3 weeks (Rohrer et al., 2003).

7.3.2.6 NT-3

Neurotrophin-3 (NT-3) has been shown to exert effects opposite from NGF in light-induced retinal degeneration by activating TrkC-induced signalling in RMG which resulted in increased release of b-FGF from RMG. This intrinsically released b-FGF may be responsible for the increased retinal defence against light toxicity (Harada et al., 2000).

7.3.2.7 BDNF

Intravitreal injection failed to protect the retina in a variety of mutant mice (LaVail et al., 1998) and in a model for autosomal dominant feline rod-cone dystrophy (Chong et al., 1999). Viral delivery of a BDNF transgene delayed degeneration induced by the Q344ter mutation in rhodopsin (Okoye et al., 2003). Likewise, prolonged release from transgenic cell transplants into the eye slowed degeneration in RCS rats (Lawrence et al., 2004) and rd1 retinal explants cultured in the presence of BDNF showed better preserved morphology (Caffe et al., 2001).

BDNF applied by intravitreal injection of the recombinant protein or released from transgenic cell transplants (Kano et al., 2002) protects the retina against 1–2 weeks of constant light exposure.

Surprisingly, the BDNF receptor, TrkB, is expressed in retinal ganglion cells (RGC), amacrine cells (Cellerino and Kohler 1997; Chaum 2003; Jelsma et al., 1993; Maisonpierre et

al., 1990) and RPE (Bennett et al., 1999); additionally TrkB is expressed by cone photoreceptors but not by rods. This indicates the existence of paracrine survival pathways in cases of BDNF-induced enhanced rod survival.

7.3.2.8 RdCVF

Rod-derived cone viability factor (RdCVF) is a truncated TRX-like protein specifically expressed by rod photoreceptors. RdCVF is hypothesized to protect cones in retinal diseases where cone loss follows secondary to rod loss. RdCVF was shown to be expressed by rods and to promote the survival of cones in the rd1 mouse (Leveillard et al., 2004).

7.3.2.9 GDNF

Glial cell-line derived neurotrophic factor (GDNF) has been shown to prolong suvival of rod photoreceptors in the rd1 mouse model of retinal degeneration (Frasson et al., 1999a). Additionally, this study demonstrated that GDNF was able to also preserve function of rods for an extended period of time. It has been hypothesized that this rod rescue may be due to an unknown paracrine effect, because (a) the GDNF receptor components could not be localized on photoreceptors (unpublished observations) and (b) RMG responded with upregulation of glial fibrillaric acidic protein (GFAP) to GDNF.

Transplantation of a Schwann-cell line producing GDNF into the RCS rat significantly slowed degeneration (Lawrence et al., 2004), virally delivered GDNF increased rod photoreceptor survival and function in the transgenic S334ter rhodopsin rat (Sanftner et al., 2001) and GDNF prolonged survival of photoreceptors in an *in vitro* culture of retinal neurons (Politi et al., 2001). Furthermore, GDNF has protective effects on retinal ganglion cells, as was shown by several studies (Klocker et al., 1997; Koeberle and Ball, 1998; Yan et al., 1999; Koeberle and Ball, 2002; Schmeer et al., 2002; Straten et al., 2002; Ishikawa et al., 2005).

8 The role of RMG in neuroprotection

Glial cells are thought to protect neurons from various neurologic insults. Retinal disease or injury is often accompanied by significant morphological, cellular and molecular changes in RMG. Some of these changes may reflect the involvement of RMG in protecting the retina from further damage.

8.1 Protection from glutamate toxicity

The excitatory amino acid neurotransmitters, and particularly glutamate, can have potent neurotoxic activity if their extracellular concentration becomes elevated in the central nervous system including the retina. Hence, excessive glutamate is thought to be responsible for a variety of acute neurologic insults, including ischemia and anoxia, hypoglycemia, trauma, and several chronic neurodegenerative diseases. The primary glutamate transporter expressed by retinal astrocytes and RMG is GLAST (Otori et al., 1994), which has been postulated to contribute to the clearance of glutamate and protect retinal ganglion cells from glutamate neurotoxicity (Kawasaki et al., 2000). It has been shown that RMG can protect against the excitotoxic effects of glutamate in the whole retina and increase survival of ganglion cells in culture (Heidinger et al., 1999; Izumi et al., 1999; Kawasaki et al., 2000). In physiological conditions, after its release from neurons, glutamate is quickly taken up by glial cells and amidated to form the non-neuroactive compound glutamine. This amidation is catalyzed by the enzyme glutamine synthetase, which is present only in glial cells and which is confined exclusively to RMG in the retina. Glutamine is then released by the glial cells and taken up by neurons for conversion back to glutamate. Glutamine synthetase is involved directly in neuroprotection since it has been found that chick retinas treated with cortisol induce expression of high levels of glutamine synthetase and as a consequence are more resistant to glutamate-induced damage (Vardimon et al., 1988). Beside, by using mixed cultures of neuronal cells seeded onto adult RMG as feeder cells, it has been demonstrated that glutamate induces an increase in glutamine synthetase activity and protection from glutamate toxicicity (Heidinger et al., 1999).

8.2 Protection from reactive oxygen species

Reactive oxygen species are generated in the retina under various conditions such as anoxia, ischemia and reperfusion. One of the crucial substances protecting the retina against reactive oxygen species is glutathione, a tripeptide constituted of glutamate, cysteine and glycine. Glutathione is synthesized extramitochondrially and transported into the

mitochondrial matrix of RMG. Glutamate is the rate-limiting substance in the glutathione synthesis. High levels of reduced glutathione have been localized to RMG (Pow and Crook 1995), suggesting that RMG play a critical role in regulating the content of potentially damaging oxidative species in the retina. Supply of reduced glutathione from RMG to retinal neurons has been proposed as a major protective mechanism against ischemia/reperfusion injury in rat retina (Schutte and Werner, 1998).

8.3 RMG-derived neurotrophic factors

Several *in vitro* studies demonstrated that cultured RMG release neuroprotective substances into the culture medium. RMG-conditioned medium increases the long term survival of developing retinal ganglion cells in culture. It has been demonstrated in cultures from adult pig retinas that factors secreted by RMG increase the survival and enhances the neuritogenesis of retinal ganglion cells (Garcia et al., 2002). Other authors have found that RMG have a neurite-promoting effect on retinal ganglion cells only when there is a direct cell-to-cell contact whereas soluble factors released by RMG do not induce significant neurite outgrowth in retinal ganglion cells (Raju and Bennett, 1986). In either case, the nature of the respective factors is unknown.

Recent work has demonstrated in *in vitro* cultures of purified cone photoreceptors from pig retinae that RMG-conditioned medium has a pronounced effect on survival. Above 60% of the cultured cones survived for more then 7 days *in vitro*, compared to only 4% in the control-medium (Balse et al., 2005). This leads to the hypothesis that RMG secrete neuroprotective factors, which have not yet been identified.

In vivo, RMG have also been implicated in determination of photoreceptor survival. It has been demonstated that RMG increase secretion of bFGF in response to injection of NT-3. The release of bFGF by RMG leads to protection of photoreceptors from light induced apoptosis (Harada et al., 2000). On the other hand, the low affinity neurotrophin receptor $p75^{NTR}$, which is expressed in RMG (Garcia et al., 2003), is activated by application of NGF and subsequently decreases bFGF production in RMG. Thus, it has been hypothesized that RMG transduce both, neuroprotective and apoptotic stimulus by either increase or decrease of secreted bFGF.

Injection of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and fibroblast growth factor (bFGF) into eyes of wildtype mice leads to increase in phosphorylated extracellular receptor kinase (pERK) and transcription factor c-fos only in cells of the inner retina and not in photoreceptors. This again indicates that these molecules

may exert their effects on photoreceptors by acting indirectly through activation of RMG (Wahlin et al., 2000).

9 GDNF signalling

9.1 GDNF family

Glial cell line-derived neurotrophic factor (GDNF) was originally purified biochemically from a rat glioma cell-line supernatant as a growth factor promoting the survival of embryonic dopaminergic neurons of midbrain (Lin et al., 1993). Later, neurturin (NRTN) was purified biochemically by virtue of its biological activity (Kotzbauer et al., 1996). The other GDNF-family ligands (GFLs) artemin (ARTN) (Baloh et al., 1998; Masure et al., 1999) and persephin (PSPN) (Milbrandt et al., 1998) were then isolated by a database search and homology cloning (Baloh et al., 2000). Analysis of the near-complete public human genome database indicates that further functional GFL members are unlikely to exist (Airaksinen and Saarma, 2002).

GFLs belong to the transforming growth factor-β (TGF-β) superfamily, containing seven cysteine residues with the same relative spacing as other members of this family. Despite low amino-acid sequence homology, GDNF and other structurally characterized members of the TGF-β superfamily have similar conformations (Ibanez, 1998). They all belong to the cysteine-knot protein family, and they function as homodimers.

GFLs are produced as a precursor protein, preproGFL. The signal sequence is cleaved upon secretion, and activation of the proGFL probably occurs by proteolytic cleavage. GFLs seem to bind heparin-sulphate side chains of extracellular-matrix proteoglygans, which might restrict their diffusion and raise their local concentration (Hamilton et al., 2001). The specific proteases that cleave and activate GFL precursors have not yet been identified.

9.2 GDNF receptors

9.2.1 GFRα coreceptors

All GFLs signal via transmembrane receptor tyrosine kinase RET (***re***arranged during ***t***ransfection), but the binding of GFLs is indirect, mediated by a family of proteins named GDNF receptor-α family. Those proteins are linked to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor. Four different GFRα receptors have been characterized

(GFRα1-4), which determine the ligand specificity of the GFRα-RET complex. Dimeric GDNF binds to GFRα-1 which then binds to RET and leads to a dimerisation of the transmembrane tyrosine kinase. This in turn leads to phosphorylation of intracellularly located tyrosine residues of RET. Neurturin (NRTN) binds to GFRα-2, artemin (ARTN) binds to GFRα-3 and persephin (PSPN) binds to GFRα-4 (see Figure 7). NRTN and ARTN may bind weakly to GFRα-1, and GDNF was shown to interact weakly with GFRα-2 and GFRα-3. PSPN, so far has been shown to bind exclusively to GFRα-4 (Airaksinen et al., 1999; Baloh et al., 2000; Lindahl et al., 2001; Takahashi, 2001) (Figure 7).

GFRα receptors are usually bound to the plasma membrane by their GPI anchor. However, it has been shown that cleavage by a yet unidentified phospholipase or protease produces soluble forms of these co-receptors (Paratcha et al., 2001). Additionally, alternative splicing can produce soluble forms of the co-receptors (shown for GFRα-4, Lindahl et al., 2001).

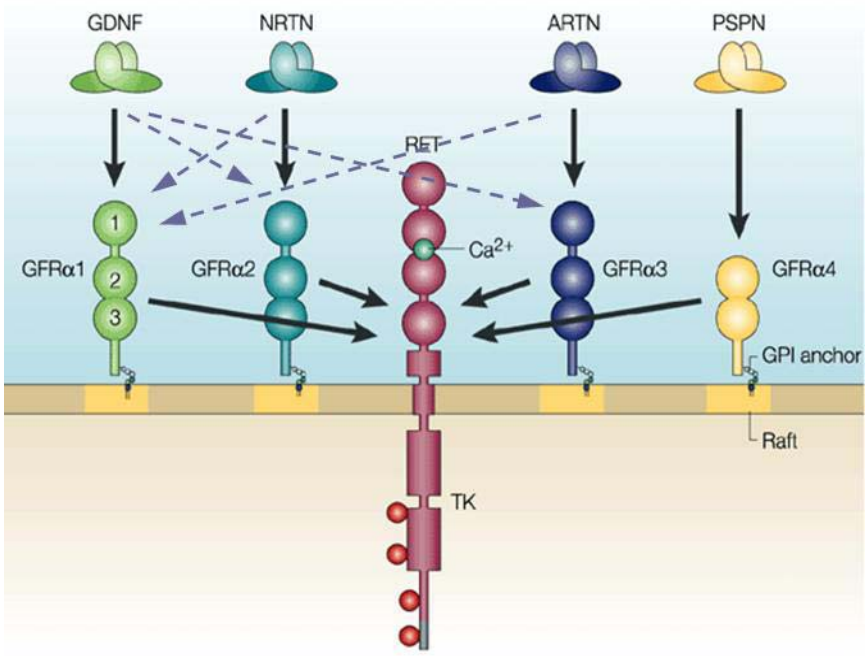

Figure 7: GDNF family ligands and receptor interactions

All GFRα receptors can bind to Ret (indicated by black arrows) and each GFRα receptor has a preferred ligand. Some GFLs can interact with 2 or 3 GFRas (dashed arrows). GFRα proteins are attached to the plasma membrane by a GPI anchor located in raft membranes. At least four tyrosine residues present on the intracellular part of RET serve as docking sites for adaptor proteins (red circles) (from Airaksinen and Saarma, 2002).

9.2.2 RET transmembrane tyrosine kinase

The RET proto-oncogene encodes a receptor tyrosine kinase with a cadherin-related motif and a cysteine-rich domain in the extracellular domain and is located on human chromosome 10q11.2 (Takahashi et al., 1988; Ishizaka et al., 1989; Takahashi et al., 1989; Iwamoto et al., 1993). Three isoforms that are generated by alternative splicing in the 3′ region have been cloned (Tahira et al., 1990; Myers et al., 1995). The three isoforms, short (RET9), middle (RET43) and long (RET51) differ in the amino acid sequence of the C-terminal tail; RET9 has nine additional amino acids from the common sequence between RET9 and RET51, while RET51 has 51 amino acids from the common sequence. Accordingly, RET43 has 43 amino acids at C-terminus not homologue to the other Ret isoforms (Ivanchuk et al., 1998). From these isoforms, RET9 and RET51 are the predominantly expressed products *in vivo*. The generation of mice that expressed either only RET9 or only RET51, demonstrated that RET9 is crucial for kidney morphogenesis and enteric nervous development, whereas RET51 is dispensable (De Graaff et al., 2001). Monoisoformic RET9 mice, which lacked RET51, were viable and appeared normal. In contrast, monoisoformic RET51 mice, which lacked RET9, had kidney hypodysplasia and lacked enteric ganglia from the colon. On the other hand, another study showed that only RET51 but not RET9 could promote the survival and tubulogenesis of mouse inner-medullary collecting duct cells in the kidney, suggesting that RET51 signalling participates in the differentiation event in later kidney organogenesis (Lee et al., 2002). Recently it was demonstrated that the signalling complex associated with RET9 was markedly different from that associated with RET51 (Tsui-Pierchala et al., 2002) thus further underlining their differential function in signalling.

9.2.3 GDNF-induced intracellular signalling

Upon GDNF stimulation, RET receptor is dimerized and specific C-terminal tyrosine residues are autophosphorylated. RET short and middle isoforms contain 16 tyrosine residues, including 10 tyrosine residues in the kinase domain, two in the juxtamembrane domains, one in the kinase insert and three in the carboxy-terminal tail, and RET long isoform has two additional tyrosines in the carboxy-terminal tail. Among these tyrosines, tyrosine 905, 1015, 1062 and 1096 represent binding sites for GRB7/10, phospholipase Cγ (PLCγ), SHC and GRB2, respectively (Pandey et al., 1995; Asai et al., 1996; Borrello et al., 1996; Arighi et al., 1997; Lorenzo et al., 1997; Ohiwa et al., 1997; Alberti et al., 1998; Durick et al., 1998; van Weering and Bos, 1998). The former three tyrosines are conserved in all three RET isoforms

but tyrosine 1096 is present in the long isoform only. Interestingly, tyrosine 1062 is so far turned out to be the binding site of at least five different docking proteins, SHC, FRS2, DOK4/5 (downstream of tyrosine kinase 4/5), IRS1/2 (insulin receptor substrate 1/2) and Enigma.

Like other receptor tyrosine kinases, RET can activate various signalling pathways including RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways (see Figure 8) (Chiariello et al., 1998; Feng et al., 1999; Hayashi et al., 2000; Melillo et al., 1999; Murakami et al., 1999a; Murakami et al., 1999b; Ohiwa et al., 1997; Segouffin-Cariou and Billaud, 2000; Soler et al., 1999; Trupp et al., 1999, van Weering, D.H. and Bos, J.L., 1997, van Weering et al., 1995; Worby et al., 1996; Xing et al., 1998). Astonishingly, all of the RAS/ERK, PI3K/AKT, p38MAPK and JNK pathways are activated mainly through tyrosine 1062 (Hayashi et al., 2000). After binding of SHC to phosphorylated tyrosine 1062, SHC further associates with GAB1/2 adaptor proteins and GRB2/SOS complex, leading to the activation of PI3K/AKT and RAS/ERK signalling pathways, respectively. GAB1/2 directly associates with p85 subunit of PI3K, resulting in the activation of PI3K (Murakami et al., 1999b; Besset et al., 2000; Hayashi et al., 2000). However, the fact that mutation of tyrosine 1062 did not completely abolish the activation of RAS/ERK and PI3K/AKT pathways suggests the presence of alternative signalling pathways. The activation of RAS/ERK and PI3K pathways via tyrosine 1062 are important for activation of CREB and NFκ-B transcription factors, respectively (Hayashi et al., 2000), as well as for PC12 cell survival (De Vita et al., 2000). In addition, RAS activation is essential for RET-induced cell differentiation in PC12 cells (Califano et al., 2000), whereas PI3K signalling independent of AKT is necessary for lamellipodia formation that is a critical event in neuritogenesis (van Weering and Bos, 1997; van Weering and Bos, 1998). It is still unknown, how phosphorylated tyrosine 1062 activates p38MAPK, JNK, and ERK5 signalling pathways (Hayashi et al., 2000; Hayashi et al., 2001). Recently, the neuron-specific adaptor protein Rai (ShcC) was found to be specifically activated by RET (Pelicci et al., 2002). Rai protein forms a constitutive complex with the p85 subunit of PI3K and the Rai-PI3K complex is recruited to phosphorylated tyrosine 1062 of RET. This triggers the activation of PI3K-AKT and promotes the neuronal cell survival. RET may potentiate prosurvival effect through the activation of Rai even in adult nervous tissues, because Rai is expressed in both developing and adult nervous system (O'Bryan et al., 1996; Sakai et al., 2000).

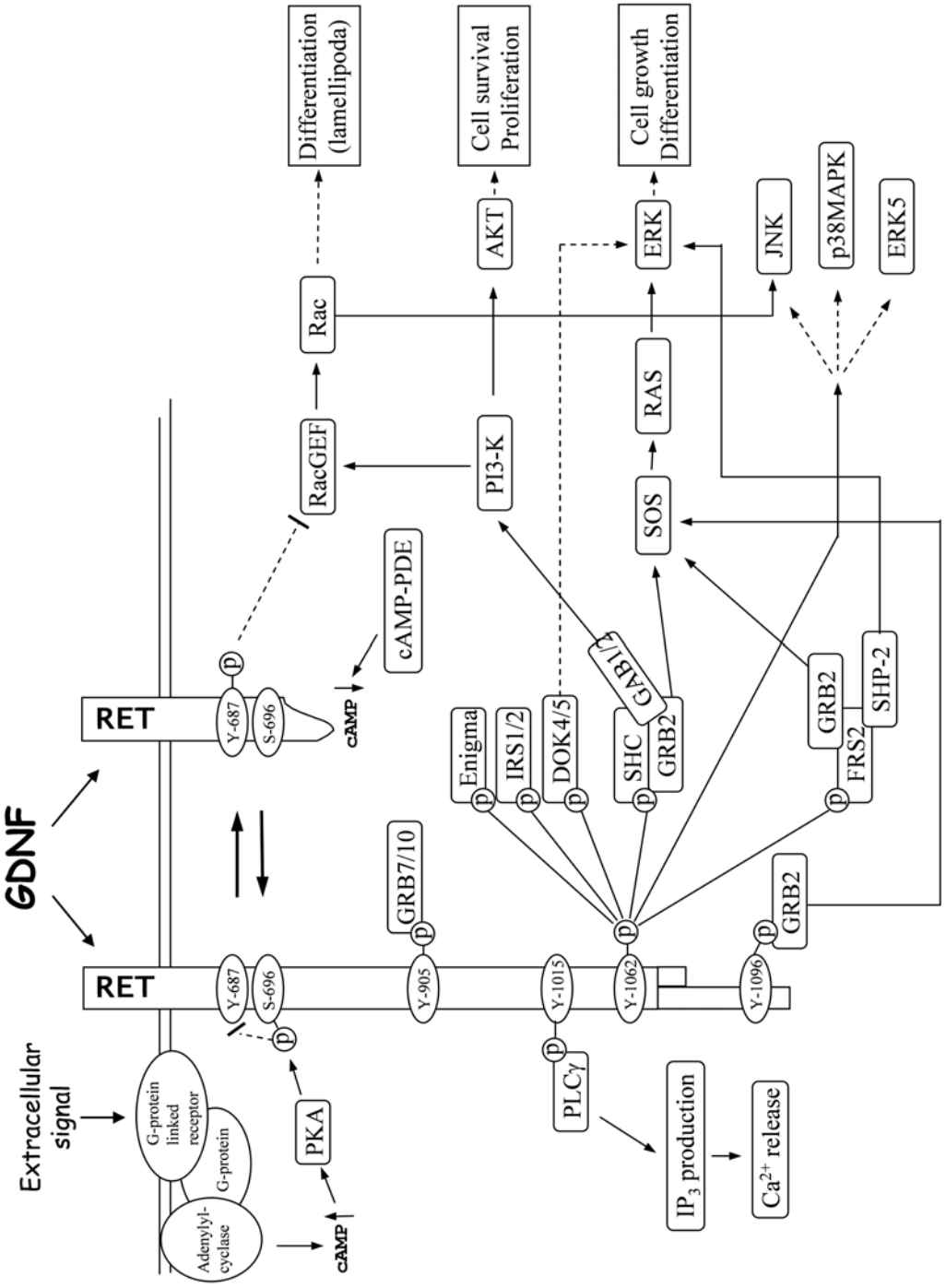

Figure 8: Intracellular signalling pathways activated by RET phosphorylation (from Ichihara et al., 2004)

9.2.4 Differential signalling inside and outside of lipid rafts

Lipid rafts are lipid micro-domains constituted of sphingolipids and cholesterol within the plasma membrane and play important roles in cellular signalling, because Src family kinases and a number of other signalling proteins are localized and enriched in the lipid rafts. Recent evidences indicate that lipid rafts are crucial for abundant biological events including growth factor-receptor signalling, cellular adhesion, synaptic transmission and membrane-associated proteolysis (Simons and Toomre, 2000). In the RET functional receptor, GFRα co-receptors are anchored to the plasma membrane by the glycosylphosphatidyl-inositol, and hereby localized mainly to the lipid rafts. It has been demonstrated that RET is localized outside of lipid rafts in the inactive form and GFRα-1 recruits RET to lipid rafts upon stimulation with GDNF (Tansey et al., 2000). Disruption of RET localisation to lipid rafts resulted in the profound attenuation of intracellular signalling events and subsequent diminished neuronal differentiation and survival, although RET was phosphorylated after the GDNF stimulation. Interestingly, it was suggested that RET which moves to lipid rafts following GDNF stimulation triggers the signal through FRS2, while activated RET located outside of rafts triggers the signal through SHC (Paratcha et al., 2001; Tansey et al., 2000). Additionally, it has been demonstrated that soluble GFRα-1, which binds GDNF and activates RET initially outside rafts, can recruit RET to the rafts with delayed kinetics (Paratcha et al., 2001). Inside or outside of rafts, or the balance between the binding to SHC and to FRS2 may be a tool to differentially influence the nature of the intracellular signalling in target cells, because FRS2 and SHC share their docking site at tyrosine 1062 in RET.

9.2.5 RET-independent GDNF signalling

9.2.5.1 The role of glycosaminoglycans

It has been demonstrated that GDNF signalling depends in addition to the direct receptors GFRα and RET on the presence of heparin sulphate glycosaminoglycans (Barnett et al., 2002; Tanaka et al., 2002). In the absence of these cell-surface molecules, GDNF-dependent RET phosphorylation and in consequence GDNF-induced axonal growth is abolished (Barnett et al., 2002). It is evident that heparin sulphate proteoglycans, such as syndecans, may be able to locally concentrate GDNF in the neighbourhood of GFRa and RET, because GFLs generally bind to heparan-sulphate side chains of extracellular-matrix proteoglygans. Application of very high concentrations of GDNF can overcome the lack of

heparin sulphates in respective cells *in vitro* and activate RET, thus underlining the hypothesis that heparin sulphates act as important local concentrators for GFLs.

Further it has been shown that GDNF is able to induce Met receptor tyrosine kinase signalling in several RET-deficient but GFRα-1 positive cells as well as in cells expressing both GDNF-receptor components (see Figure 9A) (Popsueva et al., 2003). A direct interaction between GDNF and Met is unlikely, because GDNF does not immunoprecipitate Met in these cell systems. The GDNF-induced RET-independent Met activation has been shown to be dependent on activation of Src-family kinases and may be mediated by the heparin sulphate proteoglycan syndecan (Figure 9A).

9.2.5.2 Neural cell adhesion molecule NCAM

GDNF can signal through GFRα-1 in a RET-independent way and activate Src family kinases (SFK) (Poteryaev et al., 1999; Trupp et al., 1999). In RET-deficient cells, GDNF triggers SFK activation and subsequent phosphorylation of MAPK, PLC-g, cAMP response element-binding protein (CREB) and induces c-FOS (Poteryaev et al., 1999; Trupp et al., 1999). This RET-independent signalling predicts another yet unidentified transmembrane receptor, because the GPI-anchored GFRα-1 should not be able to transmit a signal across the plasma membrane. This hypothesis is further strengthened by the fact that GFRα-1 is expressed in many tissues without coexpression of RET (Trupp et al., 1997; Kokaia et al., 1999; Ylikoski et al., 1998). Recently it has been demonstrated that neural cell adhesion molecule NCAM functions as an alternative signalling receptor for GFLs (Paratcha et al., 2003). When GFRα-1 is associated with NCAM, GDNF binds with high affinity to p140NCAM and activates the cytoplasmic Src-like kinase Fyn and focal adhesion kinase FAK (Paratcha et al., 2003) (see Figure 9B). By binding to NCAM, GDNF stimulates Schwann cell migration and axonal growth in hippocampal and cortical neurons. Even in the absence of GFRα-1, GFLs interact with NCAM with low affinity; additionally in the absence of GFLs, association of GFRα-1 with NCAM alone influences NCAM-mediated cell adhesion. This ability of GFRα-1 to modulate NCAM-mediated cell adhesion suggests distinct roles for GFRα-1-NCAM and GDNF-GFRα-1-NCAM signalling. In another study, it could be demonstrated that the effects of GDNF on midbrain dopaminergic neurons are inhibited by a NCAM-blocking antibody (Chao et al., 2003), which suggests a profound role of NCAM for GDNF-signalling even in the presence of RET.

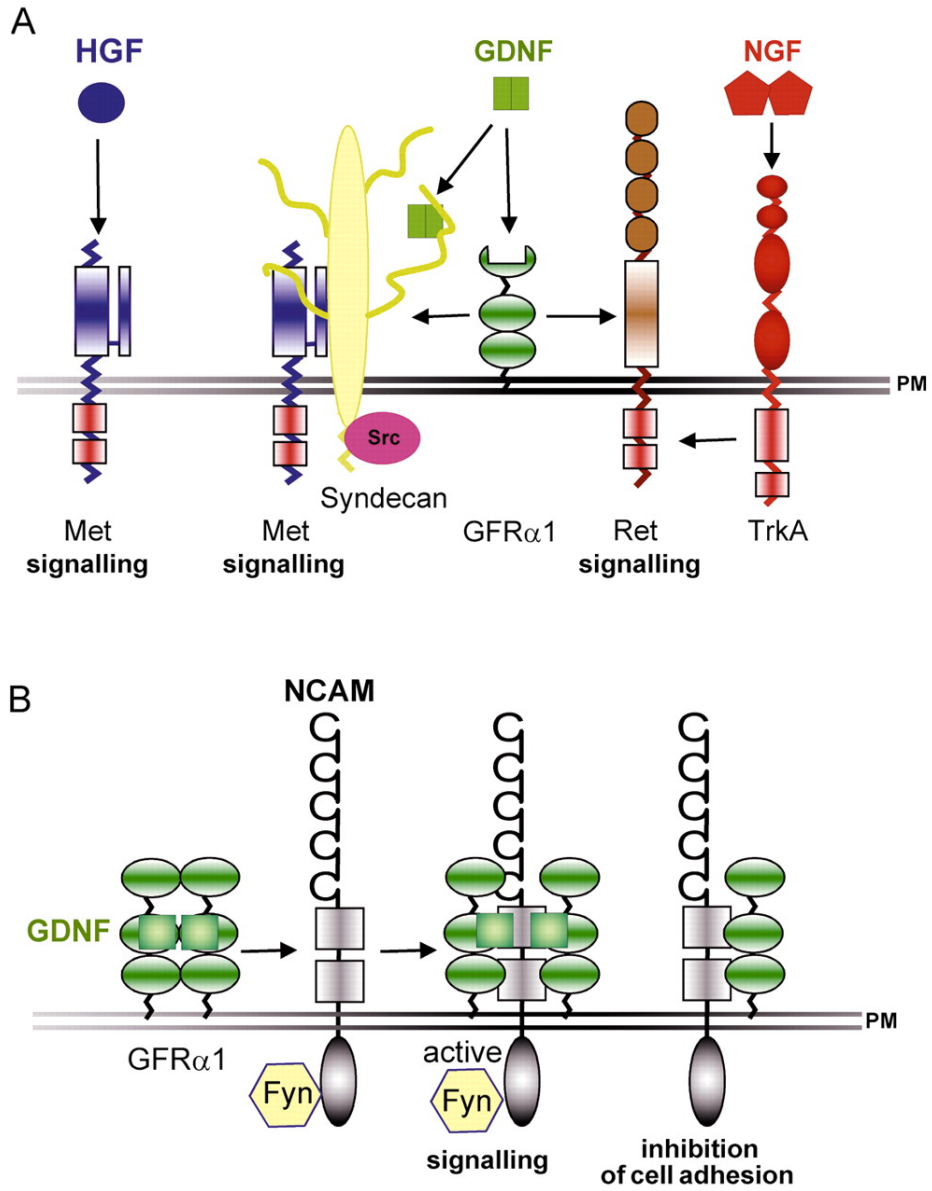

Figure 9: Ret-independent signalling for GDNF and RET activation by crosstalk with TrkA (from Sariola and Saarma, 2003)
A: GDNF promotes phosphorylation of Met. Met activation is indirect and is mediated by Src-type kinases. Evidence suggests that GDNF is locally concentrated by heparan sulphate proteoglycans, such as syndecan. By contrast, RET is phosphorylated, by an unknown mechanism, through the activation of TrkA.
B: NCAM is an alternative signalling receptor for GFLs. GDNF-GFRa complex interacts with NCAM leading to activation of Fyn, a Src-like kinase.

9.3 Crosstalk with other growth factors or receptors

9.3.1 TGFβ

Transforming growth factor betas (TGF-βs) are widely distributed and act as cytokines in development and cell-cycle control (Sporn and Roberts, 1990). Moreover, TGF-βs have been involved in the regulation of neuronal survival (Martinou et al., 1990; Chalazonitis et al., 1992; Krieglstein and Unsicker, 1994) but it has been suggested that TGF-β requires a cofactor for eliciting this neuroprotective effect. GDNF was suggested as a possible candidate cofactor, and indeed it has been demonstrated that GDNF requires TGF-β for trophic support of neurons *in vitro* (Krieglstein et al., 1998) and *in vivo* (Schober et al., 1999). TGF-β seems to be able to recruit GFRα into lipid rafts of the plasma membrane and thus enabling effective GDNF-induced ERK signalling (Schober et al., 1999).

9.3.2 BDNF

It has been suggested from experiments with knock-out mice that some neurons, namely nodose-petrosal ganglion complex (NPG) of primary cranial sensory neurons, require both, GDNF and BDNF for survival *in vivo*. Accordingly, it has been demonstrated that BDNF and GDNF exert additive survival-promoting effects on neurons *in vitro* (Erickson et al., 2001); the mechanism of this synergistic effect is unresolved.

9.3.3 NGF

Nerve growth factor (NGF) is a classical trophic factor for developing sympathetic neurons and acts through its specific receptor TrkA. There has been experimental evidence that GFLs also enhance survival of adult sympathetic neurons (reviewed by Airaksinen and Saarma, 2002). It has been demonstrated that NGF is able to phosphorylate the long isoform of RET (RET51) independently of either GFLs or GFRα coreceptors and thus maintains the trophic status of mature neurons (Tsui-Pierchala et al., 2002). This specific phosphorylation of the long RET isoform indicates that the mechanism is not dependent on simple upregulation of GDNF. It has been hypothesised that there is a direct interaction between RET51 and TrkA receptors (see Figure 9A), although the mechanism of this crosstalk is unknown.

9.3.4 cAMP

Cyclic AMP (cAMP) is a second messenger in the cAMP/Protein kinase A (PKA) signalling cascade and functions as a key regulator for neuronal survival and regeneration (Cai et al., 1999; Cui and So, 2004; Hanson et al., 1998). Intracellular cAMP concentration is

regulated through the balance between synthesis of cAMP by adenylyl cyclase and hydrolysis of cAMP by cyclic AMP phosphodiesterase. Adenylyl cyclase is activated by trimeric GTP-binding proteins (G-proteins) after stimulation of G-protein-linked receptors by specific ligands. It has been demonstated that GDNF enhances the intracellular cAMP level in neuronal cells (Engele and Franke, 1996) and regulation of cAMP level is mediated through phosphorylation of serine 696 of RET receptor (Fukuda et al., 2002). It is presumed that Ser696 is phosphorylated by PKA and a point mutation of this serine in RET completely abolished lamellipodia formation induced by GDNF (see Figure 8). However, the exact mechanism of the hypothesized crosstalk between RET and a yet unidentified G-protein-linked receptor remains unresolved.

AIM OF THE THESIS

Inherited retinal degenerations as well as degeneration processes triggered by age or environmental factors result in eventual loss of photoreceptors, which leads to loss of vision. Due to the complexity of insults leading to degeneration of photoreceptors, it is essential for development of therapeutical strategies to understand the molecular mechanisms of apoptosis in retina and further, to learn about intrinsic neuroprotective pathways. It is a paradox that in inherited retinal degenerations, disease-causing mutations are present from the beginning, but the affected cells may survive extended periods of time, as many of these diseases progress slowly. Thus, the retinal photoreceptors are obviously neuroprotected by intrinsic processes. Many neuroprotective substances, originally found and tested in brain, have been tested for their efficacy against retinal degeneration in different animal models. One of the most effective molecules tested was glial cell-line derived neurotrophic factor (GDNF), which not only preserved histology, but also function of photoreceptors when injected subretinally in rd1 mice (Frasson et al., 1999a). However, the mechanism of GDNF-induced signalling in retina is unknown. It has been speculated that retinal Mueller glial cells (RMG) might respond to GDNF, because upon injection of GDNF RMG were found to react with upregulation of glial fibrillary acidic protein (Frasson et al., 1999a). Likewise, injection of NGF and NT-3 in a light-induced animal model of retinal degeneration leads to a reaction of RMG but not photoreceptors. RMG decreased or increased the production and release of bFGF (Wahlin et al., 2000), respectively, which is a protective factor for photoreceptors. Thus, RMG may be the crucial cell modulating neuroprotection in retinal degeneration.

In order to characterize the role of RMG in mediating neuroprotection, the objectives of this thesis were:

- Establishment of primary RMG *in vitro* culturing system as a prerequisite for characterisation of GDNF-induced signalling.
- Investigation of expression of the family of GDNF ligand receptors (Ret, GFRα-1, -2, -3) in retina followed by analysis of GDNF-induced signalling in RMG.
- Screening and characterisation of factors secreted by RMG which support survival of photoreceptors *in vitro*.
- Identification of neuroprotective factors in interphotoreceptor matrix.

SUMMARY OF PAPERS

1 Proteomic profiling of primary retinal Mueller glial cells reveals a shift in expression patterns upon adaptation to *in vitro* conditions

Glia 2003 Dec; 44(3):251-63

Primary retinal Mueller glial cells (RMG) were accessed by establishing a large-scale preparation combined with a cell culture method. Porcine retinae were first processed by enzymatic trituration followed by enrichment of RMG through exploiting their ability to attach to uncoated culture plastic ware. After an initial attachment phase, RMG were purified from non-attached neurons by panning, enabling the preparation of large quantities of these primary cells as prerequisite for further investigations. Purity of RMG cultures was assessed by immunohistochemical staining with RMG-specific marker proteins (vimentin and glutamine-synthetase). Initial purity was demonstrated at above 70% and increased to above 90% after 8 days *in vitro*. Comparative analysis of protein expressions patterns by 2D-gel electrophoresis of freshly prepared RMG to those of RMG cultivated at 4, 8 and 14 days, revealed profound changes in protein expression at the various time points. Whereas a large set of proteins, including the RMG-marker protein glutamine-synthetase, were downregulated within 8 days *in vitro*, other proteins were simultaneously upregulated. Identification of regulated proteins by mass spectrometry and subsequent functional grouping revealed that downregulated proteins were specific to energy metabolism, detoxification and glutamate metabolism functions *in vivo*. In contrast, many proteins related to cytoskeletal reorganisation e.g. focal adhesion proteins were upregulated. The expression of proteins involved in intracellular signalling processes was also found altered e.g., downregulation of Raf kinase inhibitor protein leading to liberation of the MAP-kinase pathway, which mediates cell proliferation activity in response to extracellular stimuli. The herein observed profound RMG protein expression changes in response to *in vitro* culturing is proposed to reflect the transition of retinal Mueller glial cells from a highly specialized cell type in the natural surrounding to a proliferating cell under *in vitro* conditions.

2 GDNF family ligands trigger paracrine neuroprotective signalling in retinal glial cells

Manuscript submitted

Previous reports have shown that glial cell-line derived neurotrophic factor (GDNF) very effectively rescues retinal photoreceptors in an animal model of inherited retinal degeneration (Frasson, et al, 1999a). However, the mechanism underlying the GDNF-induced rescue, as well as the GDNF-receptive cell type in retina have not been yet elucidated. In order to identify the target cells of GDNF signalling in retina and examine whether retinal Mueller glial cells are involved in GDNF action, the receptor components essential for GDNF signalling were investigated *in vivo* and *in vitro*. Immunohistochemical analysis showed that not photoreceptors but retinal Mueller glial cells (RMG) expressed all components necessary for GDNF signalling: GFRα-1, transmembrane tyrosine kinase RET and additionally express the high affinity coreceptors for neurturin and artemin, GFRα-2 and GFRα-3, respectively. The expression of these components co-localised with RMG cell membranes *in situ* and the expression *in vitro* remained stable during entire cell culture experiments. Phosphorylation studies showed that rapid phosphorylation of RET receptor occurred *in vitro* following GDNF treatment and was accompanied by activation of various intracellular signalling cascades (ERK, SAPK and PKB/AKT pathways). Activation of similar signalling cascades was also found in response to neurturin and artemin. GDNF stimulation of RMG *in vitro* caused an upregulation of basic fibroblast growth factor (bFGF), which itself was found to increase photoreceptor survival *in vitro*. Furthermore, at least one of these signalling pathways (ERK) could be also activated by GDNF in distinct cells of the intact retina; these cells may likely be RMG. These results indicate that RMG are the major GDNF-receptive cells in the retina and transmit the neuroprotective effect of GDNF to photoreceptors. In this context, bFGF promoted photoreceptor survival *in vitro* and may be most likely one of the *in vivo* one factors also supporting photoreceptor survival in response to GDNF.

3 Secreted proteins from retinal Mueller glial cells enhance photoreceptor survival: an approach to detection of new candidates for neuroprotection

Manuscript

Cultured retinal Mueller glial cells (RMG) release substances into the culture medium (conditioned medium) which induce neurite sprouting retinal ganglion cells *in vitro* (Garcia et al., 2002), and enhance cone survival *in vitro* (Balse et al., 2005). Despite available information on effects, the identity of these factor(s) remains unknown. In order to define RMG-derived neuroprotective factors, a large-scale photoreceptor survival assay was developed that enabled monitoring of survival of freshly prepared porcine photoreceptors *in vitro* at extended times. RMG-conditioned medium (RMG-CM) was found to promote photoreceptor survival, but the protective activity of secreted factors was lost with prolonged RMG culturing. The activity present in RMG-CM proofed to be a protein, as demonstrated by proteinase K treatment and enriched into size-fractions above 10 kDa. Subsequent sub-fractionation experiments of RMG-CM by mono-Q FPLC demonstrated that the active factor did not bind to anionic chromatography resins and the composition of the flowthrough was determined by liquid mass spectrometry (LC-Q-TOF). Among the 23 different proteins identified from this active subfraction were three proteins which have been previously described as growth factors in other contexts: insulin-like growth factor binding protein 5 and 7 (IGFBP-5 and IGFBP-7) and connective tissue growth factor (CTGF). Stable expression of these three candidate factors in HEK293 cells yielded media enriched with the respective factor that, although inactive on its own, enhanced the protective activity of RMG when supplemented to its original conditioned medium. This indicated that the survival promoting activity in RMG-CM is multifactorial and triggered by IGFBP-5 and CTGF.

4 Proteomic analysis of porcine interphotoreceptor matrix

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The interphotoreceptor matrix (IPM) is the extracellular compartment located between the retina and retinal pigment epithelium (RPE). Due to its key location, this compartment has been speculated to be rich in neuroprotective substances. Microvilli from retinal Mueller glial cells (RMG) and RPE project into IPM, such that proteins secreted by these cells could potentially augment the neuroprotective function of this structure. This study analysed the IPM proteome and identified 140 different proteins, the majority of which had not been previously described in this structure. Since IPM preparations are easily susceptible to contamination by intracellular proteins of neighboring, fragile cells, a strategy discriminating bona fide IPM-proteins from contaminant proteins due to cell disruption had to be established. Comparison of 2D gel protein patterns between two different IPM preparation methods increased the likelyhood of predicting the presence of IPM proteins in vivo. In addition, a computational prediction method was used to score the likelyhood extracellular occurrence for the identified proteins. Combining both prediction methods generated a subset of proteins fulfilling both conditions and which were judged most likely to occur in the IPM *in vivo*. Interestingly, the majority of these have been described in the literature as playing a functional neuroprotective role but have not yet been associated to IPM. One such example, 78 kDa glucose-regulated protein (GRP78) was confirmed in the IPM *in situ* by immunohistochemical staining of porcine eye sections, thus validating the proposed prediction method.

FUTURE RESEARCH

Neuroprotection is a promising strategy for the development of therapies against neurodegenerative diseases. In order to minimally disturb the delicate balance of the retina by therapeutic intervention, it is essential to identify intrinsic neuroprotective molecules and clearly elucidate their mechanisms of action in retinal tissue.

Finding substances intrinsically neuroprotective in retina prompted us initially to investigate the retinal Mueller glial cell (RMG) secretome, since this cell type has been implicated to play a fundamental role in supporting the survival of retinal neurons (paper 3). This analysis involved a combination of biochemical purification methods with functional testing of subfractions on a herefore developed photoreceptor survival assay. Subsequent mass spectrometric protein identifications of the active RMG-secretome subfraction led to the identification of two proteins which may be regarded as candidates for neuroprotective activity: insulin-like growth factor binding protein 5 (IGFBP5) and connective tissue growth factor (CTGF). Both proteins have been reported to bind to insulin-like growth factor and modulate its activity, thus suggesting that the main activity secreted from RMG may be IGF-1 or IGF-2. Since both insulin-like growth factors have been reported to be expressed in retina and IGF-1 has beeen demonstrated to enhance survival of retinal amacrine cells, further studies are planned to investigate the survival-promoting effect of both molecules on survival of retinal photoreceptors *in vitro*, either applied alone or in combination with IGFBP5 and CTGF. Should significant neuroprotection be achieved, such agents may be further evaluated in an already existing retinal explant model (collaboration with Prof. T. VanVeen, Lund University).

Another approach to identify intrinsically active molecules involves analysis of the interphotoreceptor matrix proteome, an extracellular compartment directly surrounding photoreceptor inner and outer segments (paper 4). Several molecules, which have been described as having neuroprotective function in other contexts, were identified in this structure. Further studies are aimed at investigating the photoreceptor survival promoting potential of some of these molecules.

In this thesis, the mechanism of GDNF-mediated neuroprotective action was adressed in detail (paper 2). However, essential parts of this molecular mechanism still remain to be solved. Although an upregulation of basic fibroblast growth factor (bFGF) was found in RMG in response to GDNF, and bFGF treatment does prolong photoreceptor survival *in vitro*, it fails however to rescue photoreceptor function in the *in vivo* situation. Therefore, the existence of additional, as of yet unidentified molecules induced by GDNF, necessary and/or

essential for exerting a functional rescue of photoreceptors, is hypothesised. In order to find these molecules, transcriptome studies will be performed with RMG prepared from transgenic mouse retina. The transgenic mice express green fluorescent protein under the glial fibrillary acidic protein promoter resulting in green fluorescing RMG in the retina (mouse developed by Dr. M. Götz, ISF, GSF). Thus, primary RMG can be enriched to very high purity by FACS-sorting and immediately stimulated with GDNF. The transcriptional effects of GDNF on genes will be then monitored by Affymetrix arrays. Potential genes coding for secreted proteins as possible candidates participating in neuroprotective effects involved in GDNF-induced photoreceptor rescue will be selected. The activity of candidate proteins will be evaluated using the photoreceptor survival assay developed in this thesis.

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PAPER 1

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Proteomic Profiling of Primary Retinal Müller Glia Cells Reveals a Shift in Expression Patterns Upon Adaptation to In Vitro Conditions

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KEY WORDS retina; transdifferentiation; functional proteomics; focal adhesion; PVR

ABSTRACT Cultured primary retinal Müller glia cells (RMG), a glia cell spanning the entire neuroretina, have recently gained increased attention, especially with respect to their presumed in vivo role in supporting photoreceptor function and survival. Cultured RMG cells, however, are at risk to lose much of their in vivo features. To determine the conditions of isolated primary RMG cells best corresponding with their physiological role in the intact retina, we profiled the respective proteomes of RMG freshly isolated from intact pig eye, as well as from cultured material at different timepoints. Protein samples were separated by high-resolution two-dimensional electrophoresis (2-DE), and isolated proteins were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) peptide mass fingerprint. Compared with freshly isolated RMG, the in vitro protein expression patterns remain relatively stable for the first 3 days in culture but change dramatically thereafter. Proteins involved in specific RMG physiological functions, such as glycolysis, transmitter recycling, CO_2 siphoning, visual pigment cycle, and detoxification, are either downregulated or absent. In contrast, cytoskeletal proteins, as well as proteins involved in motility and in proliferation, are upregulated during culture. In the present report, we show for the first time, on a systematic level, that profound changes in the RMG proteome reflect transdifferentiation from a multifunctional, highly differentiated glial cell to a dedifferentiated fibroblast-like phenotype in culture.

INTRODUCTION

Retinal Müller glia (RMG) are the main glial cells of the neuronal retina. While astrocytes surround blood vessels in the nerve fiber layer, RMG are the only macroglial cell that span the entire thickness of the retina and make contact with virtually all retinal neurons; RMG also provide structural stability to the retina. Moreover, supported by an ever increasing amount of evidence (for review, see Bringmann and Reichenbach, 2001), RMG also support photoreceptor and ganglion cell survival by yet unknown mechanisms (Garcia et al., 2002), suggesting a crucial role of these cells in the maintenance of retinal functional integrity, as well as a potential role within retinal degenerative diseases and their future therapy.

RMG span a wide range of functions: (1) regulation of the extracellular homeostasis of relevant ions, including pH and water content of the extracellular space; active retinal neurons release K^+ ions, and RMG siphon K^+ from the extracellular space and redistribute it into dif-

Abbreviations used: RMG, retinal Müller glia cells; 2-DE, two-dimensional electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; GLAST, glutamate-aspartate transporter; CRALBP, cellular retinaldehyde-binding protein; α-SMA, α-smooth muscle actin; PBS, phosphate-buffered saline; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoracetic acid; PMF, peptide mass fingerprint; ppm, parts per million; GS, glutamine synthetase; GFAP, glial fibrillary acidic protein.

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ferent sinks, such as the vitreous body, blood vessels, and the subretinal space (Newman et al., 1984; Karwoski et al., 1989; Reichenbach et al., 1992); (2) delivery of trophic substances to neurons and removal of metabolic end-products (Poitry et al., 2000); and (3) metabolism of glucose to lactose, which is preferentially taken up by photoreceptors as a source of energy for their oxidative metabolism (Poitry-Yamate et al., 1995).

In addition, RMG contribute to neuronal information processes through the fast reuptake of released neurotransmitters, by providing neurons with precursors of neurotransmitters and by reabsorbing neuronally released glutamate via the glutamate-aspartate transporter (GLAST) (Otori et al., 1994; Derouiche and Rauen, 1995); the latter is expressed exclusively by RMG and astrocytes. Glutamate reuptake is crucial for the rapid termination of light-evoked activity in retinal ganglion cells (Matsui et al., 1999), as well as maintaining low extracellular glutamate levels for prevention of glutamate excitotoxicity and disturbances in neuronal processing. The main degradation pathways of glutamate in RMG is the synthesis of glutamine by glutamine synthetase (Linser and Moscona, 1979) and the synthesis of glutathione from glutamate, cysteine, and glycine; glutathione acts as an intraretinal antioxidant (Makar et al., 1994; Huster et al., 1998). RMG have been also shown to play a crucial role in trophic factor-induced signaling within the retina (Wahlin et al., 2000) and are implicated in mediating photoreceptor rescue effects of several neurotrophic factors (Frasson et al., 1999; Wahlin et al., 2001).

Cultured RMG are widely used in physiological and signal transduction studies. It has been reported, however, that characteristic RMG features may shift or disappear; for example, cellular retinaldehyde-binding protein (CRALBP) is downregulated in vitro within 2 weeks, and de novo expression of α-smooth muscle actin (α-SMA) is induced after 2 weeks in culture (Guidry, 1996). These observations, indicative of a shift in protein expression, prompted us to conduct a systematic analysis of protein expression changes on a global proteomic level and to acquire information about distinct RMG-associated changes in function. Proteins expressed in dynamic compositions determine the phenotypic expression of genomic information. An important advantage of global protein expression profiling, as compared to individual gene regulation studies, is the ability to monitor simultaneous changes in several functional groups. Thus, we compared RMG protein expression patterns, either cultured for various periods of time or freshly isolated from the intact eye. Resultant protein expression patterns were taken as a fingerprint of the physiological state of RMG, with the aim of first identifying individual changes followed by clustering protein sets attributable to specific functional networks and their corresponding physiological features. In this study, we present proteomic RMG fingerprints showing the onset and dynamics of pronounced alterations in expression patterns resulting from dramatic environmental changes that these cells are exposed to when shifted from organotypic conditions after primary isolation to prolonged, rather synthetic cell culture conditions.

MATERIALS AND METHODS

RMG Preparation and Cell Culture

Adult porcine eyes were provided by a local slaughterhouse. They were removed from the animals within 5 min after death and kept on ice in CO_2-independent medium (GIBCO) until further use. Retinae were prepared as described previously (Gaudin et al., 1996). Briefly, major blood vessels were removed and the retina was washed twice in Ringer's solution without Ca^{2+} supplemented with 2.5 mM EGTA. Dissociation of retinal tissue was obtained by treating each retina with 2.2 U of activated papain (Worthington Biochemical) for 40 min at 37°C; papain enzyme activity was stopped by the addition of DMEM with 10% fetal calf serum (FCS). Then, 160 Kunitz-units of DNase (Sigma) was added and the tissue was further dissociated by gentle trituration using a fire-polished Pasteur pipette.

Preparation of day 3–23 samples: dissociated cells were collected by centrifugation (800*g*, 5 min), resuspended in DMEM containing 10% FCS and plated directly onto cell culture plates (NUNC). The plated cells were allowed to attach for 16 h at 37°C in an incubator. The nonattached cells were then removed by gentle agitation (panning), and the purity of the cultures was assessed immunohistochemically.

Preparation of day 0 samples: the papain-digested retinal cells were layered on a 10 ml linear density gradient composed of 0–50% Percoll (Pharmacia LKB Biotechnology, Uppsala Sweden) in 0.9% NaCl and separated at 2,800*g* for 5 min. RMG were enriched to approximately 25% Percoll and subjected to a second Percoll gradient centrifugation (Guidry, 1996). Purity of RMG samples after the second gradient was also tested immunohistochemically.

Immunohistochemistry

RMG grown on glass coverslips within the culture plates or Percoll gradient-enriched RMG were spun down onto glass coverslips with a cytospin centrifuge at 1,000*g* for 5 min.

Coverslips were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 10 min at 37°C. Cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS, preincubated in PBS containing 5% FCS and 0.1% Triton X-100 (blocking buffer) for 10 min, and then incubated with one of the following antibodies in blocking buffer: anti-vimentin (Sigma, clone V9; 1:200) or anti-glutamine synthetase (BD Transduction Laboratory; 1:200) for 2 h. After washing in PBS, RMG samples were incubated with an anti-mouse alexaFluor 488 (Molecular Probes; 1:200)

secondary antibody combined with DAPI (Sigma, 1 μg/ml) in the dark for 1 h at room temperature. Coverslips were washed and cells examined with a Zeiss Fluorescence Axioskop. DAPI-positive nuclei were counted on 3 to 5 fields of 3 separate coverslips per experiment, and compared to the number of vimentin or glutamine synthetase (GS)-positive cells on the respective fields. Immunostaining experiments were conducted in triplicate.

Protein Extract Preparation and 2D Electrophoresis

Both freshly isolated (day 0) and cultured RMG cells (day 3–23) were washed three times with DMEM and once with PBS before incubating with lysis buffer (9 M urea, 2 M thiourea, 1% DTE, 4% CHAPS, and 2.5 μM of both EGTA and EDTA) and protease inhibitors (complete mini, Roche) for 10 min at room temperature. Lysates were then centrifuged through a QIAshredder Mini Column (Qiagen) and samples were stored at −70°C until further use.

Electrophoretic separation was carried out first by pre-swelling Immobiline dry strips (Pharmacia, pH 3–10 NL, 18 cm) overnight in lysis buffer containing additional 1% Pharmalyte, pH 3–10, and 0.5% bromphenol blue (BPB w/v 0.5% in water).

Protein loading was normalized by a Bradford assay (Bio-Rad); 100 μg of whole cell extracts were then applied to the pre-swollen IPG strips by sample cup loading, followed by isoelectric focusing on a Multiphor (Amersham Biosciences) at 20°C. Initial voltage was limited to 300 V (3 h) and then increased stepwise to 8,000 V, where it was held until 120 kV/h was reached.

Prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the focused IPG strips were washed in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl pH 6.8, 30% glycerol and BPB traces) for 10 min at 25°C under reducing conditions (65 mM DTE added). This was followed by another incubation under alkylating conditions (135 mM iodacetamide added) for 10 min. Equilibrated strips were loaded onto gradient SDS-PAGE gels (20.5 × 17.5 cm; 9–16%), sealed with 0.5% agarose in running buffer (384 mM glycine, 50 mM Tris, and 0.2% SDS) and electrophoresed overnight at constant 10 mA per gel (Bio-Rad Protean IIxi Multicell). Gels were silver stained according to Blum et al. (1987) and dried between cellophane sheets.

Image Analysis

Silver-stained 2-DE gels were scanned and digital images were analyzed for changes in protein pattern using the Proteom Weaver image analysis software (release 1.3; Definiens, Germany). For a qualitative evaluation of pattern changes, raw master gels were created with Z3 software (Compugen, Israel) by merging two real gel images for each master gel.

Preparation of Samples for Mass Spectrometry

Selected spots were excised from wet or dried silver-stained gels, washed for 30 min in 100 μl nanopure water, destained (Gharahdaghi et al., 1999) and dehydrated in 100 μl 40% acetonitrile (3 × 15 min). Following the removal of acetonitrile, protein samples were digested in 5–10 μl 1 mM Tris-HCl, pH 7.5, containing 0.01 μg/μl trypsin (Sequencing Grade Modified Trypsin; Promega). In-gel digestion was performed overnight at 37°C under humid conditions to prevent drying out of the samples.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) peptide mass fingerprints were obtained on a Bruker Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Scout 384 inlet. Aliquots of the digests (0.5 μl) were mixed with 0.5 μl matrix consisting of 2,5-dihydroxybenzoic acid (Sigma) (20 mg/ml in 20% acetonitrile, 0.1% TFA) and 2-hydroxy-5-methoxybenzoic acid (Fluka) (20 mg/ml in 20% acetonitrile, 0.1% TFA) in a 9:1 ratio (v/v), and spotted onto an 400-μm anchor steel target (Bruker Daltonics). During evaporation, matrix-analyte co-crystals concentrated on the hydrophilic anchor. Mass analyses obtained in the positive ion reflector mode were run manually or automatically. For calibration purposes, angiotensin-2-acetate (M_r 1046.54), substance P (M_r 1347.74), bombesin (M_r 1619.82), and ACTH 18-39 (M_r 2465.20) spectra were recorded. When required, mass spectra were internally calibrated referring to autoproteolytic trypsin fragments of 1045.564 Da and 2211.10 Da.

Database Searching

Trypsin cleaves proteins specifically at the C-terminus of lysine and arginine residues, generating a fingerprint of peptide masses (PMF) that can be located on database searches. These were performed using the Mascot software (Perkins et al., 1999) at the following parameter settings: 150 ppm mass accuracy, one miscleavage allowed, search in eukarya sequences. Peptide masses of the tryptic digests were compared to the virtually generated tryptic peptide masses of the protein databases NCBInr and MSDB. A protein was regarded as identified if four criteria were fulfilled: (1) the MOWSE score (Pappin, 1997) was above the 5% significance threshold for the respective database; (2) the matched peptide masses were abundant in the spectrum; (3) the theoretical isoelectric point and molecular weight of the search result could be accommodated with the 2-DE position of the corresponding spot; and (4) the matched sequence did not contain more than 20% uncleaved peptides. All proteins identified during this study are summarized in Table 2.

RESULTS

RMG Can Be Isolated and Maintained as a Highly Enriched Culture

During the course of experimentation, we found the panning method to be best for generating pure high-yield RMG cultures. Using this method, single retinal cells are plated following papain digestion and are then left untouched for 16 h. After this time, RMG attached to the surface and nonattached cells (mainly neuronal cells) can be washed off by slight agitation and removal of the culture medium. The RMG purity on the day of panning (day 1) was found to be greater than 80% (Fig. 1). Nevertheless, enrichment of RMG directly after the dissociation process was not possible with this method. To obtain working samples from freshly prepared RMG, cells had to be enriched by Percoll gradient centrifugation. Indeed, greater than 70% RMG purity could be reached by applying the cells on two successive Percoll gradients.

To determine the precise proportion of RMG in Percoll-purified cytospinned samples and cells grown in culture for 1–14 days, cells were stained with antibodies against vimentin and GS. Positive cells were related to the numbers of total, DAPI-positive cells (Figs. 1A and 2).

In Percoll-enriched preparations, immunohistochemistry for GS and vimentin showed that 70.4% (±3.4%) and 75.7% (±5.4%) of cells, respectively, were immunoreactive (Fig. 1A). On in vitro day 1 these proportions were 88.1% (±5.6%) and 82.0% (±8.7%), respectively, in preparations purified by panning; on in vitro day 3 the proportions were 76.9% (±2.9%) and 81.8% (±5.0%) positive cells, respectively. Although the proportion of vimentin-positive cells, between days 1 and 3 was identical, the proportion of GS-positive cells at day 3 was slightly decreased. This difference may be due to most RMG expressing GS on day 3 (cf. Fig. 4 protein spots indicated and Fig. 5A GS position of GS on day 0 2-DE gels), but some cells may already have downregulated GS expression. After 8 days in vitro GS expression is strongly downregulated, so that only 7.6% (±10.6%) of the cells are GS-reactive. Vimentin levels remain unchanged (cf. Figs. 2 and 4A). On days 8 and 14, 96.6% (±1.4%) and 99.2% (±1.3%) of the cells, respectively, were vimentin positive, whereas GS expression continued to decrease to 0.9% (±1.0%) on day 14 (Fig. 1A).

Both vimentin and GS have been also reported to be expressed by retinal astrocytes, which are found in vascularized retinae. CRALBP, another candidate RMG marker, has been reported to be transiently expressed in developing mouse retinal astrocytes (P.T. Johnson et al., 1997).

However, whereas no unambiguous marker exists to distinguish between porcine RMG and retinal astrocytes, the distinct RMG morphology maintained after papain digestion (Guidry, 1996; Sarthy and Bunt, 1982) is instrumental for validation of cell identity. Percoll-enriched RMG stained with anti-vimentin antibodies showed that 87.5% (±1.3%) of vimentin-positive cells were elongated and bipolar in shape, with extensive membrane ruffling typical for RMG (Fig. 1B). The remaining proportion of vimentin-stained cells could not be clearly classified and were thus regarded as possible candidates for retinal astrocytes.

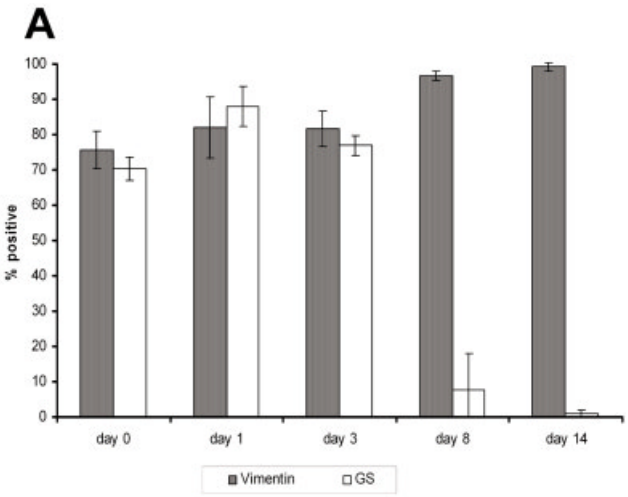


Fig. 1. Purity of freshly isolated and cultured retinal Müller glia cells (RMG). **(A)** Isolated RMG were cytospinned (day 0) or grown (days 3, 8, and 14) on glass coverslips and stained with anti-glutamine synthetase (GS) and anti-vimentin antibodies. The percentage of positive cells was calculated based on the number of total DAPI-stained nuclei on the same area of the coverslip. Error bars are SEM (n = 3). GS expression is downregulated within 14 days in vitro, whereas vimentin expression is stable and underlines the increasing purity of RMG in vitro over time. **(B)** Isolated RMG 2 h after papain digestion cytospinned on glass coverslips and labeled with anti-vimentin antibodies. RMG cells were identified by their characteristic elongated morphology. ×400. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

Protein Changes Correlate With Morphological and Histochemical Alterations in Culture

RMG begin to retrieve their elaborate processes, which are characteristic in vivo, as they are released

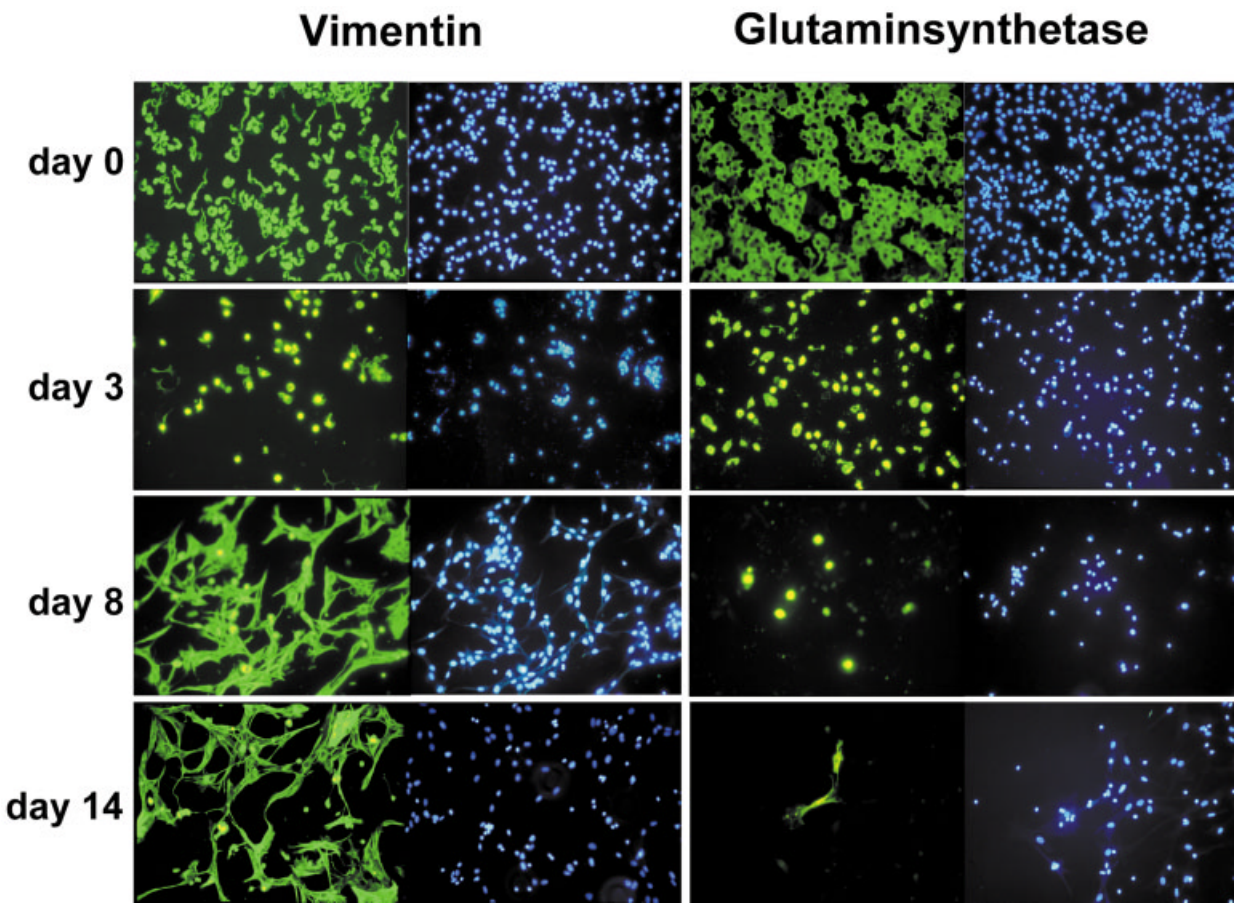


Fig. 2. Morphological changes of retinal Müller glia cells (RMG) during culture. RMG were cytospinned (day 0) or grown (days 3, 8, and 14) on glass coverslips, labeled with anti-vimentin or anti-glutamine synthetase antibodies. Nuclei were stained with DAPI. RMG show morphological changes in adaption to in vitro conditions and by entering mitotic cycles. Labeled cells on left side; nuclei of the same field on right side. ×200. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

from the retina through papain digestion. However, 2 h after digestion, at the time point when day 0 samples were prepared, RMG still showed distinct morphological features, such as an elongated cell body with a centrally located nucleus (Figs. 1B, 3A,B). After 3 days in culture most of the adherent cells showed a round shape morphology (Fig. 2). No significant changes between the 2-DE protein patterns of day 3 and day 0 were detected (Fig. 4). After 8 days in vitro, cells converted into larger, spindle-shaped, flat cells resembling cultured fibroblasts. Along with these morphological changes, the most prominent alterations in the spot pattern of the respective 2-DE gels were observed (Fig. 4). Based on morphology and the 2-DE protein pattern these cultured cells remained unchanged from day 8 to day 14 and day 23 (passage 1 cells) (Figs. 3 and 4).

Changes in Protein Pattern Are Fundamental Within the First 2 Weeks in Cell Culture

The 2-DE protein pattern of RMG lysates after 3, 8, 14, and 23 days in vitro was monitored and compared to that of freshly isolated cells (day 0). The proteome pattern changes are fundamental and the most prominent changes occurred between days 3 and 8 in culture (Fig. 4).

In an attempt to quantify changes in the proteome pattern, virtual average gels were created from three gels at each experimental time point, using Proteom Weaver software default settings (for details, see Materials and Methods). At days 0 and 14, the program detected 1,300 and 1,900 spots, respectively, from the average gels. These spots were sorted by intensity in the average gel at day 0 (defined by an internal algorithm of the software). The 300 most intensive spots were further analyzed, results showed that 29% of these spots (87 of 300) were absent on the average gel at day 14. Further, 25% of the proteins (75 of 300) at day 0 were downregulated more than two-fold, and 40.7% (122 of 300) of the spots were up- or downregulated at 2- to 0.5-fold and were therefore regarded as slightly or unchanged. In summary, 54% of the 300 most intensive spots at day 0 were downregulated by more than two-fold during the first 2 weeks in culture. In contrast, 5.3% of the spots (16 of 300) were upregu-

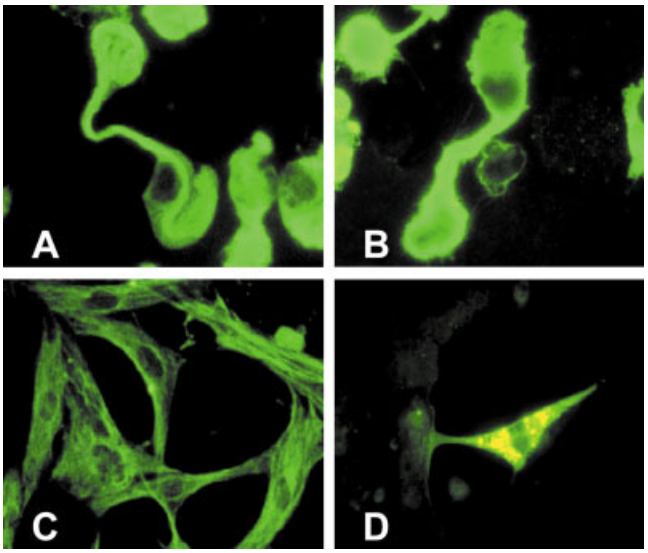


Fig. 3. Changes of retinal Müller glia cells (RMG) morphology, in detail. Examples of labeled RMG **A, C**, anti-vimentin; **B, D**, and anti-glutamine synthetase (GS) at days 0 (A,B) and 14 (C,D) at higher magnification. At day 14 in culture, several strongly GS-positive cells are still visible (D). × 600. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

lated more than twofold on the average gel at day 14 compared to the average gel at day 0.

Mass Spectrometry Identifications Reveal Downregulation of Proteins Participating in Müller Cell-Specific Function

After comparison of the RMG protein patterns from days 0 and 14, spots that were only present in day 0 gels, spots which were unchanged and spots only present or strongly upregulated at day 14 gels (Fig. 5A,B) were excised out of the gels. The proteins were subjected to tryptic digestion, peptide mass fingerprint spectra were recorded on a MALDI-TOF mass spectrometer and were then identified by comparison with public databases (for details, see Materials and Methods).

Strongly downregulated proteins sampled at day 0 included enzymes involved in glycolysis (α-enolase, fructose-bisphosphate aldolase C, GAPDH, LDH-B, 6-phosphofructokinase, and phosphoglycerate kinase) and energy metabolism (aconitate hydratase, aldehyde dehydrogenase, creatine kinase, malate dehydrogenase, phosphoglucomutase, pyruvate carboxylase, and transketolase) (Fig. 5A and Tables 1 and 2).

Also downregulated were proteins involved in visual pigment recycling (CRALBP) and enzymes involved in detoxification processes (glutathione S-transferase, peroxiredoxin 2, PHGPX nonselenium, and superoxide dismutase). Proteins involved in the phototransduction process (S-arrestin, phosducin, and recoverin) are found on the 2-DE gels of day 0 RMG and are derived most likely from contamination of these samples with photoreceptors. Glutamine synthetase (GS), the main enzyme for transmitter recycling in the RMG, was completely downregulated and disappeared after 8 days in vitro. Experiments with ^{35}S-pulse labeling on day 3 in vitro showed that even at this early time point no GS de novo synthesis could be detected (data not shown). Carbonic anhydrase II, the enzyme involved in CO_2 siphoning in vivo was also completely suppressed in culture; similarly, an enzyme from the amino acid metabolism (glutamate dehydrogenase) was also downregulated.

At day 0, strong expression of glial fibrillary acidic protein (GFAP) was found. As GFAP upregulation has been reported in response to many retinal diseases and injuries (Bignami and Dahl, 1979; Wen et al., 1995), the observed level of GFAP expression at day 0 may, at least in part, also reflect the impact of injury from tissue disruption. With advancing time in culture, GFAP was downregulated, whereas other cytoskeleton proteins, such as vimentin, cofilin, and gelsolin, remained unchanged. Another group of 10 cytoskeleton proteins was found to be strongly upregulated during culture (actin, actin-like protein 3, α-actinin 1, α-actinin 4, annexin II, L -caldesmon, HSP27, T-plastin, vinculin, and WD repeat protein 1) (Tables 1 and 2 and Fig.5B).

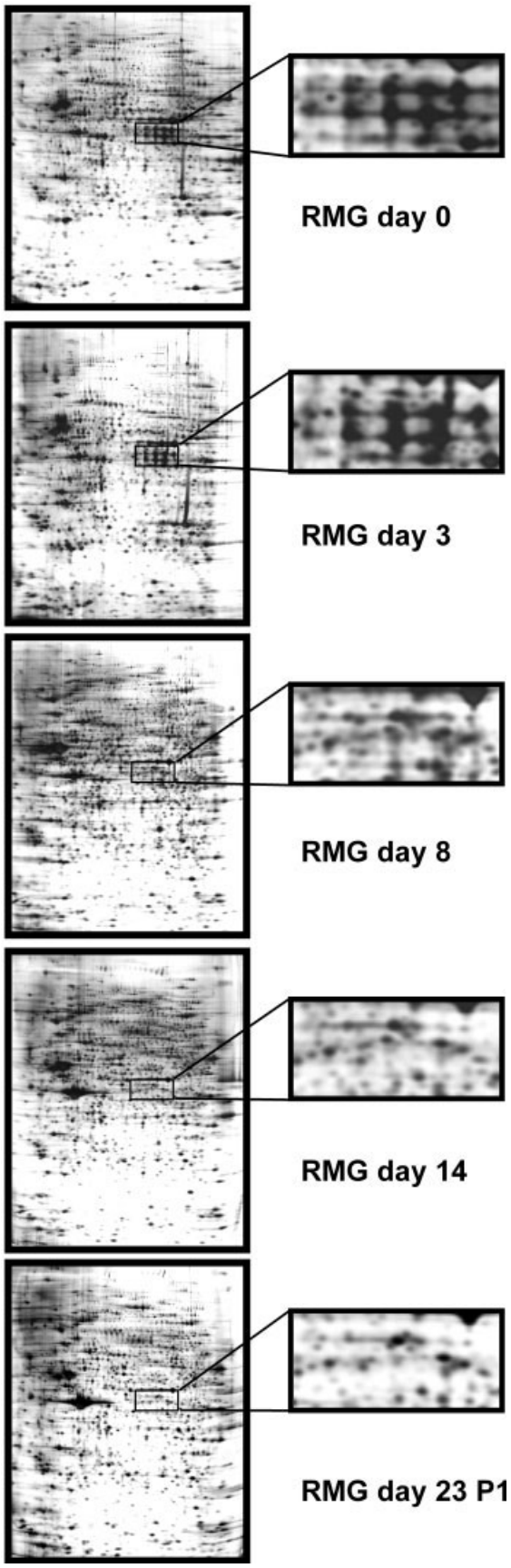
Other proteins involved in signaling processes were either downregulated (ERK2, 1433 epsilon, CRABPI, BFABP), upregulated (adenylyl cyclase-associated protein 1, LASP1, EFABP), or unchanged (rab GDP dissociation inhibitor, DJ1).

Several molecular chaperones could be also identified (endoplasmin, HSP70-2, Hsp60, Hsc73), but no changes in expression were noted. Two proteins involved in Ca^{2+} binding were found to be upregulated (calumenin and annexin V) and two unchanged (annexin VI and calreticulin).

Lastly, other identified, downregulated proteins were reticulocalbin, DPY2, hemoglobin β-chain and DDH2; unchanged were esterase D, protein disulfide isomerase, S-adenosylhomocystein hydrolase, and TER ATPase; upregulated proteins were elongation factor 2, major vault protein, ubiquitin hydrolase L1, ALG2 interacting protein, and translationally controlled tumor protein 23 (Tables 1 and 2 and Fig. 5).

DISCUSSION

Primary cells or cells derived from primary tissue are increasingly being used as replacement for intact, organotypic tissues. In addition, they often represent the only possible means of defining specific functions attributed to a specific cell within a complex tissue of cellular heterogeneity. Although this bares great potential, a major question lies in how well cultured cells represent a given cell in a given intact tissue. Here, a caveat lies in their natural plasticity in response to such stimuli as addition of culture medium, gas concentrations and serum factors, as well as removal of



natural stimuli provided by the organ context into which they are naturally embedded. It has been previously shown that avian RMG are even able to transform into phenotypic lens cells upon disruption from the intact retina (Moscona et al., 1983).

Although we took great care to isolate RMG at the highest degree of purity, we retain an initial contamination of these isolates by retinal neurons. In fact, this most likely reflects the tight initial interconnection between RMG and retinal neurons. In consequence, proteins known to be expressed in retinal neurons, most notably proteins involved in phototransduction appear as protein spots on the 2-DE gels of RMG days 0 and 3.

Phenotypic Characterization of RMG

In vascularized retinae, two types of macroglial cells are found: RMG and astrocytes (for review, see Willbold and Layer, 1998). These two cell types are potentially able to grow under in vitro conditions following cultivation of dissociated porcine retinal cells. Therefore, the ability to differentiate between RMC and astrocytes in primary cultures is of great importance. GS, a candidate marker for RMG is found expressed only in cat retina RMG (Lewis et al., 1988) but in rat retina, GS was noted in RMG and astrocytes (Derouiche and Rauen, 1995). For CRALBP, another candidate RMG marker, no expression profiling in porcine retina exists and it has been reported to be transiently expressed in developing mouse retinal astrocytes (P.T. Johnson et al., 1997). Given the lack of information on the antigenic differences between astrocytes and RMG in pig retina, we decided to base the identification of RMG following papain digestion on their morphological features. In contrast to astrocytes, RMG have long cell bodies with a centrally localized nucleus (Guidry, 1996; Sarthy and Bunt, 1982); these features can still be found about 1 h after papain digestion. We were thus able to identify most (87.5% $\pm$1.3%) vimentin-positive cells as morphologically distinct RMG when analyzed on a coverslip of a cytospinned fraction subsequent to Percoll enrichment. Cells not clearly displaying these

Fig. 4. Changes of the protein pattern of retinal Müller glia cells (RMG) with time in vitro. Images of two-dimensional electrophoresis (2-DE) gels of RMG protein extracts prepared directly after RMG preparation (day 0) or after different timepoints in vitro, as indicated above the gel images. 2-DE was performed on precast IPG strips, pH 3.5–10 NL, with 100 μg protein load and focused for a total of 120 kV/h to equilibrium; the strips were loaded on 9–16% polyacrylamide gel electrophoresis (PAGE), and proteins were separated due to their molecular weight at a constant 10 mA overnight. Gels were silver stained and dried. Each image is an average from two real gel images, which were merged into a raw master gel with Z3 software (Compugen). The main changes in protein pattern occur at 3–8 days in vitro, whereas during the first 3 days the protein expression remains relatively stable. The expression pattern at days 14 and 23 passage 1 cells does not differ. Glutamine synthetase (GS) spots are boxed and enlarged in day 0 and day 3 gels. Corresponding boxes indicated at days 8, 14, and 23 passage 1 gel display the similar position, but GS spots are no longer present.

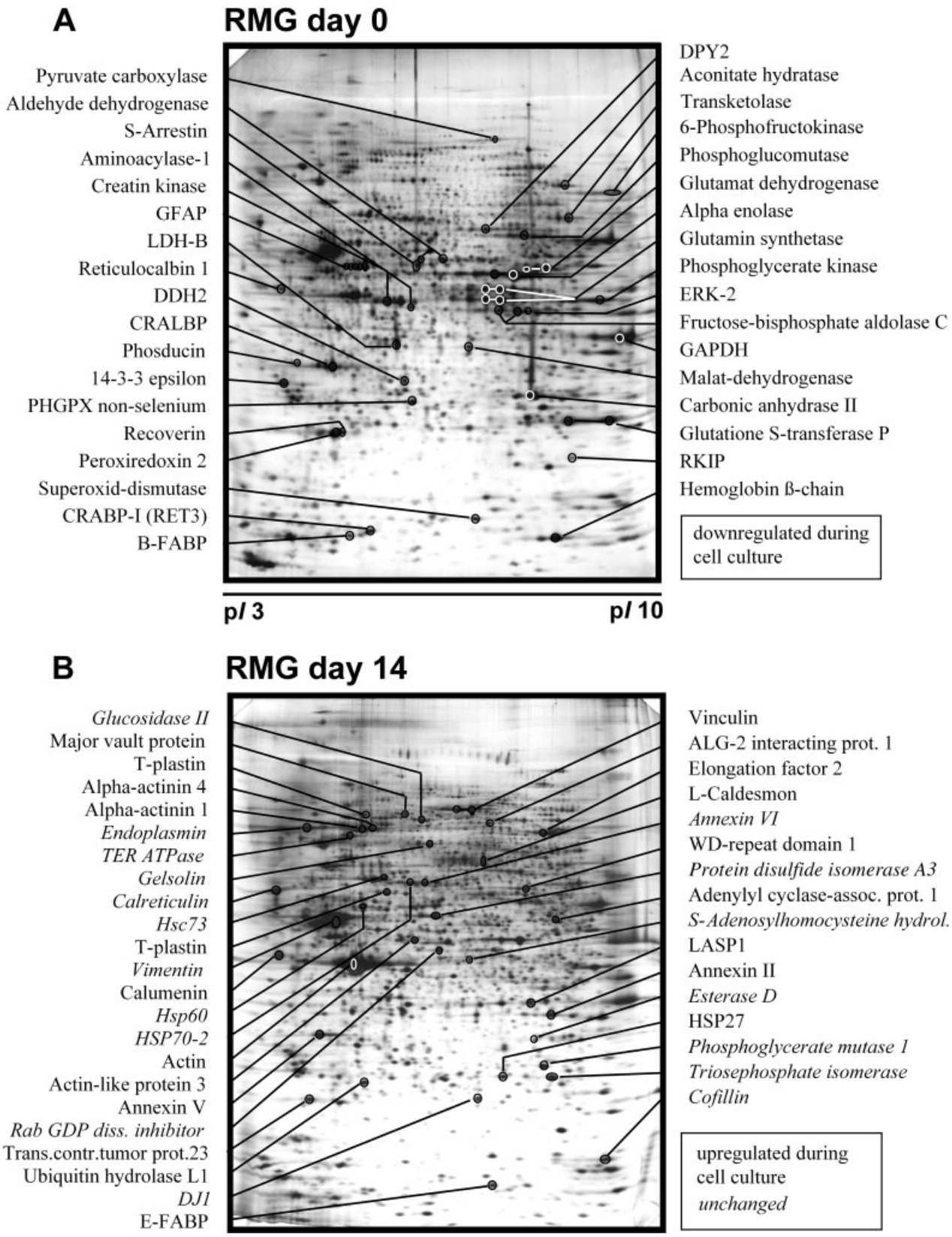


Fig. 5. Mass spectrometric protein identification. **(A)** Two-dimensional electrophoresis (2-DE) of retinal Müller glia cells (RMG) gel at day 0. The protein spots that are downregulated in vitro are circled, with their respective identification. **(B)** 2-DE RMG gel at day 14. Identification of protein spots that are upregulated in vitro indicated in roman type; identification of protein spots that are unchanged in vitro indicated italics.

TABLE 1. Functional Grouping of RMG Proteins Regulated upon Adaption to In Vitro Conditions

	Downregulated	Unchanged	Upregulated
Glycolysis	α-Enolase	Phosphoglycerate mutase	
	Fructose-bisphosphate aldolase C	Triosphosphate isomerase	
	GAPDH		
	LDH-B		
	6-Phosphofructokinase		
	Phosphoglycerate kinase		
Energy metabolism	Aconitate hydratase	Glucosidase II	
	Aldehyde dehydrogenase		
	Creatine kinase		
	Malate dehydrogenase		
	Phosphoglucomutase		
	Pyruvate carboxylase		
	Transketolase		
Visual pigment recycling	CRALBP		
Detoxification	Glutathione S-transferase P		
	Peroxiredoxin 2		
	PHGPX non-selenium		
	Superoxide dismutase		
Transmitter recycling	Glutamine synthetase		
CO_2 siphoning	Carbonic anhydrase II		
Amino acid metabolism	Glutamate dehydrogenase		
Cytoskeleton	GFAP	Cofilin	Actin
		Gelsolin	Actin-like protein 3
		Vimentin	α-Actinin 1
			α-Actinin 4
			Annexin II
			L-Caldesmon
			HSP27
			T-Plastin
			Vinculin
			WD-repeat protein 1
Chaperone		Endoplasmin	
		Hsc73	
		Hsp60	
		Hsp70-2	
Signaling	14-3-3 epsilon	Rab GDP dissociation inhibitor	Adenylyl cyclase-associated protein 1
	CRABP-1 (RET3)	DJ1	LASP-1
	B-FABP		E-FABP
	ERK-2		
	RKIP		
Ca^{2+} -binding		Annexin IV	Annexin V
		Calreticulin	Calumenin
Miscellaneous	DDH2	S-Adenosylhomocysteine hydrolase	ALG-2 interacting protein
	DPY2	Esterase D	Elongation factor 2
	Hemoglobin β-chain	Protein disulfide isomerase A3	Major vault protein
	Reticulocalbin	TER ATPase	Translationally controlled tumor protein 23
			Ubiquitin hydrolase L1

RMG, retinal Müller glia; CRALBP, cellular retinaldehyde-binding protein; GFAP, glial fibrillary acidic protein.

morphological features were regarded as potential astrocytes, although it may have been likely that these cells were either already completely rounded up or unfavorably plated on the coverslip due to sheer forces from the cytospin procedure.

Changes in RMG-Specific Protein Profiles

Our results show that several distinct interrelated functional groups of proteins attributable to specific functions of RMG demonstrated shifts in expression patterns. These alterations in proteomes suggest that in addition to the known interrelation of single proteins, at the level of functional protein networks, there is co-regulation of functionally interrelated proteins in RMG at the level of protein expression. At least six sets of markedly distinct RMG marker proteins shift in expression with prolonged culture conditions:

1. *Enzymes involved in glycolysis and energy metabolism are downregulated during culture*: This may be a consequence of the unlimited supply of nutrients in the culture medium. In vivo RMG are the principal storage site for retinal glycogen (Kuwabara and Cogan, 1961) and metabolize glucose to lactose that is subsequently taken up by photoreceptors to fuel their oxidative metabolism (Poitry-Yamate et al., 1995). Since glycolysis in RMG is activated upon NH_4-release by photoreceoptors (Tsacopoulos et al., 1997), the downregulated expression of glycolytic enzymes may be linked to the loss of close contact to photoreceptors.
2. *GS expression is reduced after only 8 days*: In vivo RMG regulate extracellular transmitter levels in the retina. This uptake is essential for terminating synaptic transmission, as well as preventing the spread of transmitters away from the synaptic cleft (E. Newman and Reichenbach, 1996). We did not

TABLE 2. Proteins From RMG Identified by Searching Peptide Mass Fingerprint Spectra in Publicly Available Databases

Top MOWSE score	Swissprot entry name	SwissProt/ Trembl accession no.	Protein name	Organism	M_r (dalton)	Sequ. cov. (%)	pI
196	143E_HUMAN	P42655	14-3-3 protein epsilon	*Homo sapiens*	29,174	52	4.63
95	RSP4_CRIGR	P38982	40S ribosomal protein SA (P40) (34/67-kDa laminin receptor)	*Cricetulus gris*	32,881	46	4.84
132	K6PF_CANFA	P5274	6-Phosphofructokinase (EC 2.7.1.11)	*Canis familiaris*	86,231	26	8.36
92	ACON_BOVIN	P20004	Aconitate hydratase, mitochondrial (EC 4.2.1.3)	*Bos taurus*	85,357	20	8.08
172	ACON_PIG	P16276	Aconitate hydratase, mitochondrial (EC 4.2.1.3)	*Sus scrofa*	83,197	30	7.42
181	ACT2_XENLA	P12557	Actin, α-sarcomeric/cardiac (α2)	*Xenopus laevis*	41,989	51	5.22
199	ARP3_HUMAN	P32391	Actin-like protein 3 (actin-related protein 3) (actin-2)	*Homo sapiens*	47,371	47	5.61
93	CAP1_RAT	Q08163	Adenylyl cyclase-associated protein 1 (CAP 1)	*Rattus norveg.*	51,547	29	7.16
114	DHAM_BOVIN	P20000	Aldehyde dehydrogenase, mitochondrial precursor (EC 1.2.1.3)	*Bos taurus*	57,129	28	7.55
104		Q9UKL5	ALG-2 interacting protein 1	*Homo sapiens*	96,079	18	6.13
141	ENOA_HUMAN	P06733	α-enolase (EC 4.2.1.11)	*Homo sapiens*	47,350	39	6.99
238	AACT_HUMAN	P12814	α-actinin 1 (αactinin cytoskeletal isoform)	*Homo sapiens*	102,974	31	5.22
266	AAC4_MOUSE	P57780	α-actinin 4 (F-actin cross- linking protein)	*Mus musculus*	104,976	36	5.24
98	ACY1_PIG	P37111	Amihoacylase-1 (EC 3.5.1.14) (ACY-1).	*Sus scrofa*	45,216	31	5.61
291	ANX2_BOVIN	P04272	Annexin II (Lipocortin II) (Calpactin I heavy chain)	*Bos taurus*	38,742	62	6.9
245	ANX5_HUMAN	P08758	Annexin V (Lipocortin V) (Endonexin II)	*Homo sapiens*	35,805	63	4.94
131	ANX6_BOVIN	P79134	Annexin VI	*Bos taurus*	34,230	14	5.4
154	CRTC_RAT	P18418	Calreticulin precursor (ERp60)	*Rattus norveg*	48,137	28	4.33
226	CRTC_RABIT	P15253	Calreticulin precursor (ERp60)	*Oryctolag. cuni.*	48,275	45	4.33
162	CALU_HUMAN	O43852	Calumenin precursor	*Homo sapiens*	37,198	41	4.47
	CAH2_HUMAN[a]	P00918	Carbonic anhydrase II (EC 4.2.1.1) (carbonate dehydratase II)	*Homo sapiens*	29,115		6.86
78	CRAL_MOUSE	Q9Z275	Cellular retinaldehyde-binding protein CRALBP	*Mus musculus*	36,277	19	4.98
87	CLI1_HUMAN	O00299	Chloride intracellular channel protein 1	*Homo sapiens*	26,922	37	5.09
220	COF1_PIG	P10668	Cofilin, nonmuscle isoform	*Sus scrofa*	18,518	67	8.16
160	KCRB_CANFA	P05124	Creatine kinase, B chain (EC 2.7.3.2) (B-CK)	*Canis familiaris*	42,960	43	5.47
155	DPY2_BOVIN	O02675	Dihydropyrimidinase-related protein-2 (DRP-2)	*Bos taurus*	62,277	44	5.95
194	DPY2_RAT	P47942	Dihydropyrimidinase-related protein-2 (DRP-2)	*Rattus norveg.*	62,277	61	5.95
82		Q99497	DJ-I protein	*Homo sapiens*	20,063	30	6.33
154	EF2_HUMAN	P13639	Elongation factor 2 (EF-2)	*Homo sapiens*	95,207	27	6.41
129	ENPL_CANFA	P41148	Endoplasmin precursor (94-kDa glucose-regulated protein)	*Canis familiaris*	92,513	23	4.74
74		Q9GJT2	Esterase D	*Sus scrofa*	31,975	18	6.54
218	IF41_RABIT	P29562	Eukaryotic initiation factor 4A-I (eIF-4A-I) (eIF4A-I)	*Oryctolag. cuni.*	44,818	42	5.32
81	FABB_HUMAN	O15540	Fatty acid-binding protein, brain (B-FABP)	*Homo sapiens*	14,862	47	5.41
68	FABE_BOVIN	P55052	Fatty acid-binding protein, epidermal	*Bos taurus*	15,350	29	7.57
94	ALFC_RAT	P09117	Fructose-bisphosphate aldolase C (EC 4.1.2.13)	*Rattus norveg.*	39,152	47	6.67
158	ALFC_HUMAN	P09972	Fructose-bisphosphate aldolase C (EC 4.1.2.13)	*Homo sapiens*	39,324	46	6.46
147	GELS_PIG	P20305	Gelsolin precursor, plasma (actin-depolymerizing factor)	*Sus scrofa*	84,775	28	5.93
201	GFAP_HUMAN	P14136	Glial fibrillary acidic protein, astrocyte (GFAP)	*Homo sapiens*	49,880	41	5.42
159		P79403	Glucosidase II	*Sus scrofa*	106,661	15	5.64
248	DHE3_MOUSE	P26443	Glutamate dehydrogenase (EC 1.4.1.3) (GDH)	*Mus musculus*	61,336	51	8.05
117	DHE3_BOVIN	P00366	Glutamate dehydrogenase (EC 1.4.1.3) (GDH)	*Bos taurus*	55,561	24	8.66
172	GLNA_PIG	P46410	Glutamine synthetase (EC 6.3.1.2) (glutamate-ammonia ligase)	*Sus scrofa*	42,030	37	6.28
143	GTP_PIG	P80031	Glutathione S-transferase P (EC 2.5.1.18) (GST P1-1)	*Sus scrofa*	23,497	50	8.07
185	G3P_PIG	P00355	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	*Sus scrofa*	35,705	32	6.9
103	HS27_CANFA	P42929	Heat-shock 27-kDa protein HSP 27	*Canis familiaris*	22,939	30	6.23
145	HS72_BOVIN	Q27965	Heat-shock 70-kDa protein 2 (HSP70-2)	*Bos taurus*	70,228	27	5.68
257	HS7C_MOUSE	P08109	Heat-shock cognate 71-kDa protein HSC73	*Mus musculus*	70,871	43	5.37
224	HS7C_HUMAN	P11142	Heat-shock cognate 71-kDa protein	*Homo sapiens*	70,898	43	5.37
176	HBB_PIG	P02067	Hemoglobin βchain	*Sus scrofa*	16,034	92	6.76
82	CH60_CRIGR	P18687	Hsp60 60 kDa heat-shock protein (Hsp60)	*Cricetulus gris*	60,988	23	5.91
161		Q99MZ8	LASP-1	*Rattus norveg*	29,970	46	6.61
169		Q28708	L-Caldesmon	*Oryctolag. cuni.*	61,482	20	8.44
144	LDHB_PIG	P00336	L-Lactate dehydrogenase B chain (EC 1.1.1.27)	*Sus scrofa*	36,481	42	5.57
111	MVP_HUMAN	Q14764	Major vault protein (MVP) (lung resistance-related protein)	*Homo sapiens*	99,327	24	5.34
131	MDHC_PIG	P11708	Malate dehydrogenase, cytoplasmic (EC 1.1.1.37)	*Sus scrofa*	36,323	31	6.15
108	MK01_BOVIN	P46196	Mitogen-activated protein kinase 1 (EC 2.7.1.-) (ERK-2)	*Bos taurus*	41,375	35	6.5
72	DDH2_HUMAN	O95865	NG,NG-dimethylarginine dimethylaminohydrol.2 (EC 3.5.3.18)	*Homo sapiens*	29,644	23	5.66
240		Q9TSX9	Nonselen. glutat.phospholip.hydroperox.peroxidase (PHGPx)	*Sus scrofa*	25,037	80	5.73
102	PDX2_PIG	P52552	Peroxiredoxin 2 (EC 1.11.1.-) (thioredoxin peroxidase 1)	*Sus scrofa*	14,163	42	4.7
71	PHOS_BOVIN	P19632	Phosducin	*Bos taurus*	28,231	24	4.92
116	PEBP_HUMAN	P30086	Phosphatidylethanolamine-binding protein RKIP	*Homo sapiens*	20,925	49	7.42
123	PGMU_MOUSE	Q9D0F9	Phosphoglucomutase (EC 5.4.2.2) (PGM)	*Mus musculus*	61,386	30	6.32
148	PGK_HORSE	P00559	Phosphoglycerate kinase (EC 2.7.2.3)	*Equus caballus*	44,842	51	8.64
184	PMG1_HUMAN	P18669	Phosphoglycerate mutase 1 (EC 5.4.2.1)(EC 3.1.3.13)PGAM-B	*Homo sapiens*	28,672	80	6.75
224	PDA3_HUMAN	P30101	Protein disulfide isomerase A3 (EC 5.3.4.1) ERp60	*Homo sapiens*	56,782	35	6.1
144	PYC_RAT	P52873	Pyruvate carboxylase, mitochondrial (EC 6.4.1.1)	*Rattus norveg*	129,689	29	6.25
122	GDIB_MOUSE	P50397	Rab GDP dissociation inhibitor β (Rab GDI β) (GDI-2)	*Mus musculus*	50,512	35	6.02
93	RECO_BOVIN	P21457	Recoverin (P26)	*Bos taurus*	23,202	30	5.32
80	RCN1_MOUSE	Q05186	Reticulocalbin 1	*Mus musculus*	38,090	30	4.7
194	RET3_HUMAN	P29762	Retinoic acid-binding protein I, cellular (CRABP-I)	*Homo sapiens*	15,434	77	5.3
131	GDIR_BOVIN	P19803	Rho GDP-dissociation inhibitor 1 (Rho GDI 1)	*Bos taurus*	23,421	58	5.12
135		CAD19504	S-adenosylhomocysteine hydrolase (EC 3.3.1.1)	*Sus scrofa*	48,176	32	6.08
119	ARRS_PIG	P79260	S-arrestin (retinol S-antigen) (48-kDa protein) (S-AG)	*Sus scrofa*	45,102	37	5.58

TABLE 2. (continued).

Top MOWSE score	Swissprot entry name	SwissProt/ Trembl accession no.	Protein name	Organism	M_r (dalton)	Sequ. cov. (%)	pI
163	GDIR_BOVIN	P38647	Stress-70 protein, mitochondrial GRP 75 (mortalin)	*Mus musculus*	73,528	29	5.81
123	SODC_PIG	p04178	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	*Sus scrofa*	15,922	61	6.04
139		S31765	T-plastin (fragment)	*Rattus norveg*	70,773	31	5.42
192	PLST_HUMAN	P13797	T-plastin	*Homo sapiens*	70,435	29	5.52
117		AAB02844	T-plastin	*Homo sapiens*	64,281	22	5.73
214	TERA_PIG	P03974	Transitional endoplasmic reticulum ATPase (TER ATPase)	*Sus scrofa*	89,288	34	5.13
93	TKT_RAT	P501137	Transketolase (EC 2.2.1.1)	*Rattus norveg*	67,643	20	7.23
99	TCTP_HUMAN	P43348	Translationally controlled tumor protein p23	*Homo sapiens*	19,697	56	4.84
147	TPIS_MOUSE	P17751	Triosephosphate isomerase (EC 5.3.1.1) (TIM)	*Mus musculus*	26,581	65	7.08
122	UBL1_HORSE	Q9GM50	Ubiquitin C-terminal hydrolase isoz.L1 EC 3.4.19.12	*Equus caballus*	25,245	48	5.14
141	VIME_CRIGR	P48670	Vimentin (fragment)	*Cricetulus gris*	51,848	31	4.94
149		Q8WMK3	Vinculin	*Sus scrofa*	116,876	22	5.89
152	WDR1_HUMAN	O75083	WD-repeat protein 1 (actin interacting protein 1) (NORI-1)	*Homo sapiens*	66,193	28	6.11

RMG, retinal Müller glia
[a]Identification by sequencing the peptide VDVLDSIK in a micromass Q-TOF mass spectrometer.

identify the main transporter for glutamate uptake in RMG (GLAST), most likely because membrane proteins are underrepresented on 2-DE gels. Since GS and GLAST have been shown to be functionally linked and their regulation synchronized (Rauen and Wiessner, 2000), the transporter may thus be downregulated as well. Heidinger et al. (1999) were able to show that cocultivation of RMG and retinal neurons resulted in a three-fold higher GS activation upon glutamate treatment than did cultivation of RMG alone. Therefore, the cocultivation of RMG with retinal neurons appear necessary and essential to yield high GS expression levels in vitro, which would suggest that together with other factors, the intimate interconnection between glia and neuron ensures functional differentiation. Limb et al. (2002) recently described a spontaneously immortalized human RMG cell line (MIO-M1) that stably retains high GS and CRALBP levels in vitro. This cell line may represent a more appropriate model to in vivo RMG than 2-week-old primary RMG itself, given that the expression of these markers for RMG differentiation would reflect a more general maintenance of the differentiated phenotype. We are currently investigating the protein expression pattern of this cell line by 2-DE gels and will conduct a detailed comparison to freshly prepared primary RMG.

3. *Proteins related to phototransduction disappear*: This in vitro downregulation of both GS and CRALBP has been observed (P.J. Linser and Moscona, 1981; Guidry, 1996; Germer et al., 1997; Winkler et al., 2000). In the present study, the loss of CRALBP, involved in the visual pigment cycle, might reflect the interrupted phototransduction cycle and might be accompanied by a downregulation of the corresponding transporters within the cell membrane. It is worthwhile to mention that downregulation of GS, CRALBP, and CAII is characteristic during gliotic processes in the retina, especially in complement to proliferative retinopathy (PVR) (Lewis et al., 1989, 1994). S-arrestin, phosducin, and recoverin are found here in freshly isolated RMG. Their appearance on 2-DE gels at days 0 and 3 most likely stems from contamination of the enriched RMG preparations with photoreceptors, but it could also be derived from endocytosized photoreceptor fragments. However, it cannot be ruled out that RMG express very minute levels of these proteins as well. As already mentioned, a proportion of contaminating cells on days 0 and 3 (24.3% and 18.2%, respectively) exists, which may include photoreceptors. After 8 days, the cultures contain only 3.4% vimentin-negative cells, but the 2-DE gels nonetheless show weaker s-arrestin and phosducin spots and a more prominent recoverin spot; these spots were absent after 2 weeks in culture.
4. *All enzymes responsible for detoxification processes in vivo appear completely downregulated after 2 weeks in culture*: Since glutamate is the rate-limiting substance in glutathione synthesis (Huster et al., 1998), in vitro conditions lacking glutamate may lead to downregulation of glutathione-producing enzymes and thus to low glutathione levels. Lacking its substrate, this may consequently lead to downregulation of glutathione S-transferase expression. Downregulation of the antioxidant enzymes superoxide dismutase, PHGPX and thioredoxin peroxidase underlying the deactivation of reactive oxygen species in vivo may result from the loss of light-induced radical production.
5. *Pattern shifts of proteins related to proliferative signaling appear inconclusive with respect to the overall changes seen after prolonged culture*: However, Raf kinase inhibitor protein (RKIP), a suppressor of Raf kinase activity, and mitogen-activated kinase (MAPK) signaling (Yeung et al., 1999), are strongly downregulated. RKIP is highly expressed in differentiated resting cells and is almost absent in highly proliferating cells and certain tumors (W. Kolch, personal communication). The MAPK pathway is a major route in the transduction of mitogen triggered signals into the nucleus. Disinhibition by RKIP downregulation after 3 days most likely liberates

this pathway in RMG for responding to serum factors. Indeed, this observation correlates with the onset of RMG proliferation in culture at this time point. Suppression of RKIP expression may be therefore a prerequisite for RMG proliferation onset in culture, whereas its expression may partly explain the absence of RMG proliferation in vivo, even when mitogens such as fibroblast growth factors are present.

6. *Expression of proteins related to cytoskeletal reorganization and dynamics increase with prolonged culturing*: Upon adaptation to cell culture, RMG begin to express vinculin, actin-related protein 3, and α-actinin 1 and 4. These are major components of focal adhesion complexes (FA), which are specialized extracellular matrix attachment and signaling organelles, located along the ventral plasma membrane of adherent cultured cells. Here, they are linked to the actin cytoskeleton via a web of membrane-associated anchor proteins (for review, see Zamir and Geiger, 2001). Vinculin, a key anchor protein, is able to cross-link F-actin with talin (R.P. Johnson and Craig, 1994; Huttelmaier et al., 1997)) upon activation by phosphatidylinositol 4,5-bisphosphate (Gilmore and Burridge, 1996), and talin docks onto membrane-spanning integrin receptors during this process (Janmey, 1994; Martel et al., 2001). FA are prerequisites for establishing cell contacts, adhesion to extracellular matrix, morphological changes, and migration. Furthermore, they promote survival signals through activation of protein kinase B (Frisch et al., 1996; Khwaja et al., 1997), and cell proliferation signals through the activation of ras (Assoian and Schwartz, 2001; Schwartz and Assoian, 2001). The upregulation of vinculin in RMG can thus be linked to proliferation and the ability of RMG to migrate. Actin-related protein 3 is one of seven proteins forming the Arp2/3 complex, which nucleates actin polymerization and thus drives membrane protrusion. Recently the presence of a transient interaction between the Arp2/3 complex with vinculin has been shown, which is stimulated by matrix adhesion and by growth factor treatment (DeMali et al., 2002). The reported upregulation of actin-related protein 3 in RMG in vitro thus correlates with the already known increase of the ability of RMG to generate tractional forces during dedifferentiation in culture (Guidry, 1997). This finding is in agreement with the premise that dedifferentiated RMG, together with RPE cells (Campochiaro, 1997; Francke et al., 2001), may participate in proliferative vitreal retinopathy (PVR), the most common complication following surgical treatment of rhegmatogeneous retinal detachment. In a parallel study from our laboratory, the effects of culture on proteomic changes in primary RPE cells were investigated. Here we found an upregulation of proteins related to cytoskeletal organization, cell shape, motility, and mediation of proliferative signal transduction (Alge et al., in press). In comparison to RMG cells in the present study, RPE cells do, however, appear to retain much more of their initial expression profile.

In summary, RMG lose their differentiated protein profile when cultured. In contrast, as some RMG markers such as vimentin do not change their expression level in vitro (Guidry, 1996), cultured primary RMG are generally considered appropriate for the study of RMG physiology in vitro. However, our results show, that this must be restricted to very early stage RMG cultures. The profound proteomic changes occurring after 3 days within the first 2 weeks in vitro indicate that RMG adaptation to culture conditions results in a transformation from a highly differentiated RMG phenotype to a much less differentiated fibroblast-like cell type. Moreover, additional studies are under way to determine how the differentiated RMG phenotype may be maintained by designing culturing conditions that more closely mimic an in vivo environment.

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PAPER 2

GDNF family ligands trigger paracrine neuroprotective signalling in retinal glial cells

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Abstract

Apoptotic cell death of photoreceptors is the final event leading to blindness in the heterogeneous group of inherited retinal degenerations. GDNF was found to rescue photoreceptor function and survival very effectively in an animal model of retinal degeneration (15). However, the cellular mechanism of GDNF action remained unresolved. We show here that in porcine retina, GDNF receptors GFRα-1 and RET are expressed on retinal Mueller glial cells (RMG) but not on photoreceptors. Additionally, RMG express the receptors for the GFL-family members Artemin and Neurturin (GFRα-2 and GFRα-3). We further investigated GDNF-, Artemin- and Neurturin-induced signalling in isolated primary RMG and demonstrate three intracellular cascades, which are activated *in vitro*: MEK/ERK, SAPK and PKB/AKT pathways with different kinetics in dependence of stimulating GFL. We correlate the findings to intact porcine retina, where GDNF induces phosphorylation of ERK in perinuclear region of RMG located in the inner nuclear layer. GDNF signalling resulted in transcriptional upregulation of bFGF, which in turn was found to support photoreceptor survival in an *in vitro* assay. We provide here a detailed model for GDNF-induced signalling in mammalian retina and propose that the GDNF-induced rescue effect on mutated photoreceptors is an indirect effect mediated by retinal Mueller glial cells.

Introduction

A major cause of blindness in the western world is the degeneration of photoreceptors (PR) as a result of point mutations in genes coding for either phototransduction-related proteins or other proteins important for retinal function (reviewed in 22). Irrespective of the diversity of mutated genes and proteins involved in this heterogeneous group of progressive retinal dystrophies with homologous phenotypes, the final event leading to blindness is apoptosis of PR. This has prompted investigation on the effects of neuroprotective agents on PR survival in animal models of retinitis pigmentosa. One of the major effective molecules discovered to rescue retinal PR was glial cell line-derived neurotrophic factor (GDNF), initially purified from a rat glioma cell-line supernatant as a trophic factor for embryonic midbrain dopamine neurons (31) and later found to have pronounced effects on other neuronal subpopulations (reviewed in (1). GDNF is a distant member of the transforming growth factor (TGF)-ß superfamily and founder protein of the GDNF family ligand (GFL), which includes neurturin (NRTN), artemin (ARTN) and persephin (PSPN). GFLs bind specific GFRα1–4 co-receptors that are either linked to the plasma membrane by a glycosyl posphatidylinositol (GPI) anchor or, by cleavage through an unknown protease, provided as a soluble co-receptor. All four different GFLs (GDNF, NRTN, ARTN, PSPN) signal via the activation of the RET receptor tyrosine kinase, a single-pass transmembrane protein containing four cadherin-like repeats in the extracellular domain and a typical intracellular tyrosine kinase domain (reviewed in (1). GDNF is widely distributed in the central and peripheral nervous systems, and is also expressed in the inner ear, olfactory epithelium, carotid body, kidney and gastrointestinal tract (35). In the eye, GDNF is primarily expressed in the retina and several investigators have shown its potential therapeutic value by providing neuroprotection in the context of retinal degeneration. GDNF has been shown to rescue retinal ganglion cells after axotomy (25, 46) and proven to be very effective in retarding retinal PR degeneration in the rd1-mouse (15).

Subretinal application of GDNF has led to decreased loss of PR, as well as a significant functional rescue, shown by recordable ERG on PN22 compared to untreated animals.

Recent evidence has proposed that neurotrophic rescue of PR may be indirect, mediated by interaction of neurotrophic factors with retinal Mueller glial cells (RMG), which in turn release or present secondary factors acting directly on PR (6, 39, 44, 45). Upon subretinal injection of GDNF in the rd1-mouse, RMG react by upregulation of glial fibrillary acidic protein, indicating that GDNF-induced regulation in RMG gene expression and subsequent effects on PR may be mediated through an indirect pathway.

To elucidate the role of RMG in GDNF-mediated protection of PR, we analyzed expression and activity of transducer elements involved in GDNF signalling in porcine retina and further analyzed candidate target genes of GDNF signalling.

Materials and Methods

Adult porcine eyes were provided by a local slaughterhouse. They were removed from the animals within 5 minutes after death and kept on ice in CO_2-independent medium (GIBCO) until further use. Within one hour after the death of the animals, the eyes were either fixed for immunohistochemistry or retinae were dissected for preparation of RMG or PR.

Immunohistochemistry

Porcine eyes were fixed in 10% formalin. For adequate and fast fixation both cornea and lens were removed and the aqueous humor was replaced by fixative. After dehydration, the eyes were embedded in paraffin.

Tissue sections (5 µm) were mounted on coated slides (Super Frost Plus), deparaffinized and rehydrated. High temperature antigen retrieval was performed using 1mM EDTA buffer pH 8.0 as described (34). Slides were rinsed in phosphate buffered saline (PBS) with 0.1% Tween20 (PBS-T, pH 7.3) and nonspecific binding was blocked with 3% BSA in PBS-T. Sections were then incubated with primary antibodies (anti-RET, anti-GFRα-1, anti-GFRα-2, anti-GFRα-3, R&D, 1:200 in 3% BSA-PBS-T) overnight at 4°C in a humid chamber, followed by incubation with alexa546 donkey anti-goat IgG conjugate (Molecular Probes, 1:200) and DAPI (Sigma, 4,6-diamidino-2-phenylindole, 200nM) for two hours at room temperature. After washing, sections were incubated for another 8 hours at 4°C with primary antibodies anti-glutamine-synthetase (Transduction Laboratories, 1:500) or anti-GLAST (Alpha Diagnostic, 1:200), followed by incubation for two hours with alexa488 anti-mouse or anti-rabbit IgG, respectively and, after final washing, mounted in FluorSave (Calbiochem). Images were obtained with a Zeiss APO-TOME.

Immunohistochemistry on GDNF-stimulated retina sections

Retinae were dissected from porcine eyes as described (14), cut into equal-sized pieces and placed into 12-well dishes containing DMEM/F12 medium (Gibco) at one piece per well. After an initial incubation phase (10 minutes), stimulation was performed with 100ng/ml GDNF (PeproTech) in DMEM/F12 or medium alone. After the indicated time points, the

medium was replaced by ice-cold paraformaldehyde (4% in PBS, 5% sucrose) and the tissue was fixed for one hour at 4°C. Cryopreservation was performed by incubation for one hour in each 5% and 10% sucrose in PBS, followed by incubation overnight in 20% sucrose in PBS, all at 4°C. The tissue samples were frozen in TissueTek on liquid nitrogen and stored at -80°C. Tissue pieces were sectioned at 10µm a cryostat, collected on gelatin-coated super-frost slides and stored at -20°C.

For immunohistochemical analysis sections were rinsed in PBS, incubated for 10 minutes in 0,1% Triton-100 in PBS, followed by blocking for 30 minutes in 3% BSA in PBS-T. Incubation with primary antibodies (anti-pERK 1:250, Cell Signaling Technology or anti-GS 1:500, BD Transduction Laboratories, diluted in 3%BSA-PBS-T) was done overnight at 4°C and then followed by incubation with alexa488/568-coupled secondary antibodies (1:200, Molecular Probes) for 2 hours at room temperature. After final washing, sections were mounted in FluorSave (Calbiochem) and images were obtained with a Zeiss AxioskopII. pERK-positive cells within the INL were counted on 2 visual fields (100x magnification) per section and 10 different sections per experiment; experiments were repeated twice.

Statistic

Statistical significance was calculated using the Peritz “f” parametric test (18).

Isolation and in vitro culturing of primary RMG

RMG were isolated from porcine eyes employing a panning method as described previously (19) and cultured in DMEM/HEPES (GIBCO) with 10% FCS to confluence (2-3 weeks). Purity of these cultures is above 97% (19).

Stimulation of RMG and preparation of cell lysates

One day prior to stimulation, RMG were washed, transferred into serum-free medium (DMEM/HEPES, Gibco) and stimulated with either GDNF (100ng/ml, PeproTech), NRTN (100ng/ml, PeproTech) or ARTN (100ng/ml, PeproTech) for the indicated time points. Inhibitors were applied 20 minutes at both prior to and during stimulation at the following

concentrations: U0126 (Promega) 10µM, LY294002 (Promega) 50µM. For phospho-tyrosine detection, stimulation was performed in the presence of phosphatase inhibitor cocktail 2 (Sigma). Stimulation was stopped by addition of liquid nitrogen and lysates were then prepared by addition of 300 µl lysis buffer (50mM Tris pH 7.4, 250mM NaCl, 25mM EDTA, 1% NP-40, 10% glycerol, phosphatase inhibitor cocktail 2 and Complete protease inhibitor cocktail, Roche) per 10cm dish. Protein content of the lysates was determined by Bradford assay.

Photoreceptor preparation and survival assay

Porcine PR were prepared as described elsewhere (14) with the slight modifications: enzymatic digestion of retinal pieces was stopped by addition of DMEM/F12 medium (Gibco) supplemented with 2% FCS. PR were collected from supernatant and plated at 1,3 x 10^5 cells per well onto pre-coated Falcon 96well plates that were first coated with p-lysine (Sigma, 2µg/cm^2, 3 hours) followed by laminin (BD Bioscience, 1µg/cm^2, overnight). Different concentrations of bFGF (20ng/ml, 100ng/ml, 500ng/ml or 1000ng/ml; purified bFGF from bovine brain, R&D) or respective GFLs (GDNF, NRTN or ARTN, all 100ng/ml, PeproTech) in DMEM/F12 medium or medium alone were applied on attached PR 20 hours after preparation. PR survival was monitored by performing an esterase calcein-fluorophore assay (Molecular Probes), whereby living cells fluoresce bright green. Fluorescence was measured daily on a different row of the plate using a fluorescence reader (BioTek, Synergy HT). All values were compared to initial fluorescence at the beginning of the survival assays. Relative fluorescence was calculated as percent of initial fluorescence, whereby every value is a mean of two to four wells per day. Every experiment was performed at least in triplicate.

Western blots

Protein lysates (10µg) of RMG were resolved on 10% SDS-PAGE and blotted semidry onto PVDF membranes. Unspecific binding was blocked for one hour with 5% BSA in TBS-T.

Blots were incubated with primary antibodies in 5% BSA-TBS-T overnight at 4°C (anti-pTyr100 1:1000, anti-pMEK1/2 1:1000, anti-pERK 1:5000, anti-pSAPK 1:2000, or anti-pAKT (Ser473) 1:1000; Cell Signaling Technology), washed and incubated in HRP-coupled secondary antibodies (Jackson Laboratories, 1:15000). Signal was developed with the ECL+ kit (Amersham) according to the manufacturer's instructions and detected on Hyperfilm ECL (Amersham). Blots were stripped and reprobed with anti-actin (Oncogene, 1:1000) or anti-vimentin (clone V9, Sigma, 1:1000) antibodies to verify equivalent protein loading.

RT-PCR

Total RNA was prepared from retina, RMG (either directly after panning procedure or from confluent cultures) or PR with RNeasy kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with RT Kit (Qiagen) and 70ng cDNA were set for PCR (annealing at 65°C, 35 cycles) with the following primers: RET for: CTGGACTCCATGGAG AACC; RET rev: TCCAGGTCTTTGCTGATGTC; GFRα-1 for: TAAAGGAAAACTACGCTGACTGCC; GFRα-1 rev: TGCCCGACACATTGGATTTC; GFRα-2 for: TTT GTC GTG AGC TCT GTG AAG C; GFRα-2 rev: GCA TGA TTG GGT CCG AGA TAA C; GFRα-3 for: AGG AGC CTT CAG TCC CCA AG; GFRα-3 rev: TGA GGC AGC GAT CCC AAT C; GDNF for: CAG TGA CTC AAA TAT GCC AGA GGA; GDNF rev: AGA TAC ATC CAC ACC TTT TAG CGG; vimentin for: GTT TCC AAG CCT GAC CTC AC; vimentin rev: AAT CTC ATC CTG CAG GCG; rhodopsin for: GGCTTCCCCATCAACTTCCTC; rhodopsin rev: GTGGCTGACTCCTGCTGCTG. Products were of expected sizes and sequenced for identity verification.

Nothern blot analysis

One day prior to stimulation, RMG were washed, transferred into serum-free medium (DMEM/HEPES) and stimulated for 24 hours with GDNF (100ng/ml, PeproTech). Northern blotting was performed as described elsewhere (37). Briefly, total RNA was prepared with RNeasy-Kit (Qiagen), examination of integrity and quantification was performed with RNA-

nano chip on a 2100 Bioanalyzer (Agilent). Equal amounts (5µg) were loaded onto formaldehyde gels, resolved and blotted. Detection of bFGF transcript was performed by generation of a bFGF-specific DIG-labeled RNA with DIG labeling Kit (Roche) using a RT-PCR product generated from RMG with the following primers: bFGF for: CAAGCGGCTGTACTGCAAAAAC; bFGF T7 promoter rev: TAATACGACTCACTATAGGATGTGGCCATTAAAATCAGCTCTT. The detected transcript was approximately 6 kbp and thus similar in size to that of the full length human transcript (6.774 kbp, ENST00000264498, http://www.ensembl.org/).

bFGF-ELISA

RMG were seeded onto 96-well culture plates at 2 x 10^4 cells per well, incubated for 24 hours in DMEM/ 25 mM HEPES in the presence of 10% FCS and then, prior to stimulation, incubated for an additional 24 hours without FCS. RMG were then left either unstimulated or stimulated with GDNF, NRTN or ARTN (all 100ng/ml, PeproTech) in the absence or presence of U0126 (10µM, Promega) for 16 hours. Medium from the wells was collected after stimulation and the amount of secreted bFGF was analyzed by a bFGF-ELISA (DuoSet, R&D) test according to the manufacturer's instructions; the experiment was performed in triplicate.

Results

GDNF receptor components are expressed on RMG in porcine retina

In order to clarify whether GDNF-mediated PR rescue effects are exerted directly or involve indirect retinal pathways, we first analyzed the expression patterns of GDNF receptor components in porcine retina.

Expression of transmembrane receptor RET was found in all retinal layers (Figure 1, A and B). Similar expression patterns were found for GFRα-1 (C and D), GFRα-2 (E and F) and GFRα-3 (G and H), thus indicating the possibility for a receptor expression on membranes of RMG, since these cells are present throughout the retina. Co-staining with the cellular RMG marker glutamine synthetase (Figure 1, A, C, E and G) supported this hypothesis. In order to localize precisely the site of expression, sections were additionally co-stained with antibodies against GLAST, a membrane glutamate receptor expressed in RMG (36, 38). Co-staining with GLAST produced exact colocalisation in overlay images, indicating that receptor components for GDNF, NRTN and ARTN are indeed confined to the RMG membrane (Figure 1, B, D, F and H). The fluorescence signal observed for PR inner and outer segments is due to autofluorescence and was revealed by comparison with negative controls (omission of primary and/or secondary antibodies, data not shown).

Expression of GDNF receptor components on isolated RMG in vitro

Since RMG in the whole retina were found to express GDNF receptor components, we further investigated the expression of these receptor components in isolated RMG. We have previously shown that RMG protein expression patterns are markedly altered upon *in vitro* culturing (19). Therefore, we addressed the question of whether the GDNF receptor components remain expressed *in vitro*.

RT-PCR analysis of mRNA prepared from porcine retina, freshly isolated RMG and RMG cultured *in vitro* for two weeks, showed that whole retina and freshly isolated RMG express RET, GFRα-1, GFRα-2 and GFRα-3 and RMG retained expression of these components

after two weeks in culture (Figure 2, A, B and C). Although GDNF itself was not expressed in freshly isolated RMG, its expression increased after two weeks *in vitro*, indicating the establishment of an autocrine activation loop. No expression of GDNF receptor components was found in freshly isolated PR (Figure 2D).

GDNF phosphorylates RMG RET receptor in vitro

In order to investigate GDNF signalling in RMG *in vitro*, we first tested GDNF-induced tyrosine phosphorylation. Western blots incubated with an antibody specific for phospho-tyrosine showed specific bands of the expected size for the RET receptor (170 and 150 kDa), appearing just 15 seconds following GDNF stimulation (Figure 3) and remained stable for 30 minutes in the presence of phosphatase inhibitors. In the absence of phosphatase inhibitors, no such phosphorylation could be detected after 30 minutes. Application of phosphatase inhibitors in the absence of GDNF did not result in higher phosphorylation. Similar results were obtained after stimulation of RMG with ARTN (data not shown).

GDNF, Neurturin and Artemin activate distinct RMG signalling cascades in vitro

Consistent with the expression of co-receptors GFRα-1, GFRα-2 and GFRα-3 in RMG, we found that all three ligands (GDNF, NRTN, ARTN) could activate signalling cascades in RMG *in vitro*. Five minutes of GDNF stimulation led to maximum phosphorylation of both MEK and ERK (Figure 4A). Similar signalling cascades were also activated following stimulation with NRTN and ARTN (Figure 4, B and C). ERK phosphorylation was completely blocked by application of the MEK inhibitor U0126. Whereas GDNF led to strong phosphorylation of SAPK after 5 minutes, stimulation with ARTN resulted in weaker phosphorylation; NRTN induced SAPK phosphorylation after 10 minutes. In all three cases, SAPK activation could be partially blocked by the PI-3 kinase inhibitor LY294002, indicating that SAPK phosphorylation was partly induced by active PI-3 kinase. SAPK phosphorylation was completely blocked by the MEK inhibitor U0126. PKB/AKT was phosphorylated by stimulation of NRTN, ARTN and GDNF at 1, 5 and 10 minutes, respectively. This

phosphorylation was completely blocked by addition of the PI-3 kinase inhibitor LY294002. PKB/AKT phosphorylation was also blocked by the MEK inhibitor U0126.

GDNF activates ERK in cells of the inner nuclear layer in retinal explants

In order to confirm that GDNF is able to induce signalling in intact retina, we dissected fresh porcine retina and immediately treated with GDNF or medium alone for 20 minutes. Tissue samples were then fixed, cryopreserved and sectioned. Immunohistochemical staining for pERK showed intensely positive areas close to nuclei of cells in the inner nuclear layer and additionally in IPL and GCL of GDNF-treated sections compared to untreated sections (Figure 5, A and B). Coimmunolabelling for glia-specific marker glutamine synthetase (GS) reveals a colocalisation of all pERK-positive signals with glial cells (Figure 5C). Quantification of pERK-positive signals within the INL revealed a significant ($p<0.001$) increase in response to GDNF stimulation (Figure 5C).

GDNF upregulates expression of bFGF in vitro

An upregulation of bFGF by RMG in response to NT-3 treatment has been demonstrated (17) and bFGF has been shown to protect PR *in vivo* (12). We therefore investigated whether GDNF treatment could increase bFGF expression in RMG. Indeed, northern blot analysis demonstrated that stimulation with GDNF for 24 hours increased bFGF transcript level in RMG (Figure 6A).

GDNF, NRTN and ARTN stimulate secretion of bFGF

In order to verify that GDNF-induced upregulation of bFGF expression also led to increased secretion of bFGF by RMG, we quantified bFGF levels in the culture medium following incubation with GDNF, NRTN and ARTN using a bFGF-ELISA. All three factors induced secretion of bFGF by RMG compared to unstimulated controls. Unstimulated RMG medium contained 33.8 pg/ml ($\pm$ 3.8) bFGF, whereas GDNF stimulation resulted in an increase of bFGF in the medium to 46.1 pg/ml ($\pm$ 3.4), NRTN to 48.1 pg/ml ($\pm$ 1.7) and ARTN to 47.7 pg/ml ($\pm$ 8.8). Moreover, bFGF secretion was dependent on active MEK as application of the

MEK inhibitor U0126 not only prevented increased secretion (Figure 6B), but also lowered secreted bFGF below the level observed for unstimulated RMG to 25.3 pg/ml (± 4.3).

bFGF prolongs survival of porcine photoreceptors in vitro

Although the results of our studies indicate that GDNF targets RMG rather than photoreceptors (PR) in porcine retina, we wanted to investigate direct protective effects of GFLs on PR. GDNF, NRTN and ARTN were applied to porcine PR *in vitro* and survival was monitored by a calcein-esterase assay. In accordance with our hypothesis, neither of the tested GFLs prolonged survival of PR compared to medium alone (Figure 7A).

In order to verify that bFGF promotes PR survival, different concentrations of bFGF were applied to porcine PR *in vitro*. We found that bFGF significantly increased numbers of surviving PR in a concentration-dependent manner. The survival-supporting effect of bFGF was already detectable after 2 days *in vitro* (data not shown) and was most pronounced after 5 days *in vitro* (Figure 7B). Concentrations of 20ng/ml did not increase survival compared to the negative control, but bFGF at 100ng/ml, 500ng/ml and 1000ng/ml robustly enhanced survival of PR.

Discussion

The large heterogeneity of retinal diseases leading to PR death and consequent loss of vision in patients requires development of alternative therapeutical strategies beyond corrective gene therapy. A promising approach has been to target dying PR with molecules known to act as neuroprotective in brain and, in such manner, delay disease progression. Although this has been achieved with several molecules in animal models of retinal degeneration (reviewed in (8), the underlying mechanisms of PR rescue in most of these studies remain unresolved.

Retinal Mueller glial cells as target cell for GDNF-induced signalling in porcine retina

One of the most effective molecules in rescuing PR function thus far has been GDNF, which rescued PR at both morphological and functional levels in the rd1 mouse model of retinal degeneration (15). The molecular mechanisms of GDNF-induced PR rescue have not been resolved to date, but are a fundamental prerequisite for therapeutic application of this molecule. In our study, we examined the expression of GDNF receptor components in the retina. The porcine retina was selected as model due to its high density of cone cells (21), as well as the close similarity between the porcine and the human eye with respect to dimension, anatomical and physiological features (33, 41).

We found that the transmembrane receptor RET, as well as GDNF co-receptor GFRα-1 were not expressed by PR but rather by RMG in porcine retina. In addition to GFRα-1, RMG express the co-receptors necessary for NRTN and ARTN signalling: GFRα-2 and GFRα-3. Studies on the expression of GDNF receptor components in rat retina localized RET and GFRα-1 in PR, inner nuclear layer, inner plexiform layer and ganglion cell layer (24) suggesting, among other cells, a localisation on RMG, whereas Harada and coworkers (16) have reported expression of GFRα-1 and GFRα-2 receptors mainly in PR. Other evidence has reported GFRα-1 expression in retinal ganglion cells, RMG and PR (26). These discrepancies have been proposed to reflect specific differences between the rat strains used for the studies

(16). Additionally, there are indications that retinas of rodents and other mammals differ in the expression and distribution of neurotrophic factor receptors. As shown for CNTFRα its expression is absent in PR of adult and developing rodent retinas, but rather consistently found in rods and cones of non-rodent mammals, including pig (4). With regards to GDNF, our findings that GFRα receptors and RET are absent from PR but abundant on RMG lead to the conclusion that, in porcine retina, the neurotrophic effect exerted by GDNF is transduced not directly to PR but indirectly via RMG.

There has been increasing evidence for the crucial role of RMG in prolonging PR survival during progressive retinal degenerations (47). Light-induced retinal degeneration studies in rats have shown that GFRα-2 expression in RMG is upregulated in response to GDNF (16), whereas others have observed an upregulation of GFRα-2 expression in response to light damage in the same model occurring in different retinal structures (PR and retinal pigment epithelium, (24)). Further, intravitreous injection of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or bFGF in eyes of wildtype or mutant rats and mice resulted in increased pERK and c-fos immunostaining in cells of the inner retina, particularly RMG but not PR (43, 44). In accordance to the hypothesis that BDNF, CNTF, and bFGF exert their effects on PR by acting indirectly through RMG activation, we propose here that a similar indirect survival-promoting pathway is induced following GDNF application.

GDNF, NRTN and ARTN-induced signalling in RMG

In order to examine molecular action of GDNF in detail, we used pure primary cell cultures of porcine RMG (19) as a tool to address GDNF-induced signalling exclusively in a GDNF-targeted retinal cell. We found that GDNF, as well as NRTN and ARTN induced several distinct signalling cascades in primary RMG: MEK/ERK, SAPK and PKB/AKT, which have been previously described to be activated by those ligands (recently reviewed in 23). However, the majority of studies were performed on cell lines, especially those expressing

constitutively active RET mutations derived from multiple endocrine neoplasia (MEN2A and MEN2B). Most interestingly, our study revealed considerable crosstalk between the activated signalling cascades. Our results with the specific MEK inhibitor (U0126; 11) demonstrated that GDNF-induced MEK activation triggers not only ERK, but also regulates SAPK phosphorylation and PKB/AKT to a lesser extent (see Figure 8). Such communication between the MEK/ERK and SAPK pathways has been also proposed in growth factor-stimulated epithelial layers of the porcine lens. There, MEK inhibition with U0126 led to decreased SAPK phosphorylation, suggesting close association of MEK/ERK with the SAPK cascade (48). Further, in our study, the specific PI-3 kinase inhibitor (LY294002; 42) blocked PKB/AKT phosphorylation completely and, in addition, reduced SAPK phosphorylation. SAPK is thus activated by at least two kinases in primary RMG: MEK and PI-3 kinase, whereby a complete block of SAPK phosphorylation occurs only through MEK inhibition; PI-3 kinase inhibition is only able to reduce SAPK phosphorylation. This indicates that MEK acts on an unidentified target kinase which itself activates PI-3 kinase. Although GDNF, NRTN and ARTN activated similar signalling cascades in this study and which is consistent with previous reports (1, 23), the time course of activation for phosphorylation of PKB/AKT was remarkably different: stimulation with NRTN, ARTN and GDNF resulted in phosphorylation of PKB/AKT after 1, 5 and 10 minutes, respectively. Such a differential time lapse in response to different GFLs has, to our knowledge, not been previously reported. The very rapid phosphorylation of PKP/AKT following NRTN stimulation may indicate recruitment of different adapter proteins to the activated RET receptor, in contrast to the considerably later activation of PKB/AKT through GDNF stimulation. Activation of PKB/AKT by phosphorylated tyrosine 1062 of RET via recruitment of docking proteins SHC, GRB2 and Gab1 or Gab2 has been reported (reviewed in (23, 32), whereby the time lapse between start of activation and onset of PKB/AKT phosphorylation was between 5 and 15 minutes (5, 20). This is in agreement with the time lapse following stimulation with GDNF in

our study. However, NRTN stimulation induces more rapid PKB/AKT phosphorylation and may indicate that stimulation with this GFL leads to either recruitment of different docking proteins or involves as of yet undefined kinases upstream of PKB/AKT. Moreover, we consistently observed GFL-associated different SAPK/JNK phosphorylation intensities: whereas GDNF induced strong phosphorylation, NRTN and ARTN resulted in reduced or very weak phosphorylation of SAPK/JNK, respectively. This indicates a differential triggering of signal intensities by different GFLs. Together, these findings underline that although these GFLs signal through the same transmembrane receptor RET (2) and induce phosphorylation at similar tyrosine residues of RET, intracellular signalling dynamics are indeed distinct.

GDNF-induced and bFGF-mediated indirect survival pathway to photoreceptors

Upon stimulation with GDNF, phosphorylated ERK is shuttled into the nucleus of cells. We found both, pERK-positive signals close to nuclei of inner nuclear layer (INL) in intact retina and additional signals in inner plexiform layer and ganglion cell layer. The pERK-positive cells within the INL show regular spacing, are all located within one row of nuclei in the INL and are most likely RMG. We could colocalize all pERK-positive labeling with glutamine synthetase, the marker for glial cells in retina (9, 30), thus confirming that ERK is selectively activated in RMG.

Given that GDNF has been shown to rescue PR in the rd1-mouse and our own results indicate that RMG are the target cells of GDNF in the porcine retina, we specifically screened for GDNF-induced expression changes in secreted neuroprotective molecules. We found that RMG indeed respond to GDNF application with an upregulation of basic fibroblast growth factor (bFGF) expression. This is in agreement with the observations of Harada and coworkers (16), who reported GDNF-induced upregulation of bFGF in rat RMG in vitro. Furthermore, subretinal injection of NT-3 prior to light-induced retinal degeneration in mice (17) resulted in an upregulation of bFGF expression in RMG. This may therefore represent a

general pathway for retinal neuroprotection (44, 47). Moreover, we found increased bFGF secretion stimulated by GDNF, NRTN and ARTN, which may support paracrine action of those factors. The increased bFGF secretion is entirely dependent on GFL-induced MEK activation, since the presence of U0126 completely abolished enhanced secretion. Since the amount of secreted bFGF decreased below control levels in presence of U0126, this indicates that the presence of RMG-derived GDNF alone triggers basic levels of bFGF secretion.

We verified that bFGF is able to prolong survival of porcine PR *in vitro*. The ability of bFGF to enhance survival of postnatal rat PR has been demonstrated (13), and indications for the long-term survival promoting effect of bFGF (after two weeks *in vitro*) on porcine PR following repeated application (40) also exist. However, the observed GDNF-induced secretion of bFGF occurred within 16 hours, and we therefore addressed the question of whether *in vitro* bFGF application would show faster survival-promoting effect in our system. Indeed, we found a pronounced and concentration-dependent effect of bFGF on survival of PR *in vitro,* detectable already two days after application (data not shown) and most pronounced after five days *in vitro*. Based on these results we propose a model for GDNF-induced neuroprotection of porcine PR: GDNF activates distinct intracellular signalling pathways in RMG, namely, MEK/ERK, AKT/PKB and SAPK/JNK. Activated MEK results in enhanced bFGF transcription and bFGF is released from RMG and subsequently acts as survival factor for neighboring PR. The close proximity between RMG and PR also probably leads to high, local concentrations of bFGF, thus enhancing its efficacy (Figure 8).

The functional importance of this model of PR support through RMG is further strengthened by the finding that RMG express three different co-receptor types for GDNF family ligand signalling, thus providing a redundancy also seen with striatal and hippocampal neurons (27). bFGF has been thus far shown to only increase PR numbers *in vitro* (13, 40) and *in vivo* (7, 28, 29), but failed to improve electroretinogram (ERG) recordings *in vivo*, indicating it is insufficient for functional rescue. Thus, we hypothesize that GDNF modulates the expression

of additional molecules in RMG, which in turn enable the preservation of PR function. Identification of these GDNF-induced molecules are a part of our ongoing and future studies, where the porcine retina represents an excellent model, as it is cone-rich and in which RMG have been recently demonstrated to secrete a factor specifically promoting cone survival *in vitro* (3). Although specific information on this factor is presently unavailable, it remains an intriguing candidate for being targeted by GDNF-induced signalling in RMG.

GDNF has been repeatedly suggested in the context of future therapeutic regimens, presented either alone or combined with a cell-based therapy/delivery. Previous studies in gene and cell therapy have revealed that application of such neurotrophic cytokines prior to an in-depth understanding of their mechanism of action bear unpredictable risks for the patients treated (10). Such, this study contributes to a better understanding of GDNF's mechanism of action on primary retinal cells and tissue, and may contribute to a more rationalized risk assessment on this promising factor for future clinical trials.

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Figure legends

Figure 1: Detection of GDNF family receptors on tissue sections of porcine retina

Porcine eyes were fixed and embedded in paraffin, sectioned (5µm) and nuclei were stained with DAPI. Nomarski image and blue fluorescence image were overlaid to visualize retinal structures (left picture in each panel). Sections are stained with primary antibodies against RET (A, B), GFRα-1 (C, D), GFRα-2 (E, F) or GFRα-3 (G, H), followed by visualisation with red fluorophore (alexa546). Additionally, all sections were stained with primary antibodies against RMG-specific proteins: anti-GS (A, C, E, G) or anti-GLAST (B, D, F, H) and visualized with green fluorophore (alexa488). Overlay images (right picture in each panel) demonstrates complete colocalisation of all GDNF family receptor components with membrane-localized RMG-specific marker, GLAST. RPE: retinal pigment epithelium; OS: outer segments of PR; IS: inner segments of PR; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

Figure 2: RT-PCR detection of receptor components

RT-PCR of RMG day 1 and day 14 (A and B, respectively) and whole porcine retina (C) show expression of RET receptor and co-receptors GFRα-1, GFRα-2 and GFRα-3. Additionally, GDNF is expressed in whole retina and its expression is induced in RMG at day 14 *in vitro*. In contrast to RMG and whole retina, isolated PR do not express any of the GDNF family receptors (D). Identity of all PCR products was verified by sequencing.

Figure 3: GDNF-induced receptor phosphorylation

GDNF induces rapid phosphorylation (after 15sec) of 2 bands with the expected size for the RET receptor (150 kDa and 170 kDa), detected with anti-phospho Tyrosine antibody (pTyr100, CST). Equal amounts of protein were loaded as demonstrated by re-incubation with anti-beta actin antibody. The data shown are representative of two independent experiments

Figure 4: GDNF, NRTN and ARTN-induced signalling in RMG

A: GDNF induces phosphorylation of both MEK and ERK after 5 minutes and the latter can be blocked with MEK inhibitor (U0126). Further, GDNF phosphorylates SAPK/JNK after 5 minutes, which can be also blocked completely by MEK inhibitor (U0126) and partially by PI3-kinase inhibitor, LY294002. In addition to activating the MAPK and SAPK pathways, a GDNF-induced activation of AKT/PKB was observed after 10 minutes treatment and which could be also blocked by PI3-kinase inhibitor and partially by MEK inhibitor.

B: Similar signalling cascades as described in A are induced by stimulation of RMG with NRTN but showing a slightly different time course: SAPK/JNK phosphorylation is observed after 10 minutes and AKT/PKB after only 1 minute of stimulation.

C: Similar signalling cascades as described in A are induced by stimulation of RMG with ARTN but showing a slightly different time course: AKT/PKB after 5 minutes of stimulation.

Equal amounts of protein were loaded in each condition as demonstrated by re-incubation with anti-vimentin antibody. The data shown are representative of at least three independent experiments of each stimulation set.

Figure 5: Detection of pERK in retina after stimulation with GDNF

A: GDNF induces ERK phosphorylation only in cell nuclei of inner nuclear layer in intact retina after stimulation for 20 minutes (right panel), as compared to unstimulated controls (left panel). OS: outer segments of PR; IS: inner segments of PR; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

B: Quantification of pERK positive cells shows significant increase after GDNF stimulation ($p<0.001$).

C: Colocalisation of pERK with glutamine synthetase (GS): all pERK-positive signals after GDNF stimulation (green) could be colocalized with the cytosolic glial-specific protein glutamine synthetase (GS, red). Stimulation of ERK (pERK, green) was found in the inner

nuclear layer (INL) in close proximity to nuclei (large arrow in upper panels and similar section enlarged in lower panels, counterstained with DAPI for visualisation of nuclei). Additionally, pERK-positive signals were found in inner plexiform layer (IPL) and ganglion cell layer (GCL); in all cases the signals colocalized with GS (open arrows, upper panels). Retinal structures are visualized with Nomarski optics (upper right panel); OS/IS: outer segments and inner segments of photoreceptors; ONL: outer nuclear layer consisting of photoreceptor nuclei.

Figure 6: GDNF, ARTN and NRTN induce secretion of bFGF

A: GDNF-induced upregulation of bFGF mRNA

RMG were either incubated in medium alone or stimulated with 100ng/ml GDNF for 24 hours, northern blot analysis was performed with 5µg RNA per lane and bFGF transcript was detected with DIG-labeled RNA probe. The experiment was repeated three times. GDNF increases bFGF transcript in RMG. Equal loading was controlled by methylene blue staining (data not shown).

B: GDNF, NRTN and ARTN-induced bFGF secretion

bFGF-ELISA of culture medium from RMG stimulated for 16 hours with GDNF, NRTN or ARTN in absence or presence of the MEK inhibitor U0126, as compared to unstimulated RMG. All three factors induce bFGF secretion and secretion of bFGF is reduced below basic level of bFGF secretion from unstimulated RMG with MEK inhibitor.

Figure 7: bFGF prolongs survival of porcine photoreceptors *in vitro*

Porcine PR were plated in 96-well culture plates, treated as indicated for five days in vitro and cell survival was monitored by an esterase calcein-fluorophore assay. Every value is a mean of two to four wells per experiment, and each experiment was performed in triplicate.

A: Neither GDNF, nor NRTN or ARTN increase survival of photoreceptors compared to medium alone.

B: bFGF increases survival of PR *in vitro* in a concentration-dependent manner: whereas 20ng/ml did not affect survival, treatment with 100, 500 and 1000ng/ml demonstrated increasing and significant effects on PR survival (* $p<0.05$, ** $p<0.01$).

Figure 8: Model of intra and inter-cellular signalling induced by GDNF in RMG

RET transmembrane tyrosine kinase is phosphorylated in response to GDNF, NRTN and ARTN. All ligands are able to induce three intracellular signalling cascades: MEK/ERK, SAPK and PKB/AKT pathways. Inhibitor experiments demonstrate that MEK acts upstream of PI-3 kinase to an unidentified target and upstream of SAPK. Both, PI-3 kinase and MEK are able to activate SAPK. Phosphorylated ERK is shuttled into the RMG nucleus where it induces increased transcription of bFGF mRNA. This leads to increased expression and secretion of bFGF protein, which then supports survival of PR which are in close proximity to RMG in intact retina.

Figure 1

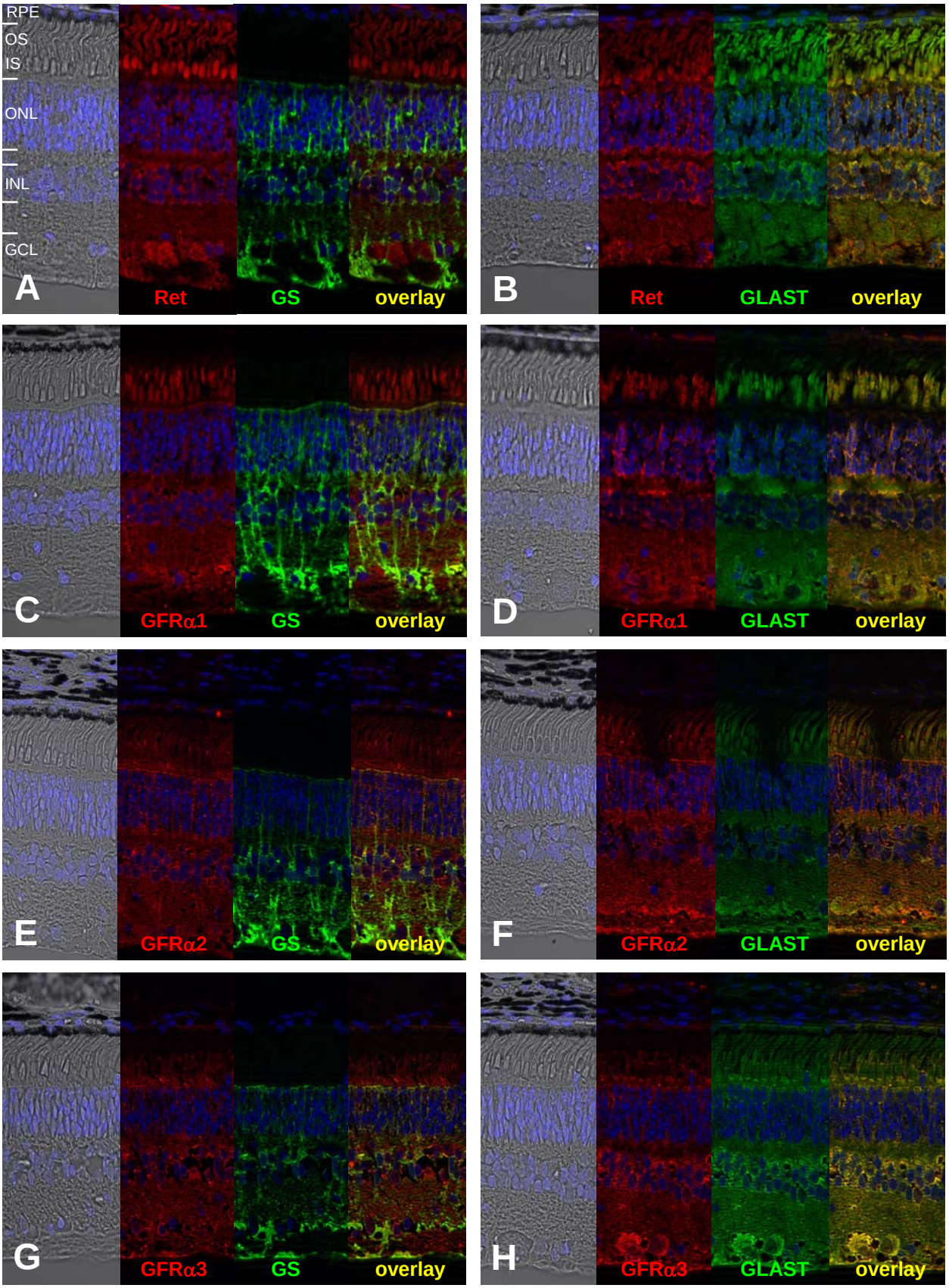
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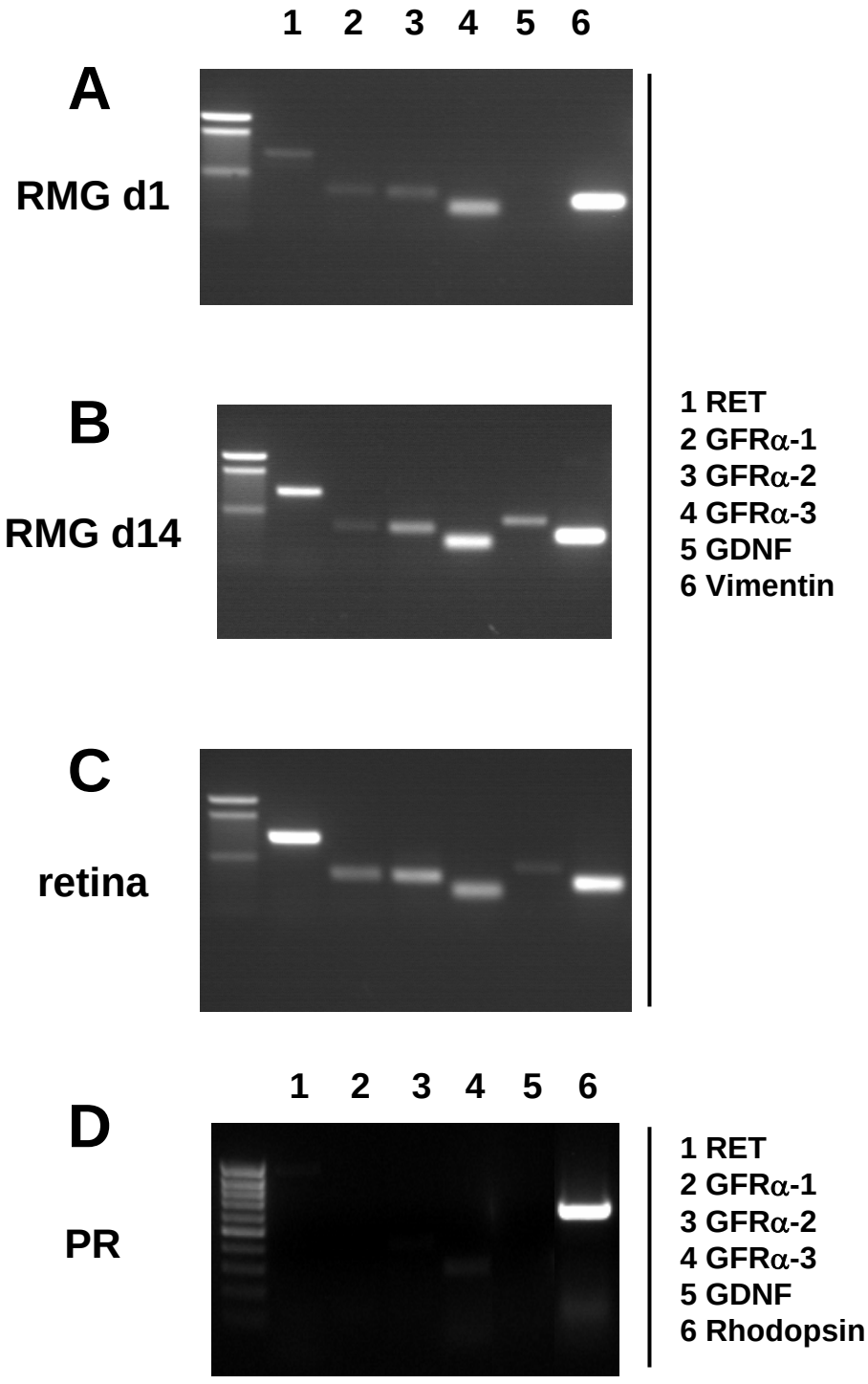
Figure 2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6 Rhodopsin

Figure 3

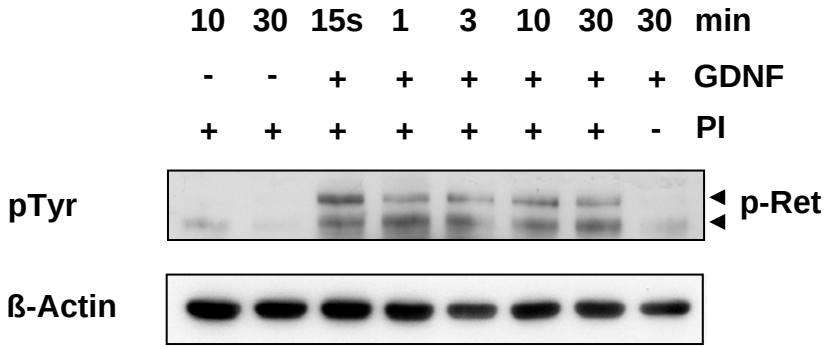

Figure 4

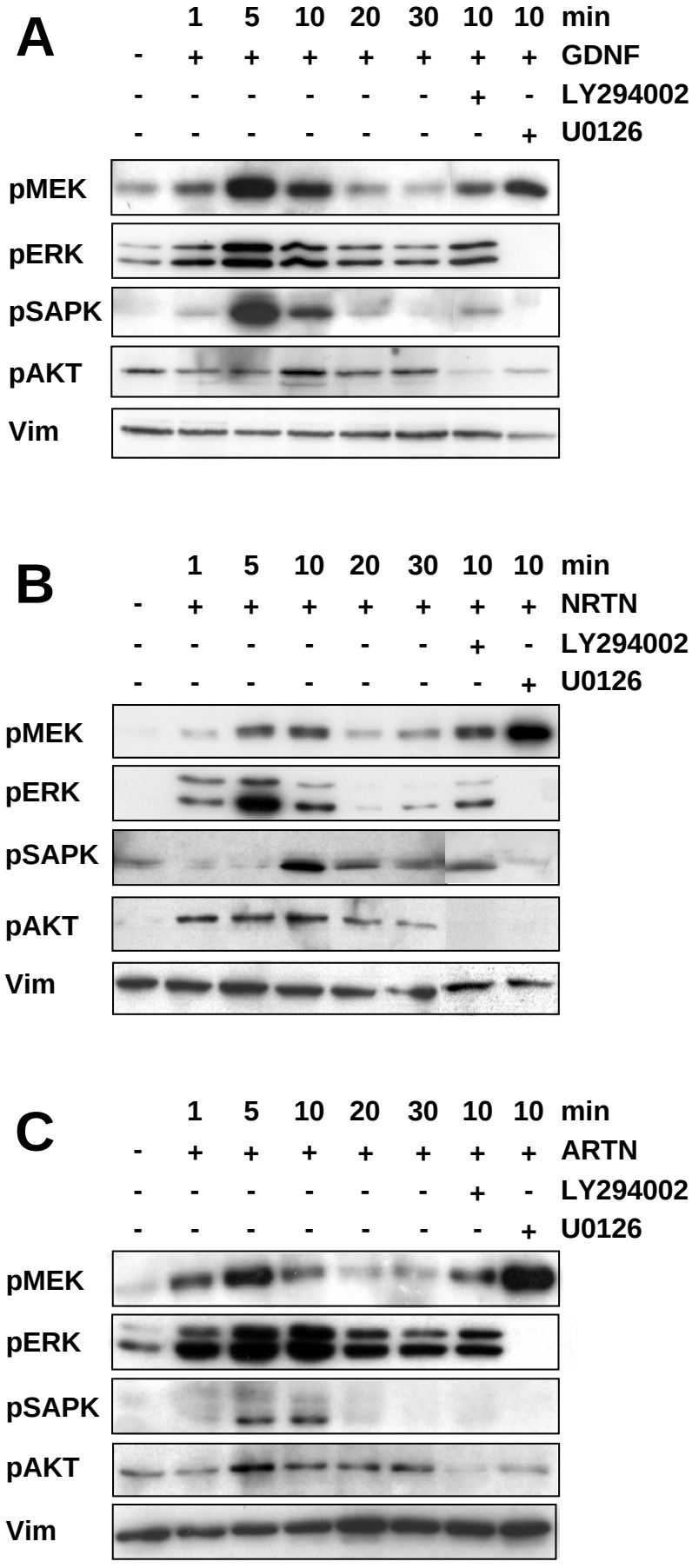
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Figure 5

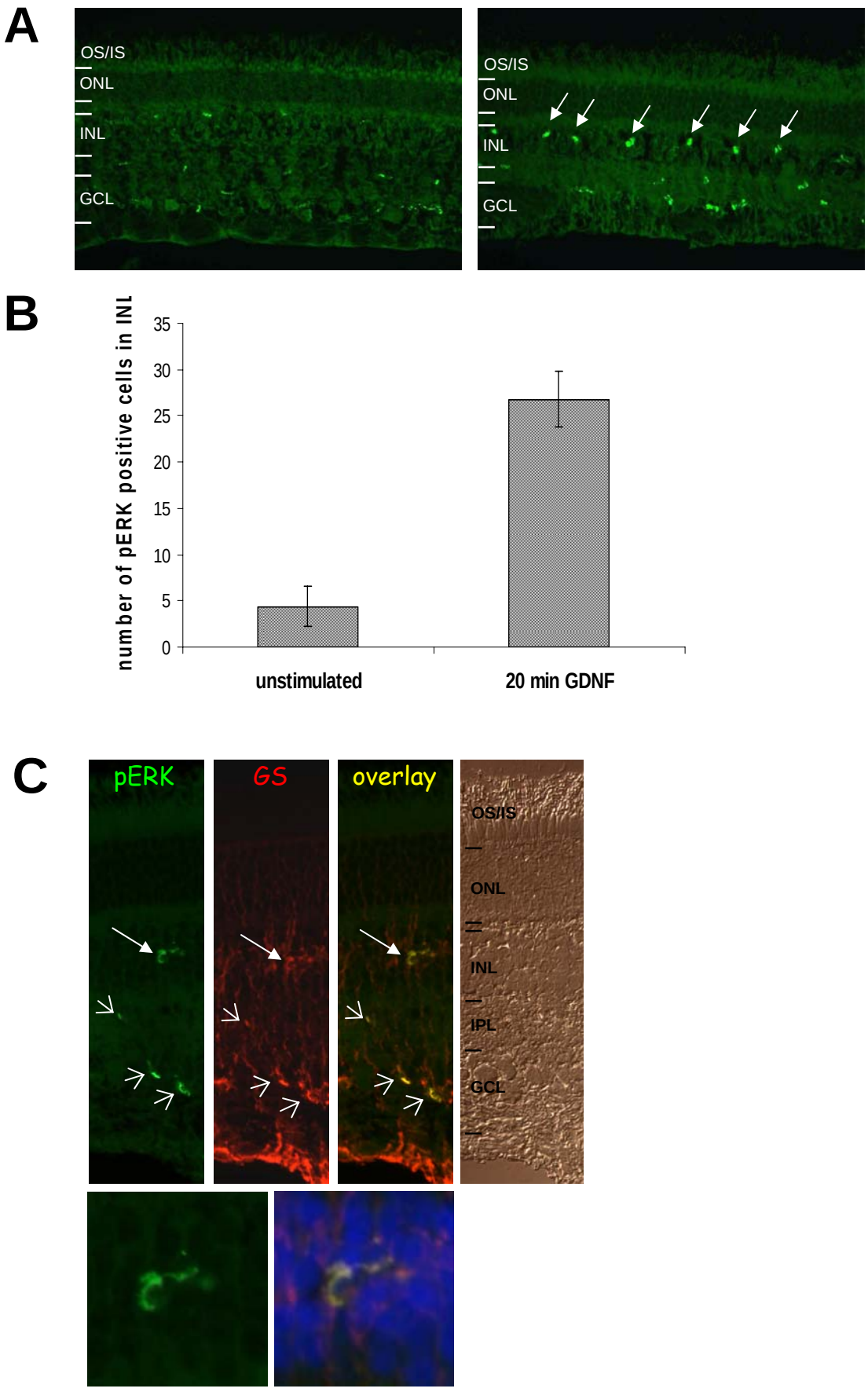
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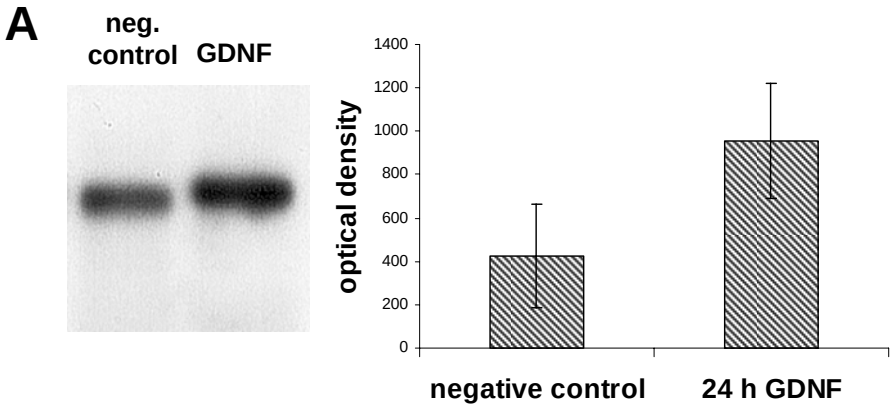
Figure 6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200
0
negative control
24 h GDNF

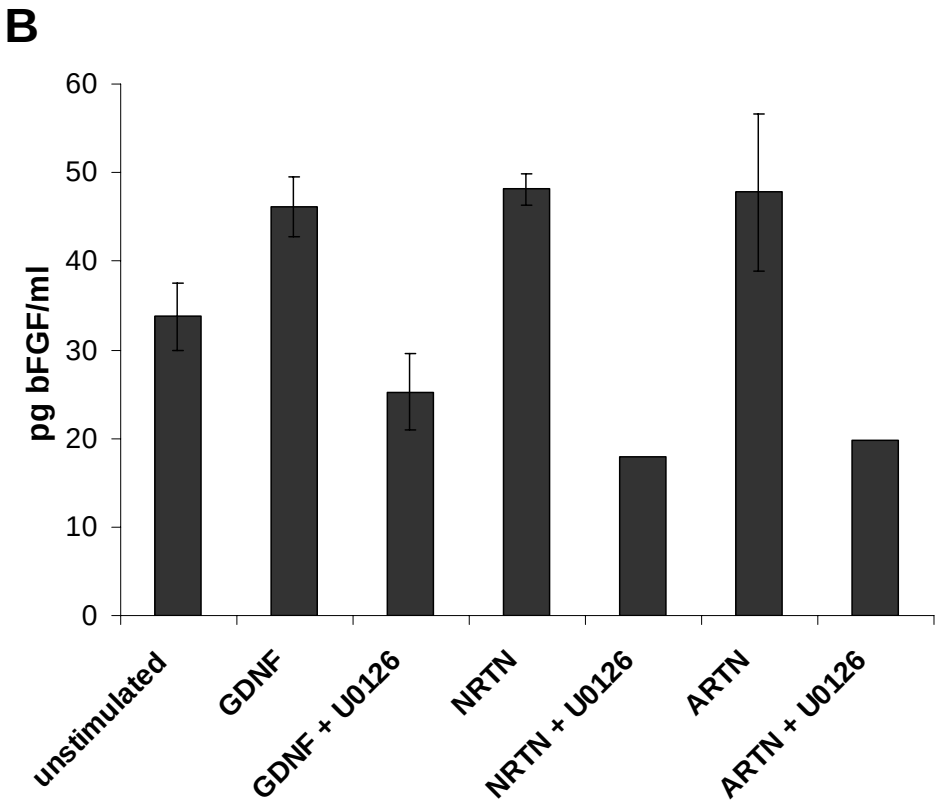
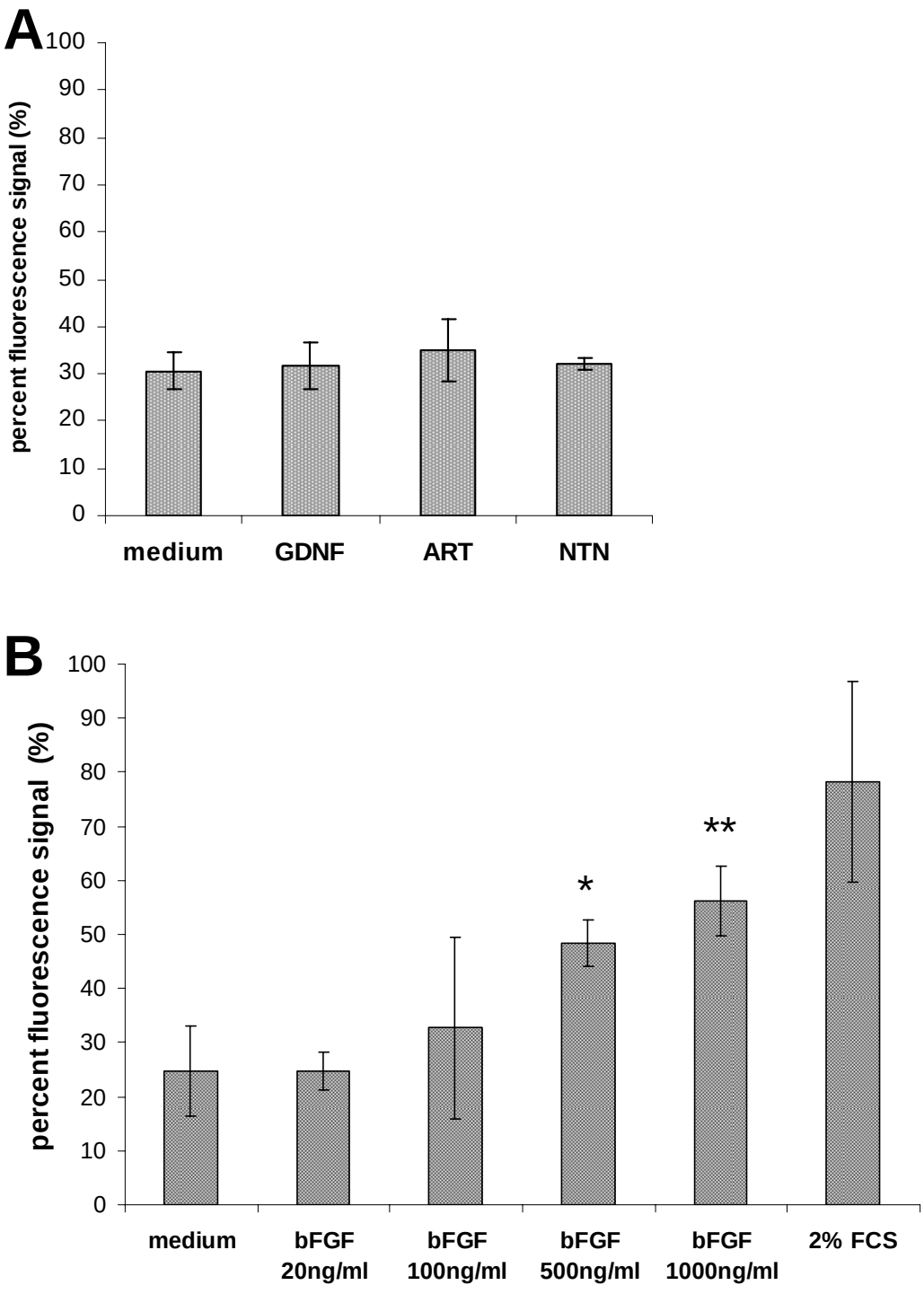
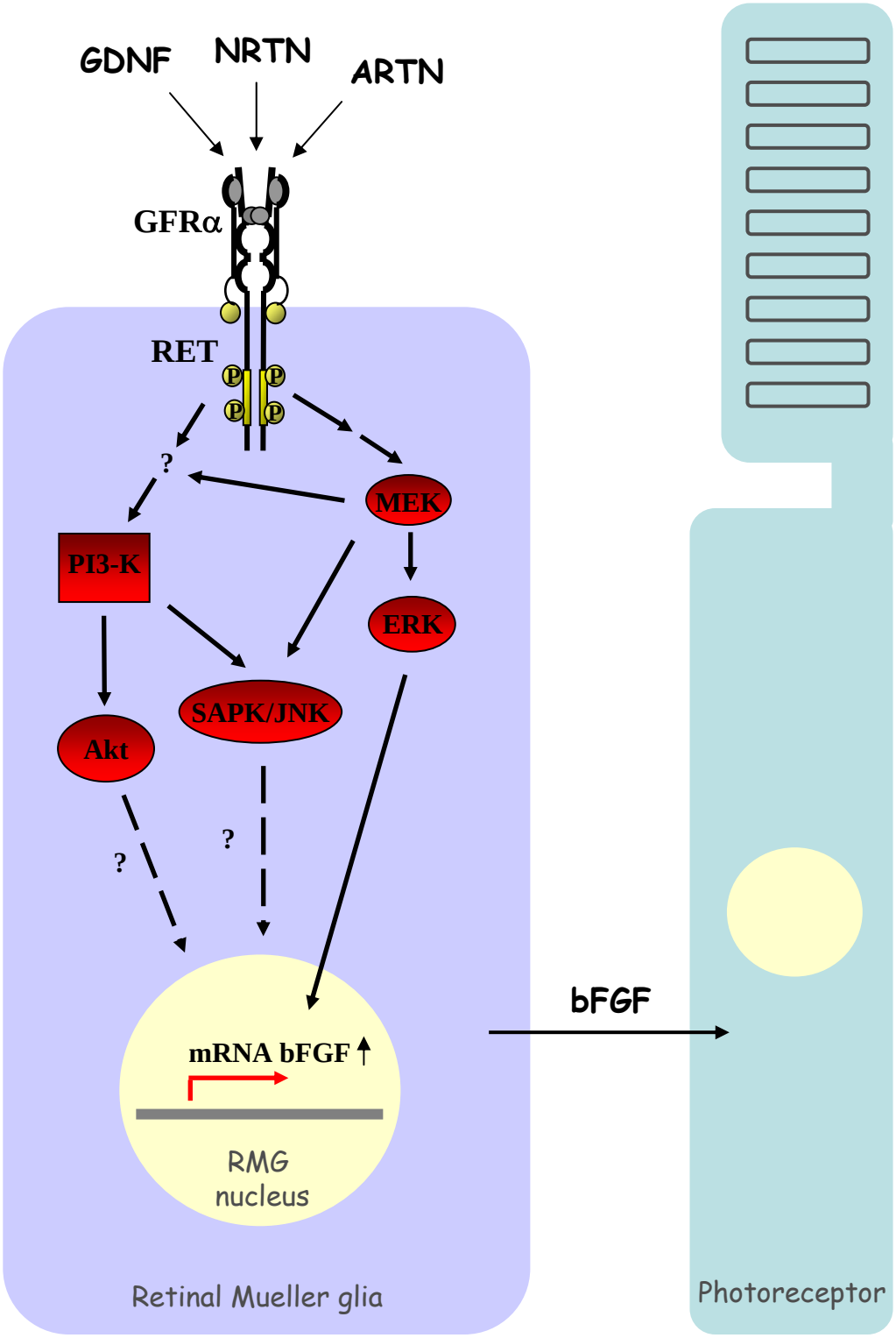

B
pg bFGF/ml
60
50
40
30
20
10
0
unstimulated
GDNF
GDNF + U0126
NRTN
NRTN + U0126
ARTN
ARTN + U0126

Figure 7


A
percent fluorescence signal (%)
100
90
80
70
60
50
40
30
20
10
0
medium
GDNF
ART
NTN
B
percent fluorescence signal (%)
100
90
80
70
60
50
40
30
20
10
0
*
**
medium
bFGF 20ng/ml
bFGF 100ng/ml
bFGF 500ng/ml
bFGF 1000ng/ml
2% FCS


GDNF
NRTN
ARTN
GFRα
RET
P
P
P
P
?
MEK
PI3-K
ERK
SAPK/JNK
Akt
?
?
mRNA bFGF
RMG
nucleus
Retinal Mueller glia
bFGF
Photoreceptor

PAPER 3

Secreted Proteins from Retinal Mueller Glial Cells Enhance Photoreceptor Survival: Identification of New Candidates for Neuroprotection

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Running Title: Secreted neuroprotective proteins from RMG

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Abstract

It has been demonstrated that cultured retinal Mueller glial cells (RMG) release substances into the culture medium (conditioned medium) that are able to induce neurite sprouting in cultures of retinal ganglion cells, and that enhance survival of cones *in vitro*. The identity of these factor(s) is unknown. In order to define RMG-derived neuroprotective factors, a large-scale photoreceptor survival assay was developed, which enabled to monitor survival of freshly prepared porcine photoreceptors in different media for extended times *in vitro*. RMG-conditioned medium (RMG-CM) was found to promote photoreceptor survival, but the secreted activity is lost with prolonged RMG culturing. The activity proofed to be a protein, with a size above 10kDa and sub-fractionation of RMG-CM by mono-Q FPLC demonstrated that the active factor does not bind effectively to anionic chromatography resins. Consequently, composition of mono-Q FPLC flowthrough was directly determined by liquid mass spectrometry (LC-Q-TOF). Two Insulin-like growth factor binding proteins (IGFBP-5 and IGFBP-7) and connective tissue growth factor (CTGF) were among the identified proteins of the active flowthrough. Stable expression of these three candidate factors in HEK293 cells produced CM enriched with the respective factor and proofed to add activity to the original RMG-CM, although not being active alone. This indicated that the survival promoting activity in RMG-CM is multi-factorial, and is triggered by IGFBP-5 and CTGF.

Introduction

Neuronal survival depends on neurotrophic factors released from surrounding tissues, and in their absence, neurons undergo programmed call death [Davies, 1994 #911]. Degeneration of retinal photoreceptor cells is a major cause of blindness, and although this degeneration is often preceded by mutation in photo transduction-relevant genes, degeneration only occurs delayed in life. An explanation for delayed apoptosis might be the existence of paracrine, neurotrophic activities produced by retinal cells, which supports photoreceptor function even in diseased-gene background.

Several efforts have been made to define those neurotrophic activities. In an *in vitro* model of sympathetic neuronal survival it has been shown that medium conditioned by chick retinal explants and medium conditioned by chick retinal Mueller glial cells (RGM) support neuronal survival [Reis, 2002 #912]. The retinal Mueller glial cells (RMG), the main glial cell in the retina, has also been shown to secrete a factor that promotes survival and neuritogenesis of retinal ganglion cells *in vitro* [Garcia, 2002 #817] and cone photoreceptors (1). However, in none of these studies the protein responsible for the neuronal survival-promoting effects has been identified.

In this study we aimed at investigating paracrine factors acting between RMG and retinal photoreceptors (RPR). RMG-conditioned medium was tested for survival-promoting activity on primary RPR in an especially here fore developed large-scale survival assay. In order to further characterise the survival-promoting activity, complexity of RMG-conditioned medium was reduced by subfractionation and finally, active subfractions were subject to mass-spectrometric identification of composing proteins. We found that active fractions of RMG-conditioned medium include at least 23 different proteins among them three potential new candidates for neuroprotective activity in the context of RPR survival: connective tissue growth factor (CTGF), insulin-like growth factor binding protein 5 (IGFBP5) and insulin-like growth factor binding protein 7 (IGFBP7). Expression cloning and retesting of these

candidates for their ability to promote RPR-survival, revealed that two of them (CTGF and IGFBP5) are able to increase RPR-survival when applied in combination with the original RMG-CM, indicating that the RMG-derived neurotrophic activity is multi-factorial.

Materials and Methods

RMG preparation

Adult porcine eyes were provided by a local slaughterhouse. They were removed from the animals within 5 minutes after death and kept on ice in CO_2-independent medium (GIBCO) until further use. Retinae were dissected from the eye and RMG were prepared as described previously [Gaudin, 1996 #266] [Hauck, 2003 #928]. Briefly, major blood vessels were removed and the retina was cut into small pieces which were washed twice in Ringer`s solution. Dissociation of retinal tissue was obtained by treating each retina with 2.2 units of activated papain (Worthington Biochemical Corporation) for 40 minutes at 37°C; papain enzyme activity was stopped by addition of DMEM-HEPES (Gibco) with 10% FCS. Then, 160 kunitzunits of DNAse (Sigma) were added and the tissue was further dissociated by gentle trituration using a fire-polished Pasteur pipette. Dissociated cells were collected by centrifugation (800 g, 5 min), resuspended in DMEM containing 10% FCS and plated directly onto cell culture plates (NUNC). The plated cells were allowed to attach for 16 hours at 37°C in an incubator. Non-attached cells were then removed by gentle agitation (panning) and RMG were cultured for 14 days to near confluence.

Photoreceptor preparation

Retina was dissected from porcine eyes as described above and photoreceptors were isolated from retina as described (2). Briefly, retinal pieces were cut and washed as described above, subsequent papain treatment was 15 minutes, reaction was stopped by addition of DMEM/NUT-MIX F12 (GIBCO) with 2% FCS. After gentle trituration, PR were collected by

centrifugation (800 g, 5 min), resuspended in DMEM/NUT-MIX F12 containing 2% FCS and plated on 96well plates, which were pre-coated with poly-D-Lysine (Sigma, 2µg/cm^2, 3 hours) and laminin (BD Bioscience, 1µg/cm^2, overnight). After incubation for 20 hours, the medium was removed and replaced by different conditioned media or subfractions.

Conditioned medium

Plates with RMG, R6-C1 or HEK293 cells were washed twice with serum free medium, incubated for 3 hours in serum free medium, then medium was replaced with DMEM/NUT-MIX F12 containing 1mM sodium pyruvate (Gibco), and penicillin/streptomycin (Gibco). Medium was conditioned for 18 hours, then filtered (<0.2µm) to remove non-adherent cells and debris and either directly applied on photoreceptors in 96wells, or subfractionated as described below.

Photoreceptor survival assay

Conditioned media from either RMG or rat embryonic cell line (negative control) were applied on attached photoreceptors 20 hours after preparation. Photoreceptor survival was monitored by performing an esterase calcein-fluorophore (Molecular Probes) assay, living cells fluoresce bright green. Fluorescence was measured daily with a different row on the plate in a micro plate fluorescence reader (MWG Biotech FL600) and values were compared to the initial fluorescence at the beginning of the survival assays. Relative fluorescence is given in percent of initial fluorescence, every value is a mean of two to four wells per day and experiment, and every experiment was performed at least three times.

Inhibitors: U0126 and LY294002 (both from Cell Signaling Technology) were added to the photoreceptor survival assay to a final concentration of 1µM and 5µM, respectively.

Proteinase K treatment: Proteinase K (Sigma) was added to RMG-conditioned medium at a final concentration of 100µg/ml, incubated for 30 minutes at 37°C and reaction was stopped by addition of 12,5µg/ml a2-macroglobulin (Serva).

Expression cloning of candidate proteins

ESTclones were ordered from RZPD: IGFBP5 (IMAGp998M039414Q3), IGFBP7 (IMAGp998E148162Q3) and CTGF (IMAGp998F1110165Q3). The cDNAs were subcloned into the pcDNA3.1 V5/His TOPO Vector (Invitrogen) by PCR-amplification (Pfx, Promega) using following primer-pairs (sense, anti-sense): 5'-AGTGCCAACCATGACCGC-3', 5'-TGCCATGTCTCCGTACATCTTCC-3' (CTGF); 5'-ACTAAGAGAAGATGGTGTTGCTC AC-3', 5'-CTCAACGTTGCTGCTGTCG-3' (IGFBP5); 5'-ACATTATACGAAGTTATGG ATCAGG-3', 5'-TAGCTCGGCACCTTCACC-5' (IGFBP7). The PCR products were purified by extraction from agarose gels using the QIAquick gel-extraction kit (Qiagen) and adenosine overhangs for TOPO-TA cloning were generated by incubation with Taq polymerase (Fermentas) at 72°C for 20min applying 0.2mM dATP (Fermentas). PCR-products were then directly used in TOPO ligation according to manufacturer's protocols (Invitrogen). Resulting clones were sequenced. HEK293 cells were transfected with expression clones (Effectene, Qiagen) and stable cell lines were generated by selection with neomycin (G418, 1500µg/ml, Calciochem). Proper secretion of the factors was monitored by western blot of conditioned media with anti-V5 antibody (Invitrogen) (data not shown).

Subfractionation of conditioned media

Size fractionation: 14ml of conditioned media were centrifuged at 5000g for 40minutes, 4°C through 50kDa or 10kDa Macrosep devices (Millipore); the remaining size-enriched concentrate was applied on the photoreceptor survival assay.

Mono-Q FPLC: prior to FPLC separation, conditioned media were concentrated and equilibrated to 30mM sodium-phosphate buffer via Amicon 10kDa filters. 10fold concentrated conditioned media were loaded onto mono-Q columns (HR_5/5,Amersham) in 30mM sodium-phosphate buffer (pH 7.5), flowthrough was collected and proteins binding to mono-Q matrix were eluted by a gradient 0-60% 1M NaCl within 25 minutes into 96well plates. Collected fractions were combined as described in results, concentrated 10fold via

Microsep 10kDa devices and rediluted 10fold in DMEM/NUT-MIX F12 before application onto photoreceptor survival assay.

1D-PAGE

Proteins from conditioned media or from mono-Q-FPLC fractions were precipitated with acetone, solved in buffer for 1D-PAGE (50mM Tris pH 7.4, 250mM NaCl, 25mM EDTA, 1% NP-40, 10% glycerol, Complete mini), protein content was measured by Bradford assay (BioRad) and samples were resolved on a 9-15% PAGE. Protein bands were visualised by silver stain.

2D-PAGE

Medium was conditioned in the presence of 0,8mCi ^{35}S (trans-^{35}S-label, ICN) per 10cm culture dish, filtered after conditioning and proteins from conditioned medium were precipitated with acetone. Precipitates were resolved in 2D-lysis buffer (9 M urea, 2 M thiourea, 1% DTE, 4% CHAPS and each 2.5 µM EGTA and EDTA) and protease inhibitors (complete mini, Roche) and protein content was determined by Bradford assay (BioRad). Electrophoretic separation was carried out as described (Hauck et al, 2003 etc) with IPGstrips pH 3-10 (Amersham Biosciences) in the first dimension and gradient SDS-PAGE gels (9–15%) in the second dimension. Gels were silver stained according to [Blum, 1987 #669] and dried between cellophane sheets. Hyperfilms (Amersham Biosciences) were exposed with dried gels for 1 week to produce autoradiographs.

Mass spectrometry

Peptide sequence information was obtained as described before (Hauck et al, 2005) by LC-coupled tandem-MS (MS/MS)-analysis on a Q-TOF2-System (Micromass, UK) coupled with a CapLC-System (Micromass, UK). Proteins from aliquots (400µl) of active FPLC-flowthrough fraction were precipitated with acetone, resolved in 50mM ammonium bicarbonate, reduced in 5mM dithiothreithole (Merck) for 30 minutes at 60°C followed by alkylation in 15mM iodoacetamide (Merck) for 30 minutes at RT. Proteins were then

subjected to tryptic digest (5ng/µl sample) at 37°C overnight. Peptide samples were acidified to a final concentration of 5% with formic acid and sonicated for 3 min. Fifteen microliter aliquots were loaded on the CapLC system, trapped on a C18-pre-column (5 µm, 100 Å PepMap-C18, LC Packings) and separated on a 75 µm-C18 column (3 µm, 100 Å PepMap-C18, LC Packings) by elution with a gradient (0-60% solution B: 95% acetonitrile, 0.1% formic acid).

Database searching

Database searches were performed using the Mascot software (3) at the following parameter settings: one miscleavage allowed, search restricted to database entries from metazoan, 0.8 Da peptide tolerance, 0.2 Da MS/MS tolerance. Peptide masses of the tryptic digests were compared to the virtually generated tryptic peptide masses of the protein databases NCBInr and MSDB.

Results

Retinal Mueller gill cell (RMG) conditioned medium promotes porcine photoreceptor survival in vitro

RMG have been suggested as important players in rescuing photoreceptors during retinal degeneration or toxic insults. It has been proposed that RMG release factors that inhibit apoptosis, but the nature of these factors remain to be elucidated. In order to define those potentially neuroprotective factors released by RMG, we designed an *in vitro* survival essay which enables to monitor the protective effects of RMG-conditioned media or subfractions on photoreceptors (see materials and methods). Photoreceptor survival is measured each day after replacing regular medium with respective conditioned media and compared to the amount of initially living cells.

RMG conditioned medium resulted in consistently higher photoreceptor survival rates than application of either conditioned medium from control cells (R6-C1, a rat embryonic cell line) or medium alone (see Fig.1). The most significant time point of photoreceptor-survival promoted by RMG compared to controls is 6 days after application of conditioned media.

The RMG-derived survival-promoting activity is lost during prolonged culturing

We have described before that RMG dedifferentiate upon prolonged culturing (4). It could thus be hypothesised that RMG might loose, along with other specific marker proteins, the photoreceptor survival-promoting activity.

We tested conditioned medium from RMG which have been cultured for 7 (data not shown), 14 and 21 days before conditioning. The constantly highest survival promoting activity was found in conditioned medium from 14 day old RMG, after 21 days in culture the activity was lost compared to the controls (see Fig.2A). Medium conditioned by 7 day old RMG also showed lower survival effects on RPR compared to day 14 conditioned medium; this was accompanied by a lower overall protein content in the conditioned medium. This probably

resulted from lower cell densities in the culture plates of 7 day-old RMG compared to 14 and 21 day-old RMG.

Survival-promoting activity from RMG is a protein, separating into size fraction above 10kDa

In order to confirm that the survival promoting activity is a protein, RMG conditioned medium was treated with proteinase K. Proteinase K treatment was stopped by addition of α2-macroglobulin, which itself proofed to be not toxic to photoreceptors *in vitro*. Proteinase K treatment completely destroyed the survival-promoting activity of RMG conditioned medium (see Fig.2A).

In order to further characterise the survival-promoting factor, the conditioned medium was fractionated into molecular weight fractions <10kDa, between 10 and 50 kDa and above 50kDa. RMG derived survival-promoting activity was enriched most compared to control in the fraction above 50kDa and to a lesser extent also in the 10kDa fraction. R6-C1 (negative control) conditioned medium showed a survival promoting effect in the size fraction below 10kDa (see Fig.2B).

Survival-promoting activity acts through PI-3 kinase dependent pathway in photoreceptors

Cell survival can be promoted by several distinct or interacting pathways. In order to define the pathways responsible for RMG-mediated prolonged survival of photoreceptors *in vitro*, inhibitors were applied along conditioned medium in the survival assay. LY294002 inhibits selectively PI3-kinase and U0126 is an inhibitor of MEK kinase. Application of LY294002 completely blocked survival-promoting activity of RMG-conditioned medium, whereas U0126 only partially reduced survival of RPR (Fig.3). In order to ensure that the application of the inhibitors itself would not interfere with RPR-survival both inhibitors were applied in combination with 2%FCS on RPR-survival assay. 2% FCS carries strong survival-promoting activities and neither LY294002 nor U0126 were able to prevent RPR-survival under those conditions (Fig.3). RPR-survival promoted by RMG-conditioned medium thus seems to

depend significantly on PI3-kinase pathway and only to a lesser extent on MEK-kinase pathway.

RMG-conditioned medium is a complex mixture of proteins

In order to define the factor responsible for RMG-derived RPR-survival, we first studied the complexity of RMG-conditioned medium. For this purpose, medium was conditioned in the presence of ^{35}S, and subsequently proteins were resolved by high-resolution 2DE-PAGE. Radioactive labelling allows detecting proteins transcribed during the conditioning process on a very sensitive level.

RMG-conditioned medium shows highly complex protein-pattern (see Fig.4). About 2200 different protein spots were detected on autoradiographs of 2D gels. Thus, a prerequisite for identifying proteins responsible for the neuroprotective effect of RMG-conditioned medium is to reduce complexity.

RMG-conditioned medium complexity can be diminished by separation on mono-Q FPLC

In order to reduce complexity of RMG-conditioned medium we chose to fractionate via mono-Q FPLC. RMG-conditioned medium gave reproducible elution profiles from mono-Q columns (see Fig5A), complexity of eluting fractions was monitored by 1DE-PAGE (see Fig.5B). A total of 33 fractions from each run were collected into 96well plates, and according to the UV280nm detection the majority of proteins are eluting into fractions 20 – 34 (see Fig.5A). 10µg of total protein from every second fraction were resolved by 1DE-PAGE and shows that prominent bands probably representing one protein species, appear in approximately 6-8 consecutive fractions.

In order to test which subfractions from FPLC separation carry the RPR-survival promoting activity, fractions were pooled as depicted in Fig.5B into 5 pools: A (fraction 1-7), B (fraction 8-13), C (fraction 14-19), D (fraction 20-26), E (27-33). Flowthrough from sample application was also collected.

Survival-promoting factor does not bind strongly to anion exchange column

All pooled fractions from mono-Q FPLC along with flowthrough were tested on RPR-survival assay for survival activity. Activity of every tested fraction was compared to an analogously treated conditioned medium from R6-C1 cell line, in order to evaluate selective RMG-derived activity.

It showed that, compared to R6-C1 conditioned medium, most of the survival-promoting activity from RMG did not bind to mono-Q sepharose. The major part of the activity reappeared in the flowthrough from FPLC, and some in pooled fraction A, which covers very low eluting salt concentrations (Fig.6). Thus it showed that RMG-derived survival-promoting activity does not bind strongly to anion exchange matrices and could be an acidic protein. The activity can thus be very effectively separated from the majority of proteins included in RMG-conditioned medium and be recovered in mono-Q sepharose flowthrough.

Identification of proteins in mono-Q flougthrough fractions

The enrichment of the survival-promoting activity in the mono-Q flowthrough, which is not very complex, makes it feasible to identify composing proteins directly and generate a list of probable candidates for this activity. Flowthrough was digested with trypsin and applied to Q-TOF-MS/MS mass spectrometry in order to identify its components.

23 different proteins were identified above significance threshold from RMG-FPLC flowthrough (Table 1). Among those proteins are 3 growth-factor related proteins: insulin-like growth factor binding protein 5 (IGFBP5), insulin-like growth factor binding protein 7 (IGFBP7) and connective tissue growth factor (CTGF) which are good candidates for the RPR-survival activity. Further, superoxide-dismutase (SOD) and thioredoxin were identified from this fraction; both proteins might protect photoreceptors by preventing oxidative damage. In order to confirm the mass spectrometric identifications, we additionally monitored the expression of five candidate proteins by RT-PCR from RMG *in vitro*. RMG were found to express CTGF, IGFBP5, IGFBP7, SOD and thioredoxin (data not shown).

Testing of identified candidate proteins for their survival-promoting activity on RPR

In order to identify which protein from active fraction is responsible for the RPR survival-promoting effect of RMG-conditioned medium, we expression-cloned CTGF, IGFBP5 and IGFBP7 into HEK293 cells and generated stable cell lines secreting the respective proteins into the cultur medium (proper secretion monitored by western blots with antibodies against V5 epitope, data not shown). Additionally, we tested commercially available superoxide dismutase (SOD) and thioredoxin (THIO) for their activity on RPR.

We found that compared to negative control (medium alone), SOD and THIO had no survival promoting effect on RPR *in vitro*. Effects of expression-cloned candidates were evaluated by comparison to empty HEK293-cell medium (Fig. 7). None of the candidates had convincing survival-promoting effects for RPR compared to negative control. However, when applied together with the original RMG-CM, CTGF and IGFBP5 could increase the survival promoting effect of RMG-CM, whereas SOD, THIO and IGFBP7 failed to do so (Fig. 7). This indicates a survival-promoting effect for CTGF and IGFBP5 which acts together with other factors present in RMG-CM.

Discussion

In this study we aimed at identifying components specifically secreted by primary retinal Mueller glial cells (RMG) which support survival of retinal photoreceptors. Therefore we developed a large-scale *in vitro* survival assay that allows screening for survival promoting activity in 96well format. We took special effort to induce objectivity for the measurement of survival by measurement of overall fluorescence per well, rather then counting single fluorescent cells per field (5), because the latter depends on subjective field selection criteria by the experimentator.

In order to select molecules specifically secreted by RMG, we compared the activity of RMG-CM initially and during factor purification to factors secreted by a fibroblast cell line (R6). Interestingly, R6-CM also displayed survival-promoting activity, but in contrast to RMG-CM, this activity is enriched in fractions <10kDa (and 50-10kDa) from size-fractionated conditioned media and the activity strongly binds to monoQ sepharose, as is demonstrated by elution from FPLC in high salt containing fractions (Pool E, Fig. 6). It could be hypothesized that this activity is bFGF, because bFGF is a small protein (17kDa) with basic properties, thus predicted to bind strongly to anionic sepharose.

The activity purified from RMG-CM is enriched in fractions above 50kDa and (50-10kDa) and does not bind to anionic resin. Moreover, the production of this activity is diminished with prolongued culturing underlining the specific expression by primary RMG. We identified 23 different proteins from FPLC-flowthrough, which was demonstrated to be the active fraction after FPLC separation of RMG-CM. We decided to test 5 candidate proteins for their activity on photoreceptor-survival: thioredoxin (THIO) and superoxide-dismutase (SOD), because both proteins are known be protective against reactive oxygen species and recently a member of thioredoxin family was identified to promotes survival of cone photoreceptors, which is derived from rod photoreceptors and (6). Connective tissue growth factor (CTGF) and insulin-like growth factor binding proteins 5 and 7 (IGFBP5, IGFBP7)

were chosen because they all are described as secreted proteins with cell growth or maintenance activity (www.expasy.org) and might thus be involved in paracrine signalling. The complexity of RMG-CM indicates that the survival promoting activity might be multi-factorial, rather than induced by one single factor. Therefore we also tested the identified candidates in combination with the original RMG-CM. Whereas THIO, SOD and IGFBP7 did not increase survival of photoreceptors *in vitro*, CTGF and IGFBP5 were found to be active when applied in combination with the original CM. This is in line with our hypothesis that the RMG-CM derived activity on photoreceptor-survival is multi-factorial. Most interestingly, there has been one previous report about the expression of six IGFBP´s (IGFBP1-6) by porcine RMG *in vitro*, where the authors found that only the expression of IGFBP5 decreased with prolonged culturing, whereas the expression of all other IGFBP´s (IGFBP-1, -2, -3, -4 and -6) increased (7). This may lead to the assumption that IGFBP5 is likely to be expressed by RMG *in vivo* and it underlines our finding that the activity (which may be related partly to IGFBP5) is lost with prolonged culturing of RMG. The importance of IGFBP5 in retina *in vivo* is further underlined by a report about its expression in developing chick retina, which points to a significant role during retinal development (8).

The two active molecules found in this study, IGFBP5 and CTGF, both carry IGF-binding domains in their amino acid sequence (www.expasy.org). IGFBP5 binds with high affinity to IGF-1, is cleaved by several serine proteases (9) (10) or by matrix metalloproteases (MMP´s) (11) and is able to associate with extracellular matrix. When associated with the extracellular matrix (ECM), it appears to be protected from proteolysis, however, once released from ECM it is degraded (12) into a 22 kDa fragment that has low affinity for IGF-1 and -2 and a smaller 14kDa fragment (11). In our study, expression of IGFPB5 in HEK293 cells resulted in conditioned media which primarily carry the uncleaved form of IGFBP5 and only a minor part of the protein is degraded (data not shown). Nevertheless, both, the uncleaved as well as the cleaved form of IGFBP5 may be responsible for the photoreceptor-survival promoting

activity desribed here. IGFBP-5 has recently been found to act in concert with IGF-2 in inducing cell proliferation of neuroblastoma cells (13).

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Figure legends

Fig. 1: Photoreceptor survival *in vitro*

A: Primary porcine photoreceptors are plated onto 96well culture dishes and after initial attachment phase in the presence of 2% FCS, medium is replaced by different conditioned media: RMG-CM, medium conditioned for 16 hours by primary retinal Mueller glial cells; R6-C1, medium conditioned for 16 hours by a rat fibroblast cell line. Negative control is medium alone. Photoreceptor survival is measured every day (from day of medium replacement: d0 till 9 days after application of different media: d9) on 2-4 wells per condition by esterase-calcein fluorophore assay, intensity of fluorescence is compared to initial fluorescence and given in percent of initial fluorescence. Values are means from three experiments.

RMG-CM (black bars) resulted in consistently higher photoreceptor survival rates than application of either conditioned medium from control cells (R6-C1, gray bars) or medium alone (white bars). The most significant time point of photoreceptor-survival promoted by RMG compared to controls is 6 days and 7 days after application of conditioned media.

B: Porcine photoreceptors from 96well survival assay stained with calcein-fluorophore 6 days after application of R6-C1 conditioned medium (negative control, left panel) or RMG-CM (right panel) (400 x magnification). In RMG-CM a large number of photoreceptors survive which results in a higher overall fluorescence that can be measured photometrically as shown in A.

Fig. 2: Activity from RMG-CM is a large protein

A: Porcine photoreceptor-survival 6 days after application of medium alone (DMEM NUT MIX), R6C1 conditioned medium (R6-C1) and RMG-CM generated from RMG 14 days *in vitro* (RMG day14) or RMG 21 days *in vitro* (RMG day21). Highest survival promoting activity was found in conditioned medium from 14 day old RMG, after 21 days in culture the activity was lost compared to controls. Further, RMG-CM day14 treated with proteinase K

(proteinase K + a2-macroglobulin) was applied to photoreceptor survival assay. Proteinase K treatment was stopped by addition of α2-macroglobulin, which itself proofed to be not toxic to photoreceptors *in vitro* (a2-macroglobulin). Proteinase K treatment completely destroyed the survival-promoting activity of RMG-CM.

B: Size-fractionation of RMG-CM

RMG-CM (day14) was enriched into molecular weight fractions <10kDa, between 10 and 50 kDa and above 50kDa. Similar fractionations were performed with R6-C1 conditioned medium. All fractions were monitored for their survival promoting activity on photoreceptors. Survival is given as relative fluorescence compared to initial fluorescence 6 days after application of samples. RMG derived survival-promoting activity was enriched most compared to control in the fraction above 50kDa and to a lesser extent also in the 10kDa fraction. R6-C1 (negative control) conditioned medium showed a survival promoting effect in the size fraction below 10kDa.

Fig. 3: Inhibition of RMG-derived survival effect with PI3-kinase inhibitor LY294002

RMG-CM (day 14) was applied to photoreceptor-survival assay either alone, or together with PI-3 kinase inhibitor LY294002 (5μM) or MEK inhibitor U0126 (1μM). Survival is given as relative fluorescence signal 6 days after application compared to initial fluorescence. Inhibitors were further applied together with medium (CDM) containing 2%FCS, in order to assess toxicity of the inhibitors in the presence of strong survival signal derived from FCS. Application of LY294002 completely blocked survival-promoting activity of RMG-conditioned medium, whereas U0126 only partially reduced survival of PR. In the presence of 2% FCS neither LY294002 nor U0126 were able to significantly reduce PR-survival.

Fig. 4: Autoradiograph of RMG-CM (day 14)

RMG-CM was prepared in the presence of 35S-methionine and 150μg total protein were separated by isoelectric focusing on pH 3-10 IPG strips in the first dimension and 12% SDS-

PAGE in the second dimension. Autoradiograph was taken from dried gel. 2200 different protein spots could be detected by this method from RMG-CM.

Fig. 5: monoQ-FPLC separation of proteins from RMG-CM

A: Proteins from RMG-CM were separated by mono-Q FPLC. RMG-CM gave reproducible elution profiles (absorbtion at 280nm, indicated as mAU) with increasing NaCl-concentrations (indicated as mS/cm) from mono-Q columns and proteins were collected in 33 fractions.

B: Proteins from every second eluting fraction were separated by 1DE-PAGE (10µg per lane) and visualised by silver staining. Additionally, proteins not binding to monoQ (flowthrough from sample application, FT) are resolved by 1DE-PAGE. Molecular weights of marker (M) are indicated. Fractions were pooled as depicted into 5 pools: A (fraction 1-7), B (fraction 8-13), C (fraction 14-19), D (fraction 20-26), E (27-33).

Fig. 6: PR-survival assay with pooled fractions from FPLC separated RMG-CM and R6C1-CM

MonoQ-FPLC separation was performed with RMG-CM (day14) and R6C1-CM (negative control) and resulting fractions were pooled as shown in Fig. 5B. Pooled fractions (A, B, C, D, E) and flowthrough were tested for survival-promoting activity in photoreceptor-survival assay. Survival is given as relative fluorescence compared to initial fluorescence 6 days after application of samples. Compared to R6-C1-CM, most of the survival-promoting activity derived from RMG reappeared in the flowthrough from FPLC separation.

Fig. 7: Survival promoting activity of IGFBP5 and CTGF

Different factors were tested for their survival-promoting activity on photoreceptors: medium alone (negative control), RMG-CM (day 14) and RMG-CM supplemented with thioredoxin (THIO, 500ng/ml), with superoxide dismutase (SOD, 500ng/ml), with IGFBP5-CM (RMG-CM + IBP5), with CTGF-CM (RMG-CM + CTGF) and with IGFBP7 (RMG-CM + IBP7). Survival is given as relative fluorescence compared to initial fluorescence 6 days after

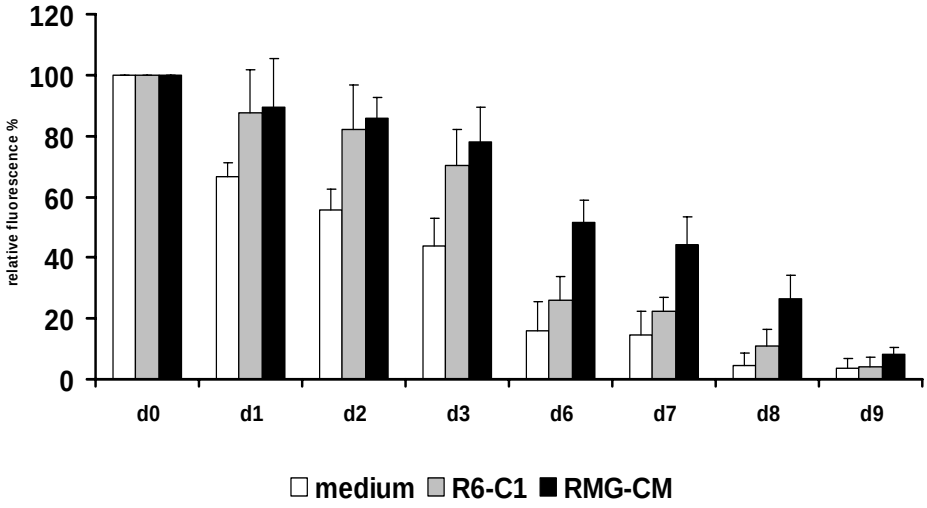
application of samples. IGFBP5 and CTGF increase the survival-promoting activity of RMG-CM.

Table 1: Proteins identified from RMG-CM FPLC-flowthrough

score	SwissProt ID	identity (with synonyms)	acc.Nr	Seq. Cov.	MG
691	CO1A2_CANFA	Collagen alpha 2(I) chain [Precursor]	O46392	16%	129835
277	ENOA_HUMAN	Alpha enolase, EC 4.2.1.11	P06733	14%	47350
225	ENOB_HUMAN	Beta enolase, EC 4.2.1.11	P13929	10%	47168
199	Q29568_PIG	Phosphopyruvate hydratase [Fragment]	Q29568	35%	16079
168	TPIS_RAT	Triosephosphate isomerase, EC 5.3.1.1, TIM	P48500	19%	27286
162	Q811C8_MOUSE	Insulin-like growth factor binding protein 7 [Fragment]	Q811C8	17%	28053
136	VINC_HUMAN	Vinculin, Metavinculin	P18206	2%	124161
124	CATD_PIG	Cathepsin D, EC 3.4.23.5	P00795	14%	37727
91	Q9GK25_PIG	Cyclophilin, EC 5.2.1.8, Peptidyl-prolyl cis-trans isomerase	Q9GK25	21%	11969
89	CO3A1_HUMAN	Collagen alpha 1(III) chain [Precursor]	P02461	1%	139696
88	PGK1_HORSE	Phosphoglycerate kinase, EC 2.7.2.3	P00559	8%	44842
70	LDHA_PIG	L-lactate dehydrogenase A chain, EC 1.1.1.27, LDH-A	P00339	7%	36749
69	TAGL_HUMAN	Transgelin	Q01995	6%	22522
57	MOES_PIG	Moesin, Membrane-organizing extension spike protein	P26042	2%	67602
55	SODC_MOUSE	Superoxide dismutase [Cu-Zn], EC 1.15.1.1	P08228	7%	15973
53	PROF1_MOUSE	Profilin I	P62962	20%	14988
48	CTGF_HUMAN	Connective tissue growth factor [Precursor]	P29279	2%	40267
47	K2C1_HUMAN	Keratin, type II cytoskeletal 1, Cytokeratin 1, K1, CK 1	P04264	3%	66018
47	IBP5_HUMAN	Insulin-like growth factor binding protein 5, IGFBP-5	P24593	5%	31576
46	ALDR_HUMAN	Aldose reductase, EC 1.1.1.21, AR, Aldehyde reductase	P15121	3%	36099
45	COF1_PIG	Cofilin, non-muscle isoform	P10668	12%	18792
44	TSP1_HUMAN	Thrombospondin-1 [Precursor]	P07996	1%	133321
43	CO4A1_HUMAN	Collagen alpha 1(IV) chain [Precursor]	P02462	<1%	161650
36	THIO_BOVIN	Thioredoxin	O97680	23%	11959

Fig. 1

A


120
100
80
60
40
20
0
relative fluorescence %
d0
d1
d2
d3
d6
d7
d8
d9
medium
R6-C1
RMG-CM

B

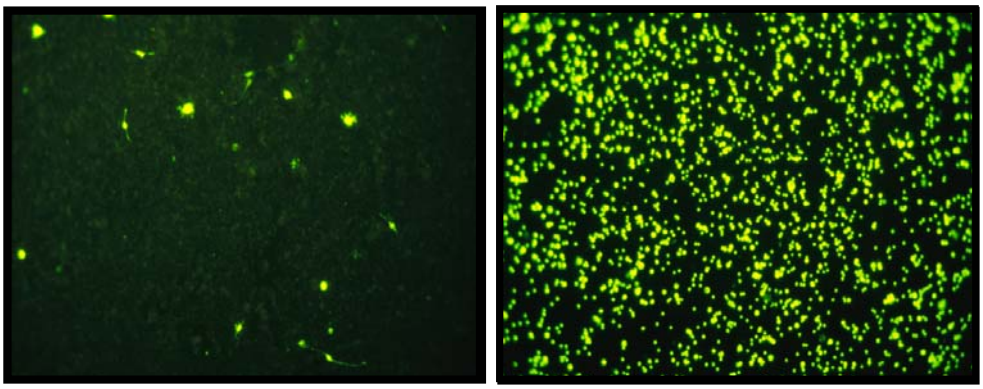
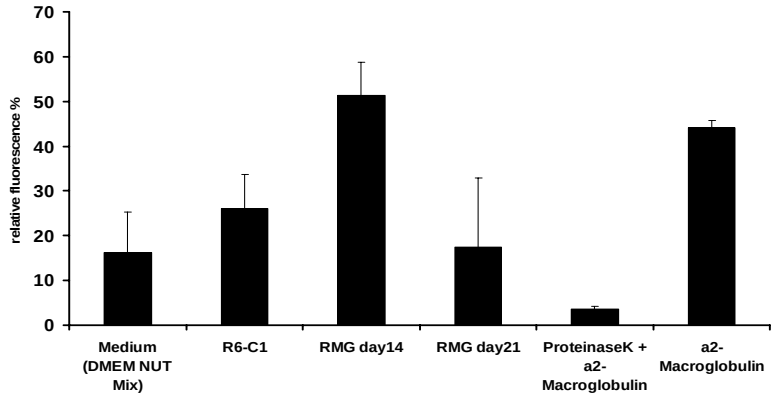


Fig. 2

A


relative fluorescence %
70
60
50
40
30
20
10
0
Medium (DMEM NUT Mix)
R6-C1
RMG day14
RMG day21
ProteinaseK + a2-Macroglobulin
a2-Macroglobulin

B

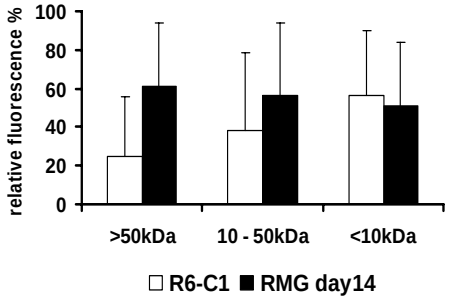

relative fluorescence %
100
80
60
40
20
0
>50kDa
10 - 50kDa
<10kDa
□ R6-C1 ■ RMG day14

Fig. 3

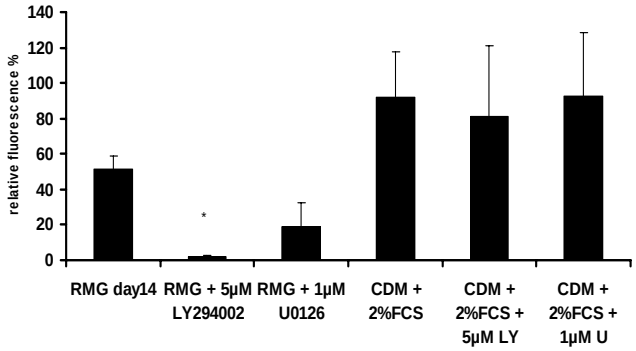
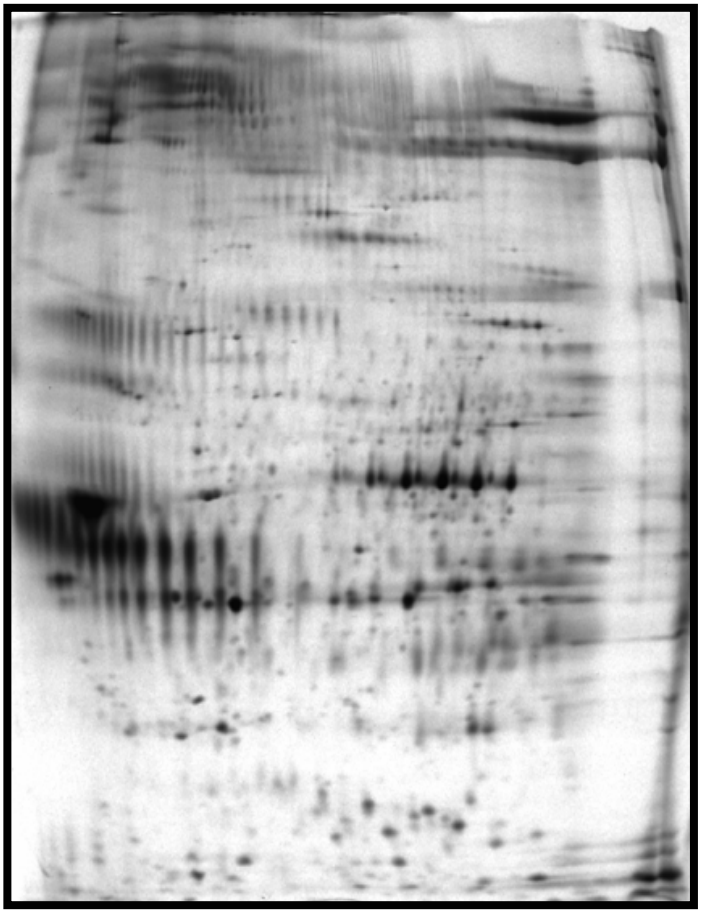

relative fluorescence %
140
120
100
80
60
40
20
0
*
RMG day14
RMG + 5µM LY294002
RMG + 1µM U0126
CDM + 2%FCS
CDM + 2%FCS + 5µM LY
CDM + 2%FCS + 1µM U

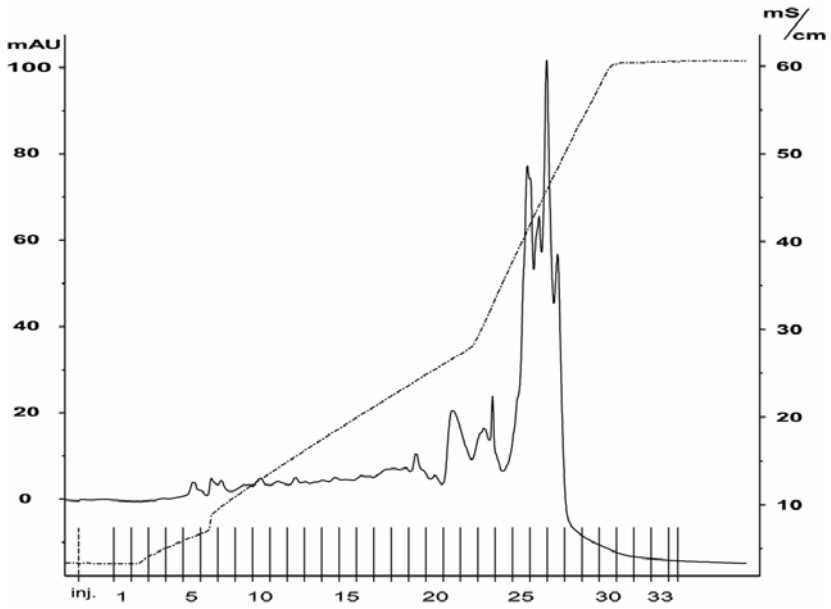
Fig. 4

RMG-conditioned medium



p*I* 3 **p*I* 10**

Fig. 5A


mAU
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80
60
40
20
0
mS/cm
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50
40
30
20
10
inj.
1
5
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15
20
25
30
33

Fig. 5B

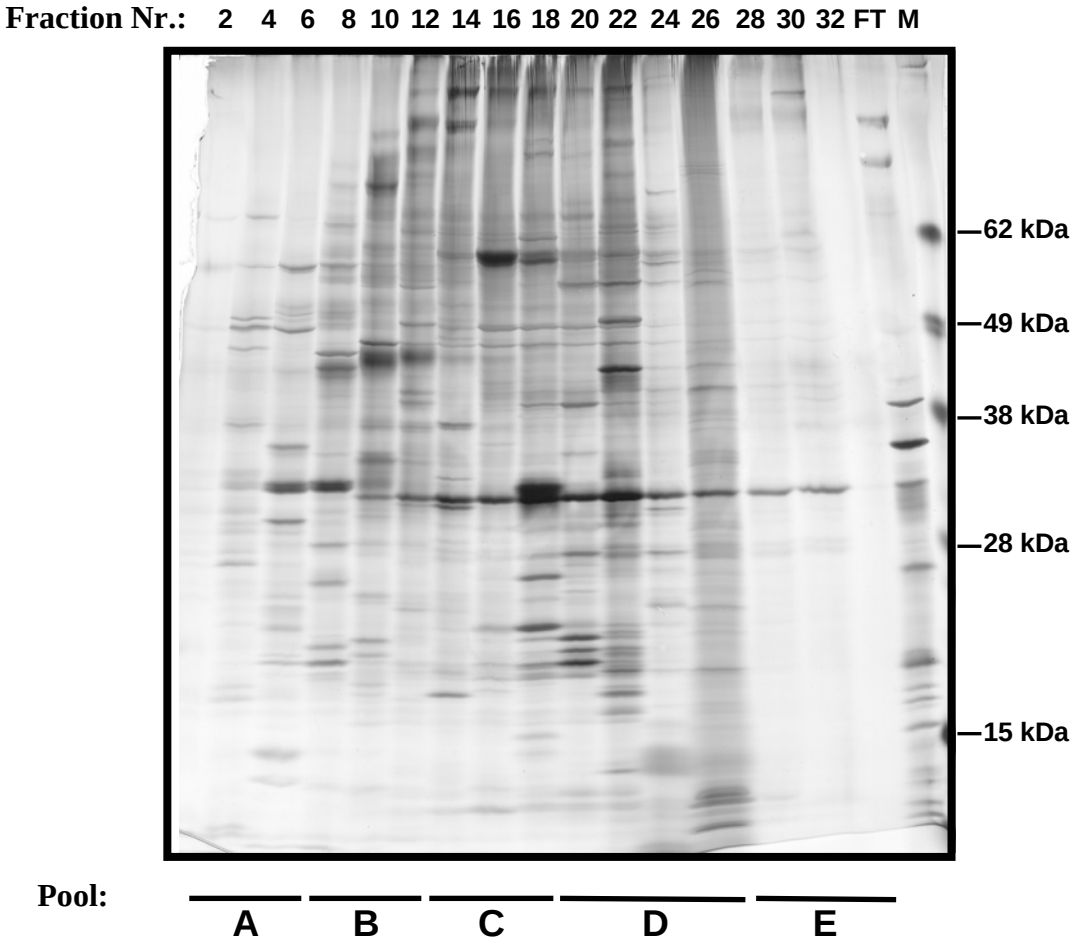

Fraction Nr.: 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 FT M
62 kDa
49 kDa
38 kDa
28 kDa
15 kDa
Pool:
A
B
C
D
E

Fig. 6

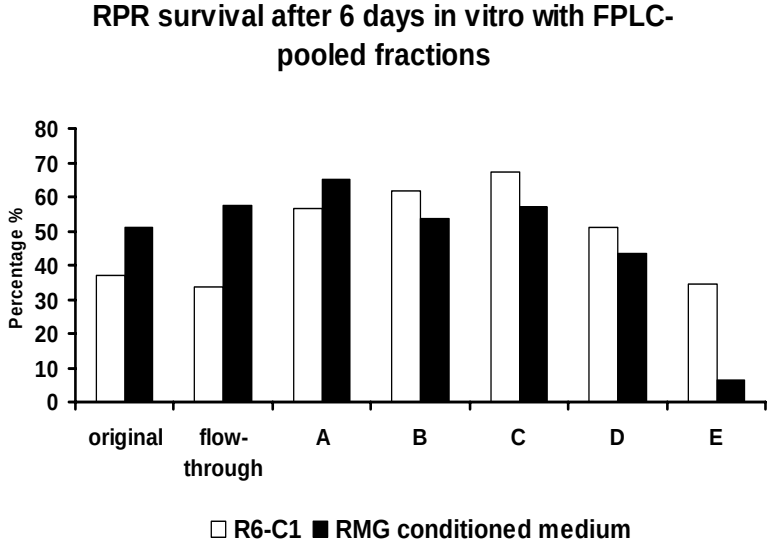
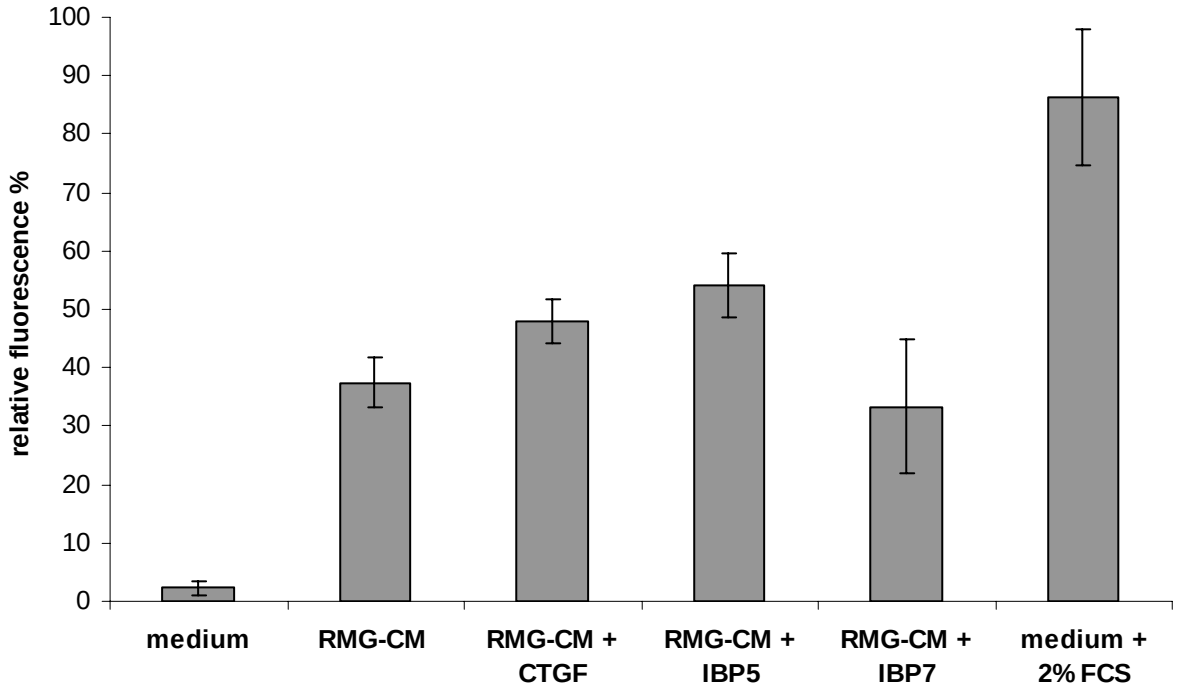

RPR survival after 6 days in vitro with FPLC-pooled fractions
Percentage %
80
70
60
50
40
30
20
10
0
original
flow-through
A
B
C
D
E
□ R6-C1 ■ RMG conditioned medium

Fig. 7


relative fluorescence %
100
90
80
70
60
50
40
30
20
10
0
medium
RMG-CM
RMG-CM + CTGF
RMG-CM + IBP5
RMG-CM + IBP7
medium + 2% FCS

PAPER 4

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REGULAR ARTICLE

Proteomic analysis of the porcine interphotoreceptor matrix

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The interphotoreceptor matrix (IPM) is located between photoreceptors and pigment epithelium in the retina and is involved in fundamental functions of the visual cycle. These include visual pigment chromophore exchange, retinal adhesion, metabolite trafficking, and growth factor presentation. In general, IPM preparations are contaminated with intracellular proteins, as has also been described for other body fluids. This study aimed at identifying new components of the IPM by discriminating between truly secreted proteins and proteins that are part of the IPM for secondary reasons. "Soluble" porcine IPM was extracted from retina and pigment epithelium with PBS by two different procedures, followed by extraction with water alone that released "insoluble" IPM matrix sheets. Samples from all preparations were separated by 2-DE and a total of 140 protein spots were identified by MALDI-TOF and/or CapLC Q-TOF MS. Although identified proteins included several already known in the IPM, the majority had not been previously described in this structure. Gene ontology classifications allocated the identified proteins into nine different functional networks. The IPM preparations also included intracellular proteins from cells adjacent to the IPM, which may have resulted from cell disruption. This underlines the experimental difficulties of a biochemical analysis of the IPM as an intact compartment. We show here a strategy for predicting the probability of identified IPM proteins occurring *in vivo* by combined high-resolution protein separation methods with computational prediction methods. Thus, a set of potentially neuroprotective proteins could be extracted, including PEA-15, peroxiredoxin 5, alpha-B-crystallin, macrophage migration inhibitory factor, 78 kDa glucose-regulated protein (GRP78), protein disulfide-isomerase, and PEP-19, which have not been previously associated with the IPM. Furthermore, with immunohistochemical staining we could confirm the localization of GRP78 in the IPM on porcine eye sections, thus validating the proposed prediction method.

Keywords:
Extracellular matrix / Eye / Neuroprotection / Retina / Secretion

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Abbreviations: GO, gene ontology; **GRP78**, 78 kDa glucose-regulated protein; **HA**, hyaluronan; **IPM**, interphotoreceptor matrix; **IRBP**, interphotoreceptor retinoid-binding protein; **MIF**, macrophage inhibitory factor; **PEA-15**, astrocytic phosphoprotein; **PEP-19**, neuron-specific protein; **PEDF**, pigment epithelium-derived factor; **PNA**, peanut agglutinin; **RMG**, retinal Mueller glia; **RPE**, retinal pigment epithelium

1 Introduction

The interphotoreceptor matrix (IPM) is a carbohydrate-rich complex occupying the extracellular compartment between the outer neural retina and the apical surface of the retinal pigment epithelium (RPE) in the vertebrate eye [1]. As such, it surrounds the elongate, light-sensitive photoreceptors extending from the outer retinal surface. Several structural and functional activities of fundamental importance to vision have been proposed to occur within the IPM, including visual pigment chromophore exchange, retinal adhesion, metabolite trafficking, photo-

receptor alignment, and cell–cell interactions, thought to be involved in photoreceptor membrane turnover (reviewed in [2]).

Initial studies of the IPM described it as unstructured and amorphous [1]. Saline rinses of the outer retinal surface removed "soluble" IPM components, such as interphotoreceptor retinoid-binding protein (IRBP), a variety of enzymes, mucins, and immunoglobins (for review, see [2]). However, later work revealed distinct matrix domains surrounding rod and cone photoreceptors that resist saline rinses of the outer retinal surface. This "insoluble" IPM can be isolated by hypotonic treatment, which frees the solid IPM from the outer retina as a relatively intact, sheet-like unit [3–5]. The "insoluble" IPM consists of a scaffold of hyaluronan (HA) to which the secreted glycoprotein SPACR and chondroitin sulfate proteoglycan SPACRCAN bind *via* HA-binding motifs in their polypeptides [6]. Other molecules containing specific HA-binding motifs have been identified in the plasma membranes of adjacent cells, *e.g.*, CD44 expressed in the apical region of retinal Mueller glia (RMG) [7] and RHAMM in the apical region of RPE [8].

A number of ubiquitous growth, survival, and neurotrophic factors have been identified in the IPM of normal and pathological retinas. Basic fibroblast growth factor has been localized to the IPM of monkeys, humans, and mice [2, 9–11] but was not detected in the IPM of rats or cows [12, 13]. Whereas insulin-like growth factor 1 (IGF-I) and insulin-like growth factor binding-protein (IGF-BP) have been found in human IPM, IGF receptors were found on rod outer segments and RPE cells, thus suggesting the presence of an outer retina autocrine/paracrine system [14]. Neuron-specific enolase (NSE), an enzyme participating in glycolysis which has been described as potential neuronal survival factor in the central nervous system, has been isolated from bovine IPM [15]. Pigment epithelium-derived factor (PEDF), a member of the serine protease inhibitor family [16], is presumably secreted by RPE cells into the IPM [17, 18] and is responsible for the neurotrophic activity of crude IPM extracts on Y79 retinoblastoma cells [19, 20]. PEDF carries a RHAMM-type motif in its primary sequence through which it binds HA, thus being strongly attached to the insoluble IPM scaffold [6].

Due to its key location and putative role in supporting photoreceptor function, additional information on IPM composition could prove useful for developing therapeutic rescue strategies against photoreceptor degeneration. This study was aimed at identifying proteins from porcine IPM in an unbiased, non-candidate approach by combining high-resolution 2-DE with mass spectrometric protein identification [21, 22]. In order to evaluate the existing IPM preparation methods, we first compared two different extraction methods for "soluble" IPM, "conventional" [23] and cannulation methods [24], followed by 2-DE analysis. The remaining "insoluble" IPM sheets, along with proteins tightly attached to these sheets, were subsequently released with water [25, 26] and also resolved by 2-DE analysis. The maps of both IPM extractions, which we hereafter refer to as "soluble" and "insoluble" IPM, were compared and the identity of 140 silver-stained protein spots was determined by MS. We present here a strategy (combination of high-resolution separations techniques with bioinformatics prediction tools) for discriminating between *de facto* secreted IPM proteins and those present in the IPM for secondary reasons. Thus, we extracted from the large number of identified proteins those most likely to be present in IPM *in vivo* and for one of these proteins we demonstrated its localization in the IPM on sections of porcine eyes.

2 Material and methods

2.1 Preparation of the IPM

Adult porcine eyes were provided by a local slaughterhouse. They were removed from the animals within 5 min after death and kept on ice in CO_2-independent medium (Gibco) until further use. Within 1 h after the death of the animals, the anterior portion of the eyes, including the lens and vitreous humor, were carefully removed and exposed retinae were then rinsed in PBS to remove any protein contaminants from the vitreous.

2.1.1 Preparation of "soluble" IPM

Two different preparation methods for "soluble" IPM were applied. For conventional "soluble" IPM preparation (adapted from [23]), retinae were carefully dissected from the eye and incubated in PBS containing protease inhibitors (Complete-mini; Roche) under gentle agitation for 20 min at 0°C. In addition, the exposed RPE layer in the eyecup was incubated in PBS containing protease inhibitors for 20 min at 4°C. After incubation, both retina and RPE samples containing the soluble IPM proteins were pooled and centrifuged for 10 min (10 000 × *g*, 4°C). The protein-containing supernatant was then precipitated with acetone [27].

Alternatively, in order to prepare "soluble" IPM free of intracellular contamination, the cannulation method was applied as described previously [23, 24]. Briefly, after careful removal of vitreous, 0.4 mL of cold PBS containing protease inhibitors was introduced through the retina into the subretinal space with a syringe forming a bleb of fluid. After 30 s of incubation the fluid was withdrawn through the introductory puncture hole. Approximately 0.3 mL of PBS-IPM lavage could be recovered from each eye; lavage from several eyes was pooled, centrifuged for 10 min (10 000 × *g*, 4°C), and the protein-containing supernatant was then precipitated with acetone.

2.1.2 Preparation of "insoluble" IPM

The dissected retinal tissues remaining from the above procedure (conventional "soluble" IPM preparation) were once again rinsed for 2 min in PBS (4°C) and subsequently incu-

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bated for 10 min in water at 0°C. This hypotonic incubation frees the "insoluble" IPM matrix sheets from the outer retina. IPM matrix sheets in water were collected along with the surrounding water and subjected to acetone precipitation. For quality control of the IPM matrix sheets, aliquots of sheets were stained with alexa488-coupled lectin-peanut agglutinin (PNA; Molecular Probes).

2.2 Preparation of protein extract and 2-DE

Acetone precipitates of "soluble" IPM (both preparation methods) and "insoluble" IPM were centrifuged at 20 000 × *g* for 30 min and the protein pellets were dissolved directly in 2-DE lysis buffer (9 M urea, 2 M thiourea, 1% DTE, 4% CHAPS, and 2.5 μM EGTA and EDTA each) and protease inhibitors; protein content was determined using the Bradford assay (Bio-Rad).

Immobiline DryStrips (IPG, Pharmacia, pH 3–10 NL, 24 cm) were swollen overnight in lysis buffer containing a 110 μg protein sample and additional 1% pharmalyte pH 3–10 (Merck) as well as 0.5% bromophenol blue. IEF of the preswollen IPG strips was performed on a Multiphor (Amersham Biosciences) at 20°C for a total of 100 kVh. Focused IPG strips were loaded onto gradient SDS-PAGE gels (9–15%) and electrophoresed at constant 3 W *per* gel overnight (Ettan; Amersham Biosciences). Gels were silver stained as described elsewhere [28] and dried between cellophane sheets.

2.3 SDS-PAGE and Western blots

"Soluble" IPM, prepared using the cannulation method, and "insoluble" IPM protein extracts in 2-DE lysis buffer were additionally solubilized by four-fold dilution with buffer containing 1% SDS. Subsequently, 20 μg or 2 μg of "soluble" IPM, prepared with the cannulation method, was resolved by SDS-PAGE and gels were stained with 0.25% Coomassie Blue R-250 [24] or silver [28], respectively. Lectin PNA-binding proteins were detected on blots as described elsewhere [25], except that separated proteins were blotted onto PVDF membranes.

2.4 Preparation of samples for MS

Selected spots were excised from dried silver-stained gels, washed for 30 min in 100 μL nanopure water, destained [29], and dehydrated in 100 μL of 40% ACN (3 × 15 min). Samples were subjected to tryptic proteolysis in 5–10 μL 1 mM Tris-HCl pH 7.5 containing 0.01 μg/μL trypsin (Sequencing Grade Modified Trypsin; Promega) overnight at 37°C.

2.5 MS

MALDI-TOF peptide mass fingerprints were obtained on a Bruker Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany). Aliquots of each tryptic digest were cocrystallized with matrix consisting of 2,5-dihydroxybenzoic acid (Sigma) (20 mg/mL in 20% ACN, 0.1% TFA) and 2-hydroxy-5-methoxybenzoic acid (Fluka) (20 mg/mL in 20% ACN, 0.1% TFA) in a 9:1 ratio v/v on 400 μm AnchorChip™ targets (Bruker Daltonics). Peptide sequence information was obtained by LC-coupled MS/MS analysis on a Q-TOF2 system (Micromass, UK) coupled with a CapLC system (Micromass). Peptide samples subjected to tryptic digests were mixed with 10 μL of 5% formic acid and sonicated for 15 min. Aliquots of 10 μL each were loaded on the CapLC system, trapped on a C18 pre-column (5 μM, 100 PepMap-C18; LC Packings), and separated on a 75 μm C18 column (3-μm, 100 PepMap-C18; LC Packings) by elution with a gradient (5–60% solution B: 95% ACN, 0.1% formic acid).

2.6 Database searching

Database searches were performed using the MASCOT software [30] (http://matrixscience.com) at the following parameter settings: one miscleavage allowed, search restricted to database entries from metazoan; MALDI: 100 ppm mass accuracy; Q-TOF: 0.8 Da peptide tolerance; 0.2 Da MS/MS tolerance. Peptide masses of the tryptic digests were compared with the virtually generated tryptic peptide masses of the protein databases NCBInr and MSDB.

Signal peptide prediction was performed with the program SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/); predictions of proteins secreted *via* non-classical secretory pathway were performed with the program SecretomeP, version 1.0 (http://www.cbs.dtu.dk/services/SecretomeP/).

2.7 Immunohistochemistry

Porcine eyes were fixed in 10% formalin. For adequate and fast fixation, the cornea and the lens were removed and the aqueous humor was replaced by fixative. After dehydration, the eyes were embedded in paraffin.

Tissue sections were cut at 5 μm either vertically or transversally at the level of photoreceptors and mounted on coated slides (Super Frost Plus), deparaffinized, and rehydrated. High-temperature antigen retrieval was performed using 1 mM EDTA buffer pH 8.0 as described elsewhere [31]. Slides were rinsed in TBS-T (pH 7.3) and non-specific binding was blocked with horse serum with 1% BSA. Sections were then incubated with primary antibody (anti-GRP78; BD Transduction Laboratories, 10 μg/mL in TBS-T) overnight at 4°C in a humid chamber. Vertical sections were then incubated with alexa488 goat anti-mouse IgG conjugate for 2 h (Molecular Probes, 1:200) and mounted in FluorSave (Calbiochem). Transversal sections were incubated with secondary antibody anti-mouse IgG biotin (Linaris, Wertingen, Germany) for 1 h (1:1000 dilution), followed by incubation with peroxidase-conjugated streptavidin (Vectastain ABC-Elite:HRP kit; Linaris). Binding was visualized with the Vector HRP substrate kit VIP, which results in violet color (Linaris). Images were obtained with a Zeiss APO-TOM and a Zeiss AxioscopII.

3 Results

3.1 Quality control of "soluble" and "insoluble" IPM preparations

For the preparation of "soluble" IPM proteins, there exist two different strategies: one is based on incubation of dissected intact retina along with the remaining RPE surface with PBS in order to extract proteins that readily solubilize in an iso-osmotic salt solution [23]. This method results in high protein yields but may interfere with the integrity of the cells bordering the IPM *in vivo*. Thus, an alternative method [24] aimed at inducing minimal damage to tissues adjacent to the IPM was applied, namely, careful subretinal injection of PBS and retraction after a short incubation through the same puncture hole (cannulation method). In this method, tissue disruption can be reduced to the puncture hole. In order to verify that the quality of the IPM prepared by the cannulation method presented here is equal to the originally published protocol, we applied 20 μg of the protein extract obtained from respective preparations onto SDS-PAGE and stained with CBB similarly to the original publication [24]. Only one prominent band was detectable in this extract (Fig. 1A, lane 1), this being in line with the original publication. This band has been shown to be formed by IRBP [24], the dominant protein present in soluble IPM. A second faint band was detected at the size of approximately 70 kDa, which can be hypothesized to be albumin by alignment of the 1-D gel (Fig. 1A) with the identifications on the 2-D gel (spots 102 and 103 in Fig. 2). Recovering the IPM by cannulation and staining the corresponding 1-D gels by a 30-fold [32] more sensitive silver stain [28] however, reveals that apart from IRBP and albumin many other protein bands are present (Fig. 1A, lane 2).

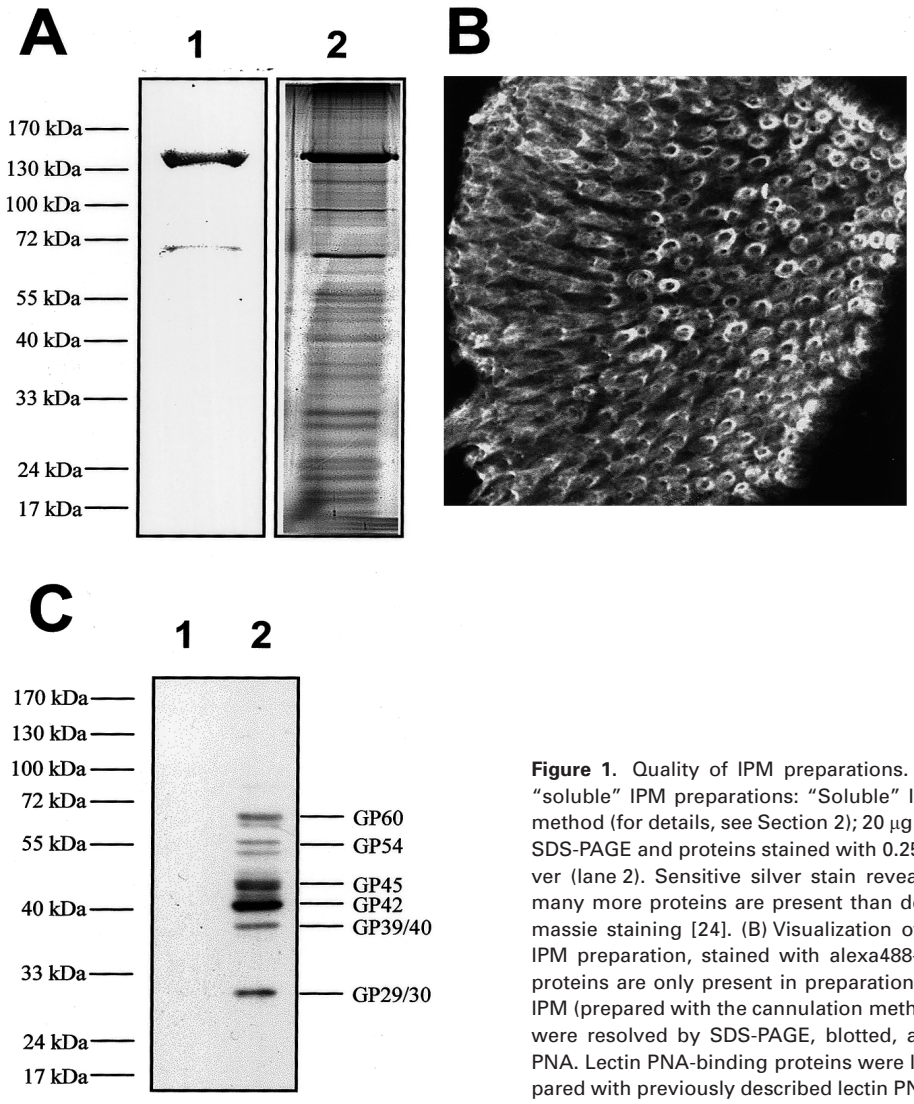

Figure 1. Quality of IPM preparations. (A) Detection of protein complexity in "soluble" IPM preparations: "Soluble" IPM was prepared with the cannulation method (for details, see Section 2); 20 μg (lane 1) or 2 μg (lane 2) was resolved by SDS-PAGE and proteins stained with 0.25% Coomassie Blue R-250 (lane 1) or silver (lane 2). Sensitive silver stain reveals that in "soluble" IPM preparations, many more proteins are present than detected with previously described Coomassie staining [24]. (B) Visualization of cone–matrix sheets from "insoluble" IPM preparation, stained with alexa488-coupled lectin PNA. (C) Lectin-binding proteins are only present in preparations of "insoluble" IPM: 2 μg of "soluble" IPM (prepared with the cannulation method; lane 1) and "insoluble" IPM (lane 2) were resolved by SDS-PAGE, blotted, and incubated with HRP-coupled lectin PNA. Lectin PNA-binding proteins were labeled according to their size and compared with previously described lectin PNA-binding glycoproteins.

"Insoluble" matrix sheets are prepared after PBS extraction of the "soluble" matrix by hypo-osmotic treatment of the retina. Cone matrix sheets swell and float off the retinal surface, and can be visualized by staining with fluorophore-coupled lectin PNA [25, 26]. Figure 1B shows an example of a cone matrix sheet present in preparations of "insoluble" IPM before being processed for 2-DE. To further confirm that preparations of "insoluble" matrix contain lectin-binding proteins, we performed SDS-PAGE of "insoluble" IPM and "soluble" IPM preparation followed by blotting and incubation with HRP-coupled lectin PNA. The resulting pattern of lectin PNA-binding bands (Fig. 1C, lane 2) appears highly similar to the pattern previously reported for porcine "insoluble" IPM [25], yielding the previously described glycoproteins GP29/30, GP39/40, GP42, GP45, GP54 and GP60. GP88, another previously described lectin PNA-binding protein, could only be detected after longer exposure of the film (data not shown). "Soluble" IPM preparation performed with the cannulation method did not show any lectin PNA-binding band (Fig. 1C, lane 1).

3.2 Proteins identified from 2-D gels of IPM preparations belong to miscellaneous functional networks

A total of 117 different proteins were identified from a total of 140 spots from "soluble" (Fig. 2) and "insoluble" IPM (Fig. 3) (a detailed list of all identifications with the respective significance is given as a supplementary table). Sixteen spots were found to include more than one protein species and 23 proteins were represented by more than one spot, indicating the existence of different isoforms and/or modifications. Gene ontology (GO) classifications allocated the identified proteins to different functional networks (Table 1), spanning the categories metabolism (27%), signaling protein (16%), chaperone (12%), protein turnover (9%), structural protein (8%), transport protein (8%), reactive oxygen metabolism (4%), Ca^{2+}-binding protein (4%), or vision (3%). Six proteins belonged to more than one category and 11 proteins, which could not be assigned to any of these categories, were thus designated as "other" (9%) (Fig. 4).

3.3 Comparison of "soluble" and "insoluble" IPM on the level of 2-D gel analysis

Comparison of "soluble" IPM prepared by cannulation and "insoluble" IPM components showed that, from a total of 117 different proteins, 42 and 43 proteins were enriched in "soluble" and "insoluble" IPM, respectively, and the remainder (32) were equally intense in both (Table 1).

Preparation of "insoluble" IPM by hypo-osmotic treatment predisposes the preparation to contamination with intracellular proteins due to cell disruption. Thus, proteins enriched in "insoluble" IPM may represent contaminants from adjacent cells (Table 1). Proteins enriched in "soluble" IPM prepared by cannulation compared to "insoluble" IPM (Table 1) are more likely to occur in the IPM compartment *in*

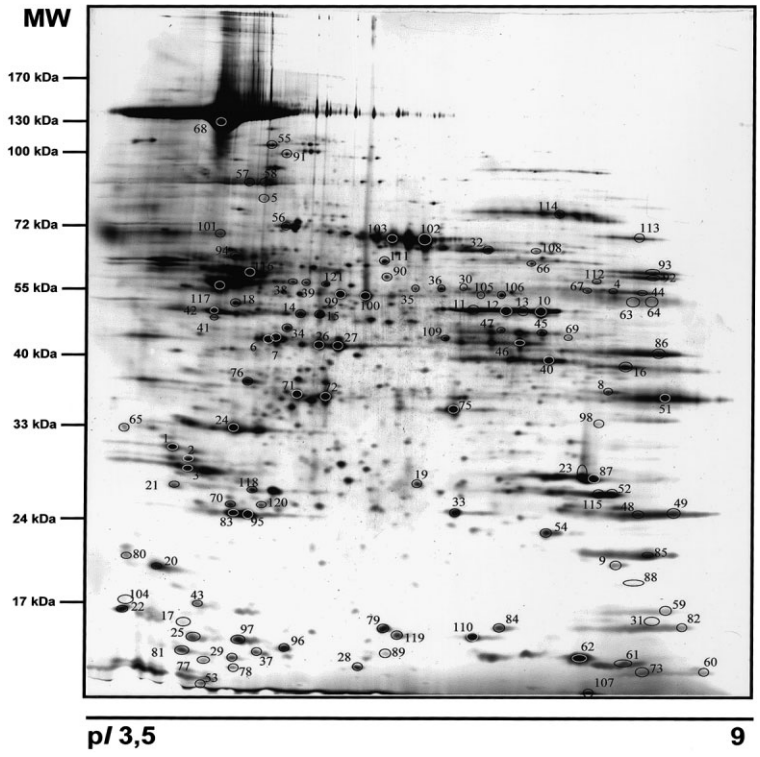

Figure 2. 2-DE analysis of "soluble" IPM prepared with the cannulation method. "Soluble" IPM was prepared with the cannulation method (for details, see Section 2). 2-DE was performed on precast IPG strips pH 3–10 NL (24 cm) with 110 μg protein load and focused for a total of 100 kVh to equilibrium. The strips were loaded on 9–15% SDS-PAGE and proteins were resolved at constant 3 W overnight. Proteins were visualized by silver staining. Spots which were excised and identified by MS are circled and numbered. These are also presented in Table 1 and in the supplementary table.

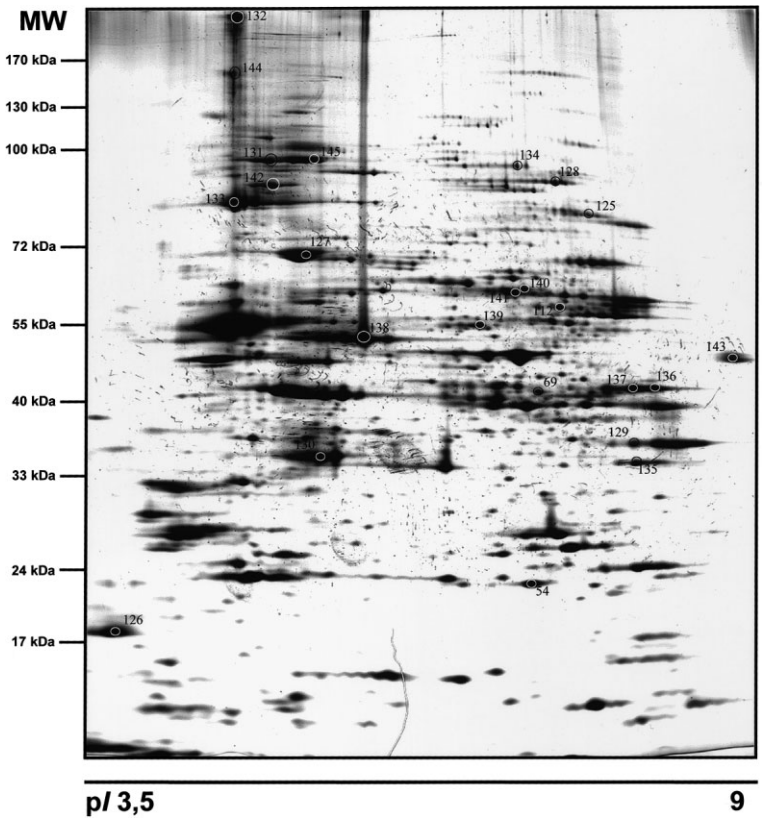

Figure 3. 2-DE analysis of "insoluble" IPM. "Insoluble" IPM sheets along with associated proteins were extracted from porcine retinae by hypo-osmotic treatment. 2-DE of this extract was performed as described in Fig. 2. Protein spots which were excised and identified by MS are circled and numbered; they are also presented in Table 1 and in the supplementary table.

Table 1. Proteins identified from the IPM

Identity	Accession ID	Spot ID gel	Enriched in soluble IPM	Enriched in insoluble IPM	SecretomeP score
Metabolism					
3-Phosphoglycerate dehydrogenase	Q8C603	4		x	0.531
Aconitate hydratase, mitochondrial, (EC 4.2.1.3)	ACON_PIG	125		x	0.361
Alcohol dehydrogenase (NADP+), EC 1.1.1.2	AKA1_PIG	8	x		0.511
Aldehyde dehydrogenase family 7 member A1, EC 1.2.1.3	D7A1_HUMAN	106	x		0.600
Alpha enolase, EC 4.2.1.11	ENOA_HUMAN	10, 11, 12, 13			0.492
Aspartate aminotransferase, cytoplasmic, EC 2.6.1.1	AATC_PIG	16	x		0.428
ATP synthase beta chain, mitochondrial, EC 3.6.3.14	ATPB_HUMAN	18			0.591
Carbonic anhydrase II, EC 4.2.1.1	CAH2_SHEEP	23			0.494
Carbonyl reductase (NADPH) 1, EC 1.1.1.184	DHCA_HUMAN	23			0.614
Creatine kinase, B chain, EC 2.7.3.2	KCRB_CANFA	6, 26, 27		x	0.260
Cytochrome *c* oxidase polypeptide Va EC 1.9.3.1	COXA_BOVIN	29			0.420
Fructose-bisphosphate aldolase C, EC 4.1.2.13	ALFC_HUMAN	40		x	0.312
Gamma enolase, EC 4.2.1.11, NSE	ENOG_MOUSE	41, 42		x	0.595
Glucose-6-phosphate isomerase, EC 5.3.1.9	G6PI_PIG	44		x	0.497
Glutamine synthetase, EC 6.3.1.2	GLNA_PIG	45, 46, 47			0.385
Glyceraldehyde 3-phosphate dehydrogenase, EC 1.2.1.12	G3P_PIG	51, 129	x		0.522
Guanylate kinase, EC 2.7.4.8	KGUA_PIG	54		x	0.829
Hexokinase, type I, EC 2.7.1.1	HXK1_MOUSE	134		x	0.352
Inosine-5′-monophosphate dehydrogenase 1, EC 1.1.1.205	IMD1_MOUSE	4, 66, 67			0.448
Isocitrate dehydrogenase (NADP) cytoplasmic, EC 1.1.1.42	IDHC_MICOH	69		x	0.532
Lactoylglutathione lyase, EC 4.4.1.5	LGUL_HUMAN	70		x	0.428
L-lactate dehydrogenase A chain EC 1.1.1.27	LDHA_PIG	135		x	0.625

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Table 1. Continued

Identity	Accession ID	Spot ID gel	Enriched in soluble IPM	Enriched in insoluble IPM	SecretomeP score
L-lactate dehydrogenase B chain	LDHB_PIG	71, 72, 130			0.607
Malate dehydrogenase, cytoplasmic, EC 1.1.1.37	MDHC_PIG	75			0.372
Phosphoglycerate kinase 1, EC 2.7.2.3	PGK1_HUMAN	86			0.398
Phosphoglycerate kinase EC 2.7.2.3	PGK_CRIGR	136, 137			0.411
Phosphoglycerate mutase 1, EC 5.4.2.1, EC 5.4.2.4	PMG1_HUMAN	87		x	0.381
Pyruvate kinase, M1 isozyme, EC 2.7.1.40	KPYM_FELCA	92			0.430
Pyruvate kinase, isozymes M1/M2, EC 2.7.1.40	KPYM_RAT	93			0.469
Succinyl-CoA ligase (ADP-forming) beta-chain, EC 6.2.1.5	SCB1_PIG	109	x		0.415
Triosephosphate isomerase EC 5.3.1.1., TIM	TPIS_HUMAN	52, 115			0.391
Vacuolar ATP synthase subunit B, brain isof. EC 3.6.3.14	VAB2_BOVIN	121	x		0.633
Transketolase EC 2.2.1.1	TKT_MOUSE	113		x	0.484
Signaling					
14-3-3 protein epsilon, Protein kinase C inhibitor protein-1	143E_HUMAN	1		x	0.330
14-3-3 protein gamma	143G_BOVIN	2		x	0.297
14-3-3 protein zeta/delta, Protein kinase C inhibitor protein-1	143Z_HUMAN	3		x	0.252
Astrocytic phosphoprotein PEA-15	PE15_MOUSE	17	x		0.898
Dihydropyrimidinase related protein-2, CRMP-2	DPY2_HUMAN	32	x		0.411
DJ-1 protein	Q99497	33			0.493
GTP-binding nuclear protein RAN, Ran GTPase	RAN_HUMAN	52			0.582
Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	GBB1_HUMAN	71, 130			0.420
Guanine nucleotide-binding protein G(T) gamma-T1 subunit	GBG1_BOVIN	53	x		0.645
Inhibitor-1 of protein phosphatase type 2A	Q8HY67	65			0.062
Nucleoside diphosphate kinase B, EC 2.7.4.6	NDKB_HUMAN	79		x	0.330
Parathymosin	THYP_BOVIN	80			0.669
Rab GDP dissociation inhibitor alpha, Rab GDI alpha	GDIA_RAT	94			0.441
Serine/threonine protein phosphatase 2A, 65 kDa reg. subunit A	2AAA_PIG	101			0.563
Calmodulin	CALM_HUMAN	22	x		0.706
Similar to calmodulin 2, Phosphorylase kinase, delta	Q9BRL5	104	x		0.738
Protein disulfide-isomerase, EC 5.3.4.1, ER60 precursor	PDA3_HUMAN	90	x		0.707
Retinoic acid-binding protein I, cellular, (CRABP-I)	RET3_HUMAN	96	x		0.696
Chaperone					
78 kDa glucose-regulated protein (Precursor), GRP 78	GR78_HUMAN	5	x		0.745
Alpha-B-crystallin chain	CRAB_HUMAN	9	x		0.864
dnaK-type molecular chaperone	HS7C_HUMAN	56, 127		x	0.229
Fascin, Singed-like protein, 55 kDa actin bundling protein	FSC1_HUMAN	30, 35, 36	x		0.390
FK506-binding protein 4, EC 5.2.1.8	FKB4_RABIT	38, 39			0.155
Heat shock 70 kDa protein 4	HS74_HUMAN	55,131		x	0.223
Heat shock protein HSP 90-alpha	HS9A_PIG	132, 133		x	0.173
Heat shock protein, HSP 90-alpha, HSP 86	HS9A_HUMAN	57, 58		x	0.172
Peptidylprolyl isomerase, EC 5.2.1.8 A	PPIA_BOVIN	82		x	0.338
Stress-induced phosphoprotein 1	IEFS_HUMAN	108		x	0.344
T-complex protein 1, alpha subunit A	TCP1_MOUSE	111			0.503
T-complex protein 1, beta subunit, TCP-1-beta	TCPB_HUMAN	139		x	0.446
T-complex protein 1, eta subunit, TCP-1-eta	TCPH_HUMAN	112		x	0.382
T-complex protein 1, gamma subunit, TCP-1-gamma	TCPG_HUMAN	140		x	0.484
T-complex protein 1, zeta subunit, TCP-1-zeta	TCPZ_RABIT	141		x	0.477
Transitional endoplasmic reticulum ATPase, TER ATPase	TERA_PIG	142		x	0.168
Protein turnover					
Cytosol aminopeptidase, EC 3.4.11.1	AMPL_BOVIN	30, 36	x		0.398
Elongation factor 2 (EF-2)	EF2_HUMAN	128		x	0.384
Eukaryotic initiation factor 4A-II, eIF4A-II, eIF-4A-II	IF42_MOUSE	34	x		0.494
Puromycin-sensitive aminopeptidase EC 3.4.11.	PSA_HUMAN	91	x		0.436
Similar to ubiquitin/ribosomal fusion protein (Fragment)	Q862M4	107	x		0.693
Translation elongation factor eEF-1 alpha-1 chain	EF11_HUMAN	143		x	0.155

Table 1. Continued

Identity	Accession ID	Spot ID gel	Enriched in soluble IPM	Enriched in insoluble IPM	SecretomeP score
Ubiquitin carboxyl-terminal hydrolase isozyme L1, EC 3.4.19.12	UBL1_MOUSE	118	x		0.540
Ubiquitin-activating enzyme E1	UBA1_HUMAN	145		x	0.530
Ubiquitin-conjugating enzyme E2 N EC 6.3.2.19	UBCN_HUMAN	119	x		0.730
Ubiquitin-conjugating enzyme E2-25 kDa	UBC1_HUMAN	120	x		0.627
Structure					
Actin, cytoplasmic type 5	ACT5_CHICK	6, 7		x	0.502
Coactosin-like protein	COAC_HUMAN	25	x		0.765
Cofilin, non-muscle isoform	COF1_MOUSE	59	x		0.629
Beta crystallin A3	CRBA_BOVIN	19	x		0.491
Destrin, actin-depolymerizing factor	DEST_HUMAN	31	x		0.622
Profilin II	PRO2_HUMAN	89			0.549
Tubulin alpha-1 chain	TBA1_HUMAN	116		x	0.472
Tubulin beta chain	TBB_PIG	15			0.526
Tubulin beta-2 chain	TBB2_HUMAN	117		x	0.501
Tubulin beta-4 chain	TBB4_MOUSE	14, 144		x	0.503
Protein and metabolite transport					
Apolipoprotein A-I (Precursor), Apo-AI	APA1_PIG	70	x		0.883
Cellular retinaldehyde-binding protein, CRALBP	CRAL_BOVIN	24	x		0.510
Fatty acid-binding protein, brain, B-FABP	FABB_HUMAN	37	x		0.450
Hemoglobin alpha chain	HBA_PIG	59, 60		x	0.348
Hemoglobin beta chain	HBB_PIG	61, 62			0.464
Phosphatidylethanolamine-binding protein PEBP	PEBP_HUMAN	85	x		0.654
Retinol-binding protein I, cellular; CRBP	RET1_BOVIN	97	x		0.381
Serum albumin (Precursor) (Fragment)	ALBU_PIG	102, 103	x		0.592
Transferrin, Serotransferrin	TRFE_PIG	114	x		0.379
Interphotoreceptor retinoid-binding protein (Precursor), IRBP	IRBP_BOVIN	68	x		0.569
Reactive oxygen metabolism/detoxification					
Glutathione *S*-transferase P, EC 2.5.1.18, GST class-pi	GTP_PIG	48, 49		x	0.338
Peroxiredoxin 2 (Fragment) EC 1.11.1	PDX2_PIG	83	x		0.629
Peroxiredoxin 5	Q9GLW8	84	x		0.888
Phospholipid hydroperoxide glutathione peroxidase, EC 1.11.1.9	GPX4_PIG	88			0.868
Superoxide dismutase (Cu-Zn) EC 1.15.1.1	SODC_PIG	110	x		0.549
Ca^{2+}-binding					
Calbindin, Vitamin D-dependent calcium-binding protein	CABV_HUMAN	21		x	0.402
Parvalbumin alpha	PRVA_MACFU	81			0.178
Recoverin, P26	RECO_BOVIN	83, 95		x	0.297
Vision					
Arrestin-C (ARR3 gene)	CAD92082	14, 15		x	0.501
S-arrestin, retinal S-antigen	ARRS_PIG	47, 99, 100, 138		x	0.375
Other					
Beta-synuclein	SYUB_HUMAN	20, 126		x	0.136
Cystatin B	CYTB_PIG	28			0.417
Gamma-synuclein, Synoretin	SYUG_HUMAN	43			0.110
Histidine triad nucleotide-binding protein 1	HNT1_MOUSE	62			0.790
Immunoglobulin gamma-chain	AAA51295	63, 64	x		0.801
Macrophage migration inhibitory factor (Fragment)	MIF_HUMAN	73	x		0.747
Mu crystallin	CRYM_RAT	76			0.619
Myotrophin	MTPN_HUMAN	77			0.330
Neuron-specific protein PEP-19	PE19_HUMAN	78	x		0.723
Ribose-phosphate pyrophosphokinase I, EC 2.7.6.1	KPR1_HUMAN	98		x	0.428
Selenium binding protein 1	Q96GX7	105, 106	x		0.496

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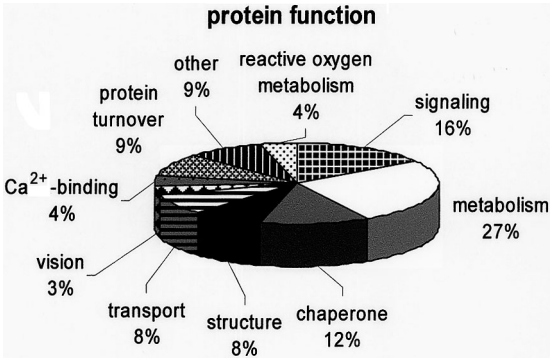

Figure 4. Allocation of all identified proteins from the IPM to functional groups.

situ, although intracellular contamination cannot be ruled out in this preparation method as well (see below). In order to further confirm that proteins enriched in "soluble IPM" are likely to occur in the IPM *in vivo*, we predicted the existence of a secretion-signal peptide with the program SignalP [33]. Only three proteins were predicted to carry a signal peptide for secretion: albumin, apolipoprotein A-I, and IRBP (Fig. 5A). Another two proteins, transferrin and macrophage migration inhibitory factor (MIF), were classified as extracellular according to GO. The major parts of the identified proteins, however, are listed as intracellular proteins or proteins with unknown cellular component. As MIF is one of only 13 proteins known to be secreted by an unconventional pathway without signal peptide [34, 35], we decided to predict the probability of all identified proteins occurring in an extracellular compartment with the program SecretomeP. This program was recently designed to predict secreted proteins without signal peptides, according to properties of the mature protein, such as number of positively charged residues, low complexity regions, number of atoms, and predicted propeptides [35]. We found that 49 of 117 proteins were predicted to be located extracellularly with a score above 0.5 (Fig. 5A and Table 1). The distribution of all scores (Fig. 5B) resembles a normal distribution (maximum frequency at 0.5) with no bias toward extracellular proteins. In order to verify our hypothesis that proteins enriched in "soluble" IPM preparations are most likely to occur in the IPM *in vivo* (see also above), the population of proteins was divided into three groups: those most prominent in "soluble" IPM, those most prominent in "insoluble" IPM, and the remainder not clearly assignable to either group (Table 1). The score distributions of those subsets clearly show a shift to higher scores for proteins enriched in "soluble" IPM with a maximum frequency at scores around 0.7 and lower scores (maximum frequency at scores around 0.4) for proteins enriched in "insoluble" IPM (Fig. 5c). This underlines the hypothesis that the subset of proteins enriched in "soluble" IPM preparations is actually present in the IPM *in vivo*.

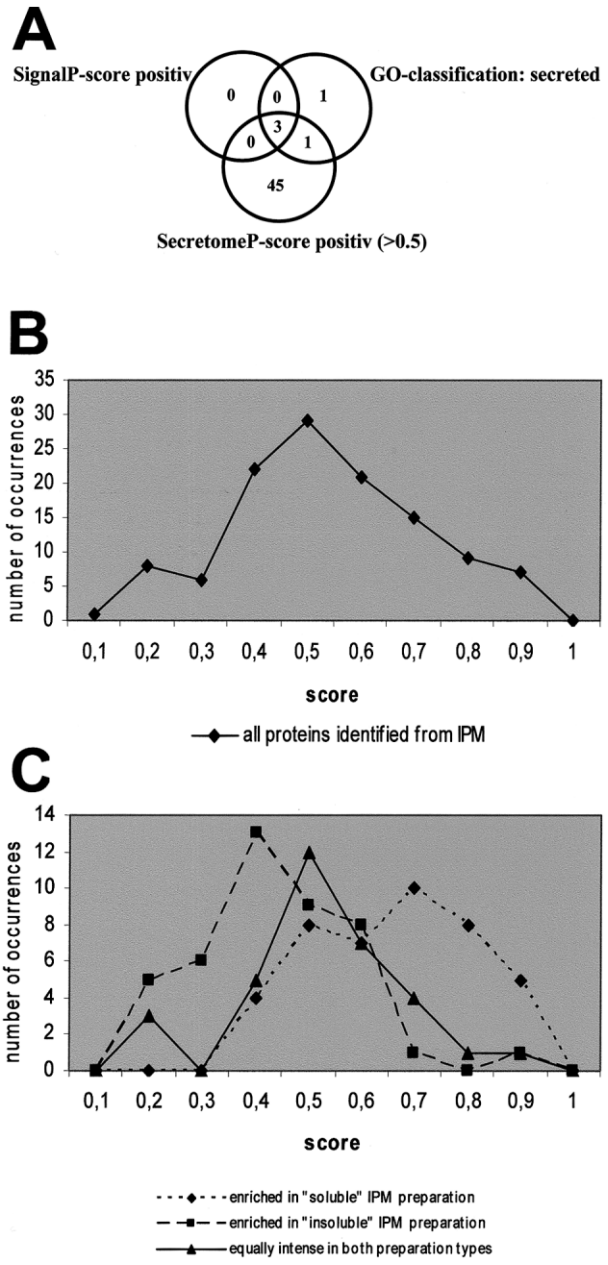

Figure 5. SignalP and SecretomeP score predictions for proteins identified from the IPM. (A) Three proteins identified from the IPM carry a signal peptide (SignalP-score positive), another two proteins are classified as being secreted according to gene ontology classification and 48 proteins are predicted to occur extracellularly according to SecretomeP (Secretome P-score positive, $0 < 0.5$). (B) Distribution of SecretomeP scores for all identified proteins peaks at 0.5. (C) Distribution of SecretomeP scores for different subsets of the identified proteins: proteins enriched in "soluble" IPM (dotted line with rhombus) have significantly higher SecretomeP scores (maximum frequency at 0.7) than proteins enriched in "insoluble" IPM (dashed line with rectangles, maximum frequency at 0.4). Distribution of SecretomeP scores for proteins found to be equally intense in both preparations (line with triangles) is similar to distribution of all proteins in (B) (maximum frequency 0.5).

3.4 The IPM contains potentially neuroprotective proteins

In order to extract from the large list of identified proteins those that most likely occur in the IPM *in vivo*, we selected for proteins that are (i) enriched in "soluble" IPM and (ii) are predicted to occur extracellularly with a very high SecretomeP score (>0.700). This filtering generated a subset of 13 proteins, 12 of them not previously linked to the IPM (Table 2). One protein from this subset, namely immunoglobulin, has been previously shown to be present in the IPM [2]. Seven of these proteins have been described in the literature to act in an anti-apoptotic or neuroprotective manner: namely, astrocytic phosphoprotein (PEA)-15 (score 0.898), peroxiredoxin 5 (score 0.888), alpha-B-crystallin chain (score 0.864), MIF (score 0.747), GRP78 (score 0.745), neuron-specific protein PEP-19 (score 0.723), and protein disulfide-isomerase ER60 (score 0.707).

Moreover, we could identify the cysteine protease inhibitor cystatin B for the first time in ocular tissue; however, the SecretomeP score for this protein is only 0.417. Cystatin B knock-out mice show neuronal atrophy and apoptosis [36].

3.5 GRP78 is located in the IPM on porcine eye sections

In order to verify the prediction method for extracellular localization of newly identified proteins in the IPM, we sectioned porcine eyes and stained the sections for GRP78. Special care was taken to preserve the fragile structure of the IPM and adjacent tissue. Sections were cut vertically and GRP78 localization was detected with fluorescent secondary antibody as shown in Fig. 6. GRP78 immunoreactivity was found in all layers of the retina and the RPE, which was in line with the ubiquitous expression of this protein. However, the strongest immunoreactivity on the sections was indeed found in the IPM. We further wanted to inquire whether GRP78 is localized extracellularly or within photoreceptors. As inner and outer segments of photoreceptors show high auto-fluorescence, GRP78 immunoreactivity on transversally cut sections was detected with a violet color reaction. Transversally cut photoreceptor segments (Fig. 6G, open arrow) show no immunoreactivity for GRP78 within the cells, whereas strong immunoreactivity was found between the segments (Fig. 6G, closed arrows), thus verifying the extracellular localization of GRP78 in the IPM.

4 Discussion

The aim of this study was to profile and characterize the IPM for previously unknown components and to elucidate potentially novel functions in this structure. We applied high-resolution 2-DE and subsequent MS identification to reveal the IPM composition in more detail than described so far.

The most noteworthy finding was the striking protein complexity of the IPM, as well as its high content of intracellular proteins, a finding that cannot be satisfactorily explained by contamination due to preparation alone. In our work, we compared "soluble" IPM samples prepared by conventional and cannulation methods, the latter designed to minimize cell disruption [23, 24]. Interestingly, using 2-DE analysis we found high proportions of intracellular proteins in samples derived from both preparation methods, although 1-D Western blots with a cellular RMG marker protein (glutamine-synthetase) and a photoreceptor marker protein (arrestin) did not reveal this contamination (data not shown). Thus, we show here that even the gentlest IPM preparation method leads to some disruption of adjacent cells with subsequent "spilling out" of intracellular proteins. Contamination with intracellular proteins also occurs in

Table 2. Proteins enriched in "soluble" IPM with SecretomeP scores above 0.7

Identity	Accession ID	SecretomeP score	GO classification entry: extracellular (*via* human ortholog, ensembl entry)
Astrocytic phosphoprotein PEA-15	PE15_MOUSE	0.898	No
Peroxiredoxin 5	Q9GLW8	0.888	No
Apolipoprotein A-I (Precursor), Apo-AI	APA1_PIG	0.883	GO:0005576 extracellular
Alpha-B-crystallin chain	CRAB_HUMAN	0.864	No
Immunoglobulin gamma-chain	AAA51295	0.801	No
Coactosin-like protein	COAC_HUMAN	0.765	No
Macrophage migration inhibitory factor (Fragment)	MIF_HUMAN	0.747	GO:0005615, extracellular space
78 kDa glucose-regulated protein (Precursor), GRP 78	GR78_HUMAN	0.745	No
Similar to calmodulin 2, Phosphorylase kinase, delta	Q9BRL5	0.738	No
Ubiquitin-conjugating enzyme E2 N EC 6.3.2.19	UBCN_HUMAN	0.730	No
Neuron-specific protein PEP-19	PE19_HUMAN	0.723	No
Protein disulfide-isomerase, EC 5.3.4.1, ER60 precursor	PDA3_HUMAN	0.707	No
Calmodulin	CALM_HUMAN	0.706	No

Proteomics 2005, *5,* 3623–3636

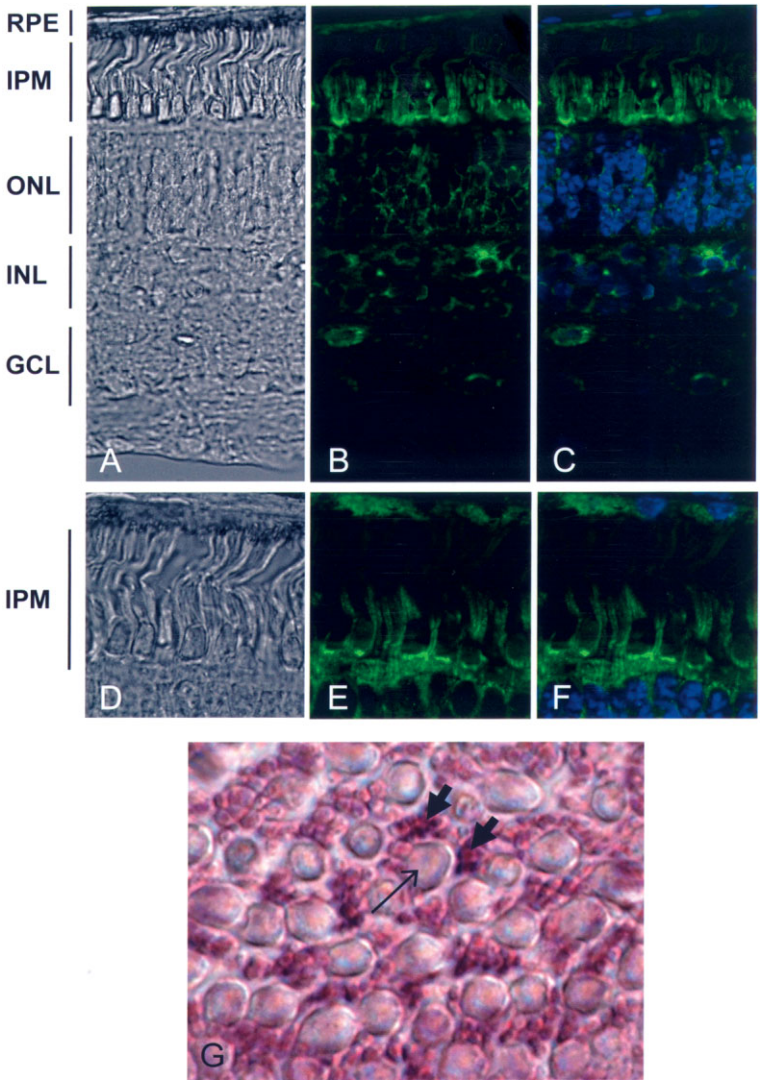

Figure 6. Immunohistochemical stainings for GRP78 on porcine eye sections. Sections (5 μm) of porcine eyes labeled with mouse anti-GRP78 antibody. (A–F) Vertical sections; (G) transversal section. GRP78 immunostaining (shown in green, panels B, C, E, F) is found in all layers of retina and RPE (overlay with DAPI-stained nuclei in panels C and F). Staining is most intense on the level of the IPM (compare to Nomarski image, panels A and D). (A–C) Magnification 400×; (D–F) magnified view (Adobe Photoshop) of corresponding sections. (G) Transversal section of porcine eye at the level of the IPM; GRP78 immunoreactivity is visualized by violet staining. Photoreceptor outer segments (open arrow) are free of staining, while outside of the photoreceptor segments (closed arrows) intense GRP78 immunoreactivity is observed (magnification 600×).

several other extracellular compartments, and has been discovered due to the increasing sensitivity of proteomics methods, *e.g.*, in plasma [37, 38], in cerebrospinal fluid [39], and in lymph [40]. Nevertheless, the high resolution of 2-DE allows for an extensive comparison of spot intensities between "soluble" and "insoluble" IPM preparations. Thus, the identified proteins can be grouped into subsets: one which contains proteins enriched in "soluble" IPM and another containing proteins enriched in "insoluble" IPM preparations. As the preparation method for "insoluble" IPM is comparatively harsh (with hypo-osmotic treatment of the dissected retina), proteins enriched in these preparations more likely derive from intracellular contamination. We then predicted the probability of every identified protein occurring in extracellular compartments with the prediction program SecretomeP [35]. The resultant score distribution for all proteins peaked at 0.5, whereas the score distribution of the two subsets of proteins showed a clear separation of maxima. Proteins enriched in "soluble" IPM have significantly higher scores than proteins enriched in "insoluble" IPM preparations and are thus likely to be present in the IPM *in vivo*.

We verified that one of the proteins enriched in "soluble" IPM and carrying a high SecretomeP score (0.745), namely GRP78, is indeed localized to the IPM *in vivo* by means of immunostaining on sections of porcine eyes. GRP78 is a constitutively expressed resident protein of the endoplasmic reticulum (ER) of all eukaryotic cells and is involved in polypeptide translocation, protein folding, and presumably protein degradation (for review, see [41]). However, there has been one report so far on GRP78 being secreted from intact cells into culture medium [42], so that, together with our results, an extracellular occurrence can be assumed. This significantly underlines the fact that the applied filter method can correctly predict protein localization in the IPM *in vivo*.

Within the subset of proteins enriched in "soluble" IPM are proteins with very high probability scores for an extracellular location. One of them is MIF, which was identified here for the first time from IPM preparations. MIF is one of very few proteins already known to be secreted by a non-classical secretory pathway [34, 35]. The existence of MIF in "soluble" IPM preparations indicates that proteins might be shuttled into this compartment not only *via* the classical but also *via* a non-classical secretory pathway.

The protein with the highest SecretomeP score (0.898) was astrocytic phosphoprotein PEA-15, although so far PEA-15 was only known to be located intracellularly. However, the enrichment of PEA-15 in "soluble" IPM, together with the high SecretomeP score, suggests that this might be a candidate for a non-classically secreted protein. PEA-15 was first identified as an abundant phosphoprotein in brain astrocytes and subsequently found to be widely expressed in different tissues, including retinal Mueller glia [43]. PEA-15 is regulated by multiple calcium-dependent phosphorylation pathways that account for its differently phosphorylated forms [44–47]. In our IPM 2-DE proteome map, the p*I* of the spot identified as PEA-15 was approximately 4 and might thus represent this protein in a phosphorylated form. Phosphorylated PEA-15 inhibits TNF-alpha-induced apoptosis in astrocytes in a PKC-dependent manner [48–50]. Most interestingly, PEA-15 levels are lower in proliferating than in non-proliferating astrocytes *in vitro* [43] and PEA-15 knock-out mice exhibit a marked increase in cell proliferation in several lineages, including astrocytes, lymphocytes, and hepatocytes [51]. Although PEA-15 function in the IPM remains highly speculative, one could expect that increased expression of this protein would hinder retinal and RPE cell proliferation, which would be deleterious to retinal function. Thus, PEA-15 may be a candidate for therapeutic intervention in proliferative vitreo-retinopathy.

The second highest SecretomeP score was found for peroxiredoxin 5 (score 0.888), the most recently identified member of the peroxiredoxin family [52]. It has been shown to protect cells from reactive oxygen-induced apoptosis [53] and protects *Xenopus* embryos from alcohol-induced anomalies [54]. Peroxiredoxin 5 has been described to be intracellularly localized to mitochondria, peroxisomes, the cytosol, and, to a lesser extent, the nucleus [55], but its finding in the IPM is the first indication of an extracellular localization.

Alpha-B-crystallin (SecretomeP score 0.864), a member of the small HSP family identified here from the IPM, has been shown to protect cultured cells from apoptosis induced by staurosporine [56], okadaic acid [57], UVA irradiation [58], TNF-alpha and Fas [56], and oxidative stress [59]. Elevated levels of alpha-B-crystallin have been described in several neurodegenerative diseases (for review, see [60]). In the rd-mouse, an animal model for inherited retinal degeneration, photoreceptor death is accompanied by elevated alpha-B-crystallin in retinal glial cells and photoreceptors [61]. Identification of alpha-B-crystallin along with many other HSPs in the IPM might implicate its potential involvement in anti-apoptotic processes and in prevention from neurodegeneration in diseased retinae.

PEP-19 (SecretomeP score 0.723), together with neuromodulin (GAP-43) and neurogranin (RC3), belongs to the neuron-specific calmodulin-binding proteins. All three have been proposed to play a role in calcium homeostasis by regulating calmodulin. In contrast to GAP-43 and RC3, PEP-19 is not inactivated by phosphorylation through PKC, and should thus remain an active calmodulin regulator even under excitotoxic conditions. In this respect, PEP-19 expression might lead to an enhanced survival of neurons exposed to insults associated with the pathophysiology of neurodegenerative processes.

In this study, many new compounds of "soluble" IPM were identified, and a noticeable number of them can be linked to neuroprotective mechanisms. Further, the combination of high-resolution protein separation methods in conjunction with computational prediction methods allowed us to overcome the unresolved problem of intracellular contamination in preparations of this fragile extracellular compartment.

However, there are clearly limitations within the applied methods. None of the proteoglycans known to build together with HA the scaffold of solid IPM sheets, was identified on 2-DE gels of "insoluble" IPM, although we showed that PNA-binding proteins were detectable on 1-D gels of these preparations. IEF in combination with SDS-PAGE might selectively exclude these large scaffold proteins and favor the contaminating intracellular proteins. Additionally, PEDF, a potent neuroprotective factor expressed by retinal pigment epithelium and secreted into the IPM (reviewed in [62]), was not among the selected candidates for identification. PEDF is known to bind tightly to the scaffold proteins of insoluble IPM [6] and might thus be retained from separation. For further investigation of the composition of insoluble IPM matrix sheets, it will be necessary to apply different strategies additionally to those described here.

Future studies are needed to validate the proper localization of all the newly identified IPM components as true components of the IPM *in situ*. However, the current panel of constituents and especially the new candidate components of the IPM strengthen the view that this acellular retinal compartment serves diverse functions. Besides inhibiting neoangiogenesis, it appears likely that the IPM actively protects photoreceptors from metabolic stress. In addition, it seems to provide yet unknown anti-apoptotic signals by neuroactive molecules that have not yet been characterized in detail with respect to their function in the retina.

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INTERACTION PROTEOME, LSHG-CT-2003-505520; and by funding from the German Federal Ministry of Education and Research BMBF-Proteomics 031U108A/031U208A.

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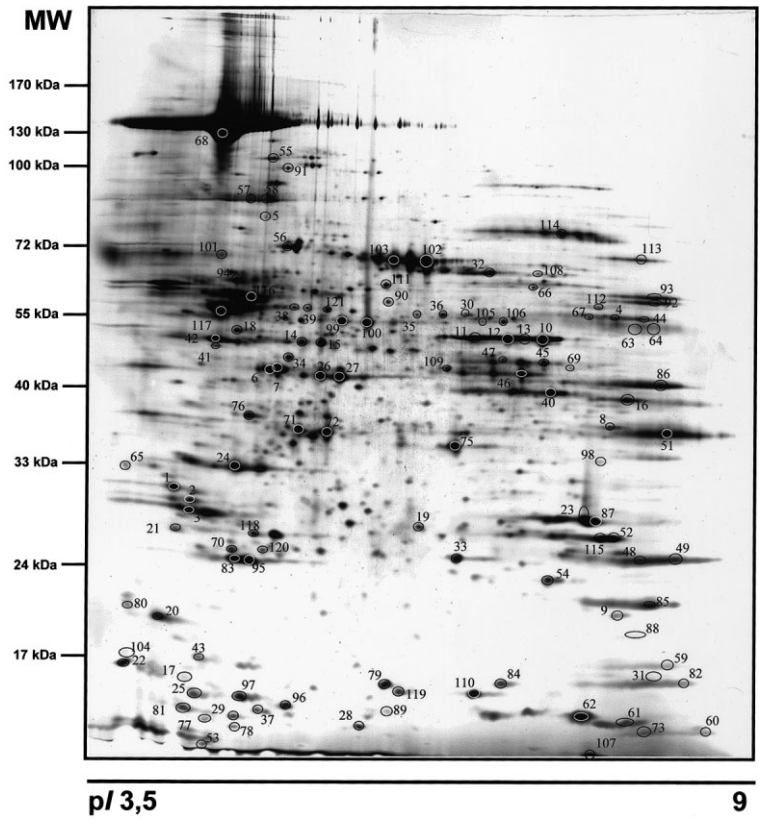
Proteomics 2005, *5,* 4637

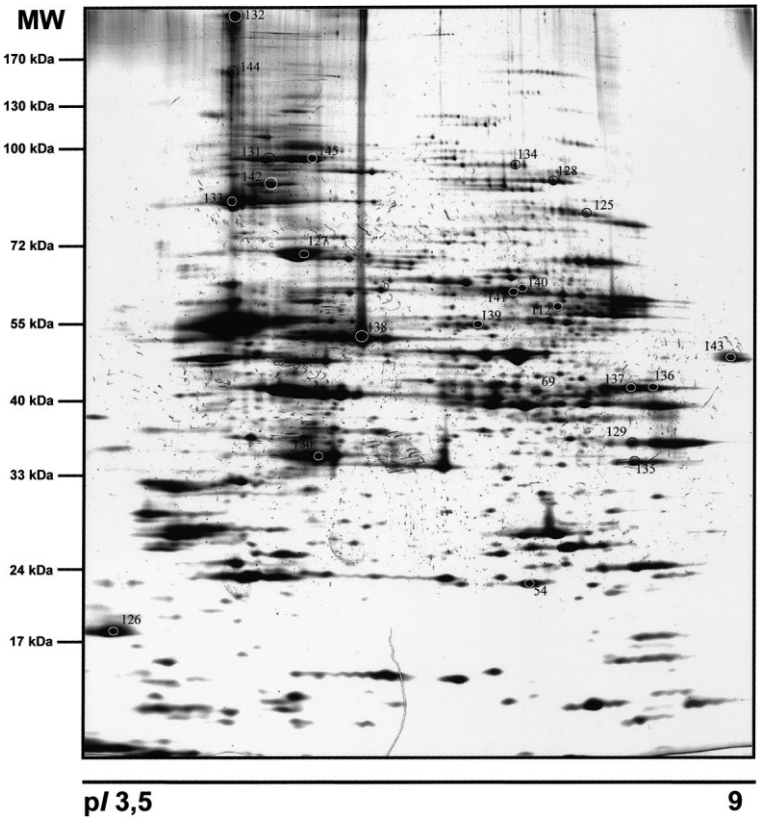
Erratum

Proteomic analysis of the porcine interphotoreceptor matrix

Stefanie M. Hauck *et al.*, vol. 5, issue 14, pp. 3623–3636
DOI 10.1002/pmic.200401223

The gels in figures 2 and 3 were been reproduced in different sizes so that a direct comparsion between them was not possible. Below are the correctly sized figures.





APPENDIX

A *Curriculum vitae*

Stefanie Monika Hauck
geboren am 30. Mai 1965
verheiratet, zwei Kinder

Schul- und Berufsausbildung

1971-1975	Grundschule Germering
1975-1981	Max-Born Gymnasium Germering
1981-1983	staatliche Berufsfachschule für MTLA, München
1988-1991	zweiter Bildungsweg: Münchenkolleg
1991	Abitur

Hochschulstudium

1992-1995	Studium der Biologie an der Universität Regensburg
1995-1999	Studium der Biologie an der Ludwig-Maximilians-Universität München; Abschluss: Diplom
1999	Diplomarbeit an der LMU München, Fakultät Biologie, PD Dr. L. Eichacker: „2D-Elektrophorese und massenspektrometrische Identifikation von plastidären Proteinen der Gerste (Hordeum vulgare)

Forschung

1983-1988	MTLA im Institut für Physiologische Chemie, Prof. Krietsch
2000-2001	Diplom-Biologin am Institut für Humangenetik, Klinische Kooperationsgruppe Ophthalmogenetik, Dr. Ueffing
seit 2001	Promotion am Institut für Humangenetik, Dr. Ueffing „Neuroprotective pathways in the retina: analysis of GDNF-mediated signalling in retinal Mueller glial cells (RMG) and screening for RMG-derived neurotrophic factors”

B *Presentations and other publications*

Stefanie M. Hauck, Sabine Suppmann and Marius Ueffing
"Proteome Analysis of Retinal Mueller Glia" Leipzig, February 2002, invited by Prof. A. Reichenbach (oral presentation)

Stefanie M. Hauck, Sabine Suppmann, Thomas Meitinger and Marius Ueffing
"Secreted Proteins from Mueller Glia and Photoreceptors: Candidates for Cell to Cell Communication" [ARVO abstract 2002, Nr. 3623] (poster presentation)

Sabine Suppmann, Hans Zischka, Jürgen Schoch, **Stefanie M. Hauck**, Thomas Meitinger and Marius Ueffing
"A high resolution protein map of human neural retina in comparison to murine, bovine and avian retinae" [ARVO abstract, 2002, Nr. 3632] (poster presentation)

Stefanie M Hauck, Sabine Suppmann and Marius Ueffing
"Proteomic profiling of primary retinal mueller glia cells reveals a shift in expression patterns upon adaptation to *in vitro* conditions" [ARVO abstract, 2003, Nr. 4561] (poster presentation)

Stefanie M. Hauck, Sabine Suppmann, Per Ekström, Poonam Ajuha, Theo van Veen and Marius Ueffing
"Comparative Proteome Analysis of *rd* versus *wt* Retina" BMBF-Proteomics-meeting, München, January 2004 (oral presentation)

Stefanie M. Hauck, Johannes Gloeckner, Claudia Alge and Marius Ueffing
"Retinal degeneration – investigating related molecular processes" PRORET-meeting, Lund, March 2004 (oral presentation)

Stefanie M. Hauck, Margaret Harley, Stephanie Schöffmann, Johannes Gloeckner and Marius Ueffing
"Secreted proteins from retinal Mueller glia enhance photoreceptor survival: an approach to detection of new candidates for neuroprotection" [ARVO abstract, 2004, Nr. 849] (poster presentation)

Francois Paquet-Durand, Seifolla Azadi, **Stefanie M. Hauck**, Marius Ueffing, N. Chadderton, Pete Humphries, Theo van Veen and Per Ekström
"Calpain-like activity correlates with photoreceptor cell death in the rd1 mouse" [ARVO abstract, 2005, Nr. 1664] (poster presentation)

Stefanie M. Hauck, Norbert Kinkl, Stephanie Schöffmann and Marius Ueffing
"GDNF-induced signalling in retinal glial cells" Pro Retina Research-colloquium, Potsdam, April 2005 (poster presentation)

other publications:

Potten H, Jendraschak E, **Hauck S**, Amar LC, Avner P, Mullhofer G
"Molecular cloning and sequencing of a murine pgk-1 pseudogene family" Gene 1988 Nov 30; 71(2):461-71

Alge CS, Suppmann S, Priglinger SG, Neubauer AS, May CA, **Hauck S**, Welge-Lussen U, Ueffing M, Kampik A
"Comparative proteome analysis of native differentiated and cultured dedifferentiated human RPE cells" Invest Ophthalmol Vis Sci. 2003 Aug; 44(8):3629-41

Sabine Suppmann, Jürgen Schoch, Magdalena Swiatek-deLange, **Stefanie M Hauck**, Hans Zischka and Marius Ueffing
"Interspecies comparison of retinal proteome profiles reveals major differences and significant discrepancies to predicted protein expression levels" (manuscript, submitted)

Stefanie M. Hauck, Per Ekström, Poonam Ahuja-Jensen, Sabine Suppmann, Froncois Paquet-Durand Theo van Veen and Marius Ueffing
"Differential Modification of Phosducin Protein in Degenerating rd1 Retina is Associated with Constitutively Active CaMKII in Rod Outer Segments" Mol Cell Proteomics. 2005 epub Oct 26

C *Acknowledgements*

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My special thanks to Dr. Sabine Suppmann for convincing me to proceed to the dissertation, very constructive and inspiring two years of collaboration and two very adventurous trips to the ARVO conference in Florida.

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I want to thank all my colleagues for a very inspiring and amicable working atmosphere, namely, Dr. Magdalena Swiatek-deLange for another two trips to Florida and for her straight-forward and uncomplicated character which enables so much progress in joint-projects; Dr. Norbert Kinkl, for being so different to me and thus being so precious as a discussion partner, Dr. Johannes Glöckner for never-ending support on computer matters and on Q-TOF, Margaret Harley for her work during her trainee period in our lab, Monika Beer, Ralf Braun, Andrea Meixner, Dr. Hans Zischka and Dr. Matthias Bauer for numerous discussions and conversations.

Further, I want to thank Dr. Ursula Olazabal for her endless support with bureaucratic matters, English language editing of my manuscripts and posters and for introducing me to “Salsa in Florida”.

I had and still have a very inspiring cooperation with Dr. Cornelia Deeg from the Department of Veterinary Medicine and I want to thank her for the many joint-projects we started and for her major input to improving my immunohistochemical techniques. Last but not least, she accelerated me to finishing this thesis and sponsored my trip to ARVO conference 2005!

I want to thank PD Dr. Lutz Eichacker for his willingness to support this thesis, which is outside of the scope of his work and for his constructive discussions.

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All the other members of the Institute for answering numerous questions.

Finally, I want to thank my husband for his endless support, tolerance and belief in my abilities;
and my children for enduring my absence and still thinking that I am a wonderful mother!

To my father, in memory of my mother

PAPER 1	"Proteomic Profiling of Primary Retinal Müller Glia Cells Reveals a Shift in Expression Patterns Upon Adaptation to In Vitro Conditions" GLIA 44:251-263 (2003)		
Autoren:	**Stefanie M. Hauck**	**Sabine Suppmann**	**Marius Ueffing**
Projektmanagment	80%	10%	10%
praktische Arbeiten	100%		
Manuskripterstellung	90%		10%
wissenschaftliche Leitung und Konzeptplanung	20%		80%
Bestätigung durch Unterschrift			

PAPER 2	"GDNF family ligands trigger paracrine neuroprotective signalling in retinal glial cells" submitted					
Autoren:	**Stefanie M. Hauck**	**Norbert Kinkl**	**Cornelia Deeg**	**Magdalena Swiatek-DeLange**	**Stephanie Schöffmann**	**Marius Ueffing**
Projektmanagment	75%					25%
praktische Arbeiten	50%		5%	5%	40%	
Manuskripterstellung	80%	10%				10%
wissenschaftliche Leitung und Konzeptplanung	10%					90%
Bestätigung durch Unterschrift						

PAPER 3	"Secreted Proteins from Retinal Mueller Glia Enhance Photoreceptor Survival: an Approach to Detection of New Candidates for Neuroprotection" submitted				
Autoren:	**Stefanie M. Hauck**	**Johannes Glöckner**	**Margaret Harley**	**Stephanie Schöffmann**	**Marius Ueffing**
Projektmanagment	80%	10%			10%
praktische Arbeiten	35%	5%	20%	40%	
Manuskripterstellung	85%	5%			10%
wissenschaftliche Leitung und Konzeptplanung	10%				90%
Bestätigung durch Unterschrift					

PAPER 4	"Proteomic Analysis of Porcine Interphotoreceptor Matrix" Proteomics. 2005 Sep;5(14):3623-36					
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