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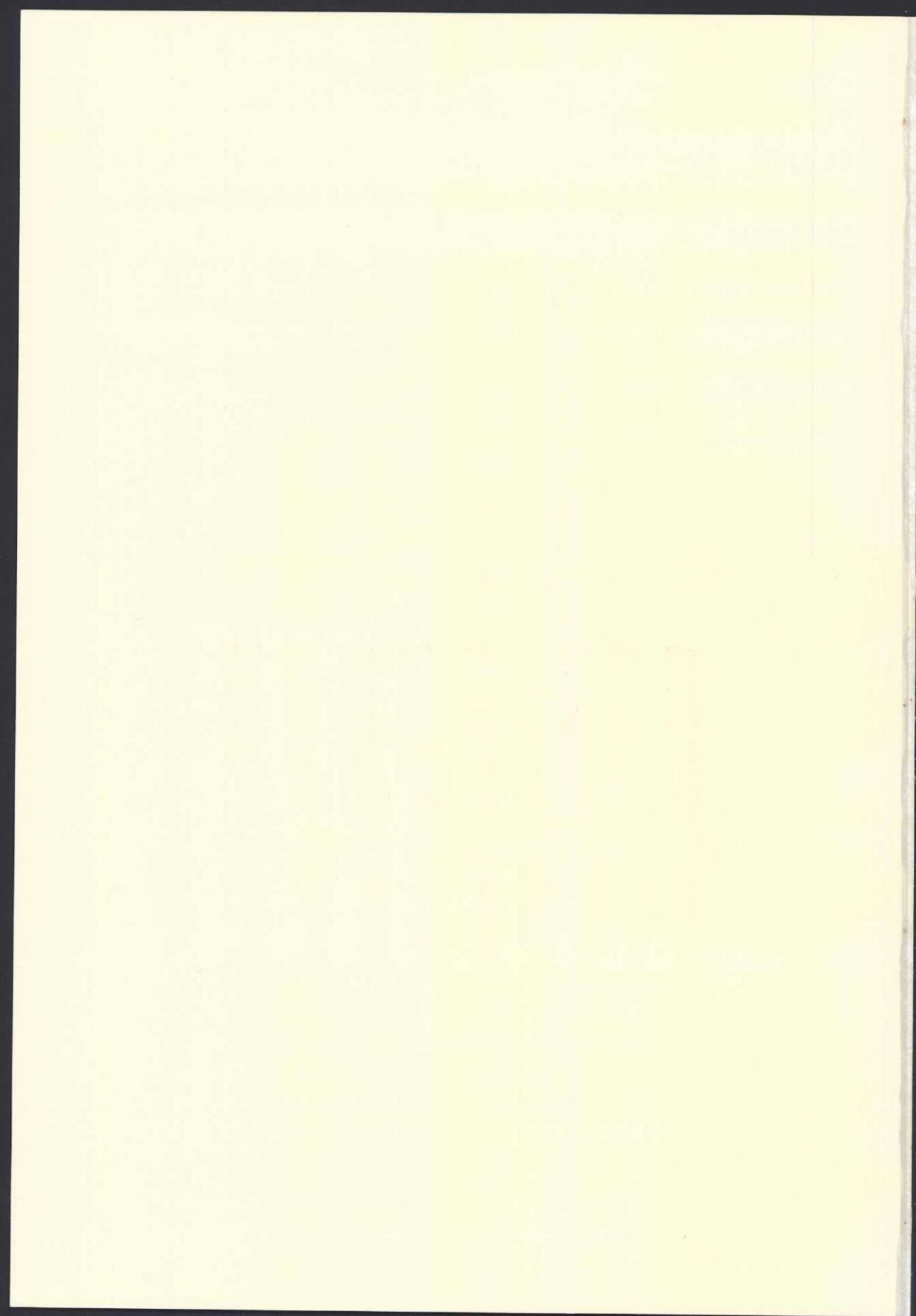
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Photoreceptor development and
degeneration in retinal organ culture
Effects of neurotrophic factors

Annika Söderpalm

Göteborg University 1999



Göteborg University
Faculty of Natural Sciences

**Photoreceptor development and degeneration in retinal
organ culture
Effects of neurotrophic factors**

Annika Söderpalm

Akademisk avhandling

för filosofie doktorexamen i zoomorfologi, som enligt beslut av ordföranden i
beredningsgruppen för biologi
kommer att försvaras offentligt fredagen den 24 september 1999, kl 10:00 i föreläsningssalen,
Zoologiska institutionen, Medicinaregatan 18, Göteborg
Fakultetsopponent är Dr Lyn Beazley, Professor of Zoology, Zoology department, the
University of Western Australia, Nedlands, Western Australia.

Göteborg 1999



ISBN 91-628-3723-0

Photoreceptor development and degeneration in retinal organ culture. Effects of neurotrophic factors.

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Key words: retina, retinoic acid, 9CRA, ATRA, apoptosis, caspase, organ culture, CNTF, BDNF, retinal pigment epithelium, TUNEL.

Abstract

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The cells differentiated and expressed cell-specific markers in the explants and irrespective of the presence of RPE all retinal layers developed. Both rods and cones developed and expressed visual pigment, with the exception of the middle-wave sensitive cone pigment. Due to optic nerve transection there was a high number of apoptotic cells in the ganglion cell layer during the first postnatal week. In addition, caspase-3, -8 and -9 activity was significantly elevated in explants 18 hours after explantation compared to age-matched retinas *in vivo* that had low steady-state levels of caspase-3, -8 and -9 activity throughout the experimental period.

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CNTF had a slight protective effect on photoreceptors in the *rd* retinal explants. This effect was profoundly increased by combining it with BDNF. Neither Cyclosporin A nor DEVD-fmk had any effect on cell survival in the *rd* retina.

The main conclusions of this study were that retinoic acid induced rod-selective apoptosis in neonatal mouse retina with RPE attached, and that caspase-3 and -9 were involved in this, effect whereas in explants without RPE it advanced opsin expression. CNTF and BDNF in combination had a better survival promoting effect on the *rd* retina than these substances administered alone.

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This thesis is based on the following manuscripts, referred to by their roman numerals in the text:

I. Selective development of one cone photoreceptor type in retinal organ culture. A. Söderpalm, A. Szél, A. R. Caffé, T. van Veen. *Investigative Ophthalmology and Visual Science* 1994; 35: 3910-3921.

II. Effect of survival factors and RPE proximity on rod photoreceptor rescue and differentiation. A. R. Caffé, A. K. Söderpalm, I. Holmqvist, and T. van Veen. Submitted to *Investigative Ophthalmology and Visual Science*.

III. Retinoic Acid Produces Rod Photoreceptor Selective Apoptosis in the Developing Mammalian Retina. A. Söderpalm, D. A. Fox, J.- O. Karlsson, T. van Veen. In press. *Investigative Ophthalmology and Visual Science*.

IV. 9-cis Retinoic Acid in Combination with Retinal Pigment Epithelium Induces Apoptosis in Cultured Retinal Explants Only During Early Postnatal Development. A. K. Söderpalm, J.- O. Karlsson, A. R. Caffé, T. van Veen. Submitted to *Developmental Brain Research*.

V. 9-cis Retinoic Acid Activates Caspase-3 and Caspase-9 in Cultured Neonatal Mouse Retina. A. K. Söderpalm, J.- O. Karlsson, D. A. Fox, T. van Veen. Submitted to *Experimental Eye Research*.

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I. Introduction

1. Retinal development and organization

The retina is the part of the eye where light-detection occurs, and where the first processing of the visual information takes place. It is a two-layered structure developed from lateral outgrowths of the forebrain vesicle. The outer layer is the retinal pigment epithelium (RPE) and the inner layer is the multi-layered neural retina that contains the photoreceptors and four other neuronal cell types: the bipolar, horizontal, amacrine and ganglion cell types. Apart from neuronal cells the retina also contains various glial cells of which the Müller cells are dominating.

1.1 Proliferation of retinal cells

Several developmental processes including proliferation, migration, differentiation and apoptosis, or programmed cell death (PCD) form the neural retina in the mouse retina. Proliferation starts in the embryonal period and continues after birth. Cells undergoing mitosis are found from embryonic day 11 until postnatal day 11 (PN11) (Sidman, 1961; Young, 1985). During proliferation of the retina the cells move back and fourth through the undifferentiated layers of the retina and all the cell divisions take place at the ventricular surface of the retina. When a cell has divided it can either migrate to its final destination and differentiate, or it can migrate towards the vitreal surface of the retina and start DNA synthesis for another mitotic division once back at the ventricular surface. The retina matures from the centre to the periphery. The first cells to stop dividing become ganglion cells, amacrine cells and cone photoreceptor cells, whereas bipolar cells and rod photoreceptor cells keep going through several additional rounds of mitosis before they migrate to the inner nuclear (INL) and outer nuclear layers (ONL) respectively, and differentiate. Figure 1.

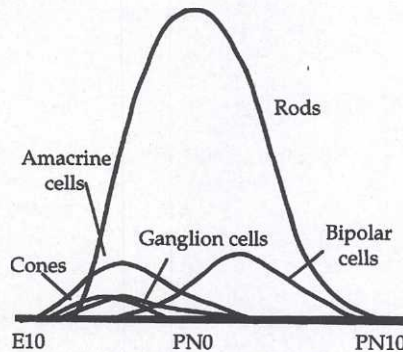


Figure 1. Differentiation of cell types in the mouse retina. Modified from Young, 1985.

At the time of birth the neural retina consists of a ganglion cell layer (GCL), separated by the inner plexiform layer (IPL) from the rest of the retina which at this stage is a thick layer of undifferentiated cells. As the majority of photoreceptors and bipolar cells reach their final destinations the outer plexiform layer (OPL) becomes visible on PN4-5. The ONL and the INL are fully separated on PN8, and at this age the retina contains all the layers of the adult retina.

1.2 Photoreceptor development

The cones are all formed before birth, whereas the rods are formed over a longer period of time. Only about half of the rods are generated during embryonic development. The number of cones reaching final mitosis in the central retina reach a peak on embryonal day 13-14, and in the periphery on embryonal day 15 (Carter-Dawson and LaVail, 1979b). Rods, however, are generated from embryonic day 13 until PN5 in the central retina and PN9 in the periphery (Young, 1985; Carter-Dawson and LaVail, 1979b). The number of rods generated reach a peak at PN0 in the centre and at PN0-4 in the periphery (Carter-Dawson and LaVail, 1979b). The discs of the outer segments (OS) start to form on PN5-6 (Olney, 1968; Sanyal and Bal, 1973). Despite the fact that cones become postmitotic earlier than the rods they do not start to express photopigment until PN4 whereas opsin expression in rods can be detected as early as PN3 (Szél, et al., 1993a).

Approximately 3% of the photoreceptors in the mouse retina are cones. The mouse retina has two cone types that express photopigments with different wave-length sensitivities. The M-cones are sensitive to light of middle-long wavelengths (green light) and comprise the majority of cones, whereas the S-cones that are sensitive to light of short wave-lengths (blue or UV-light) only represent about 10% of the cones (Jacobs, et al., 1991; Szél, et al., 1993b; Szél, et al., 1993a). The two cone types are topographically separated in the mouse retina. The M-cones exclusively occupy the dorsal part of the retina, whereas the S-cones are found all over the retina but with a much higher density in the ventral part. The border between the two cone fields is a slightly sloping line from the dorsal nasal quadrant towards the ventral temporal quadrant (Szél, et al., 1992; Calderone and Jacobs, 1995).

1.3 The retinal pigment epithelium

The outer layer of the retina, the RPE, is a single layer of melanin containing cells that interdigit the photoreceptor outer segments giving them mechanical and nutritional support (Berman, et al., 1974; Carter-Dawson and LaVail, 1979a). Together with choroidal endothelial cells the RPE also constitute a basement membrane, Bruch's membrane, which provides the blood-retina barrier. The RPE is crucial to normal development (Raymond and Jackson, 1995) and function of the retina. Several candidate substances for the trophic effects are synthesized by the cells of the RPE (Hollyfield and Witkowsky, 1974; Spoerri, et al., 1988; Pepperberg, et al., 1991; Tombran-Tink, et al., 1991; Sheedlo, et al., 1998).

1.4 Photoreceptor development and degeneration in the rd mouse

In addition to studying normal retinal development, development of the retina in the retinal degeneration (*rd*) mouse was studied in this thesis. The *rd* mouse has a selective degeneration of photoreceptors (Keeler, 1924; Sidman and Green, 1965). Since the same mutation causes autosomal recessive retinitis pigmentosa in the human it is used as a model to study human retinal degenerations. The mutation is located in the cGMP phosphodiesterase beta subunit gene (Bowes, et al., 1990) and leads to the build up of cGMP in the cytoplasm which results in apoptotic death of the cell (Chang, et al., 1993). Development of the *rd* retina progresses comparable to, but somewhat slower than the normal retina until PN10. The separation of the INL and the ONL occurs a few days later than in the normal retina and the inner segments grow slower. The outer segments develop but are small and contain fewer discs than wild-type photoreceptors. On PN10 the outer segments reach their maximum development. After this age the outer segments become disorganized and a rapid degeneration of the rods begin (Sanyal and Bal, 1973). On PN26, in the adult retina, all rods are gone and the ONL consists of only one layer of cone cells without inner and outer segments. The cones progressively decrease in number over the following months (Carter-Dawson, et al., 1978).

1.5 Apoptosis in the developing retina

Apart from cell proliferation and differentiation, another process with a significant role in the maturation of the retina is programmed cell death (PCD) or apoptosis. The pattern of PCD in the developing retina largely follows the pattern of cell generation. Most PCD's occur during the two first postnatal weeks but the peak of ganglion cell PCD occurs already at PN2-5. When the OPL forms, a wave of apoptotic cell death occurs as photoreceptors on the inner side of the OPL move across it. Rods already present in the ONL, however, die very sporadically and over a longer period of time (Young, 1984). The function of apoptosis during development is not quite understood, but it is crucial in neural development, as shown with mutant mice lacking functional caspase-3. These mutants die perinatally with an excess of cells in the CNS (Kuida, et al., 1996).

2. Mechanism of apoptosis

Apoptosis is genetically programmed. It is an active energy dependent process involving gene transcription and protein synthesis, and it occurs very fast. The cell dies and disappears completely within 12-18 hours (Wyllie, et al., 1980). Apoptosis involves shrinkage of the cell with cytoplasmic and nuclear condensation. The plasma membrane of the dying cell is kept intact, and the cell is phagocytosed by neighboring cells without eliciting an inflammatory response (Kerr, et al., 1972). This is in contrast to necrotic cell death where the plasma membrane becomes disrupted leading to leakage of the cell content and damage to surrounding cells (Wyllie, et al., 1980).

The apoptotic process involves three phases (Kroemer, et al., 1995). In the first phase, apoptosis is triggered by various death-inducing stimuli, in the second,

the effector phase, the intrinsic mechanism of the cell is activated and the cell becomes committed to die, and in the third phase degradation of the cell occurs. Activation of caspases is a central event in the apoptotic process. The caspases belong to a group of cysteine containing proteases that specifically cleave aspartic acid residues in their target proteins. More than 10 mammalian caspases have been identified and they are thought to activate each other in an amplifying cascade (Cohen, 1997; Nicholson and Thornberry, 1997). Some caspases are upstream caspases (eg caspases-2, -8, -9 and -10). They contain long pro-domains which enable protein-protein interactions and they possess autocatalytic activity. In response to for example cell-surface signalling they are dimerized and hereby activated. Downstream, or effector caspases (eg caspases-3, -6 and -7), have short pro-domains and depend on other caspases for their activation. They are activated later in the apoptotic process and are responsible for the cleavage of substrates involved in the apoptotic degradation process (Rudel and Bokoch, 1997; Thornberry, et al., 1997; Enari, et al., 1998). Induction of apoptosis can involve stimulation of death-receptors in the tumour necrosis factor (TNF) receptor family such as the Fas receptor on the cell surface. Liganded Fas receptors bind adapter proteins called FADD/MORT1 (Boldin, et al., 1995; Chinnaiyan, et al., 1995) and Flash (Imai, et al., 1999) on the cytoplasmic side and aggregate. This complex attracts procaspase-8 (Ashkenazi and Dixit, 1998) which undergoes autoactivation generating active caspase-8, which subsequently, by cleaving effector caspases, induces a cascade of caspase activation (Green, 1998).

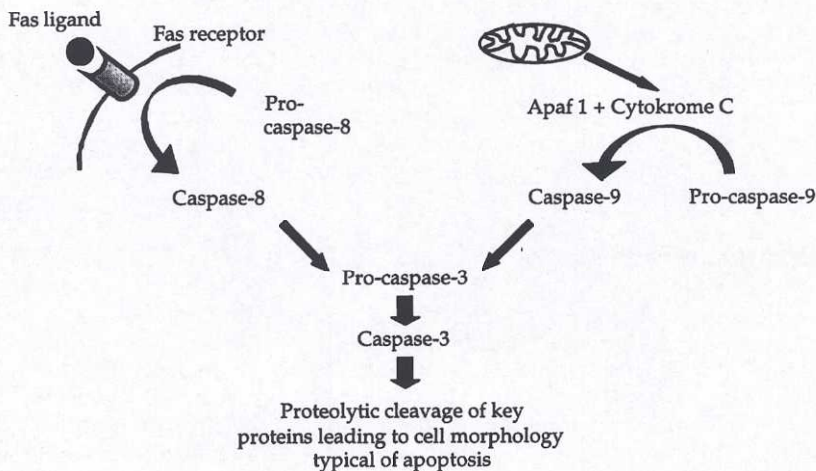


Figure 2. The two pathways of caspase activation and induction of apoptosis.

Presently, two ways of induction of apoptosis are known. Apart from induction by Fas activation, it can also occur via a pathway involving cytochrome c release from mitochondria. The released cytochrome c acts as cofactor to, and forms a complex with, the adapter protein apoptosis protease activating factor 1 (Apaf1) and procaspase-9, which becomes activated to cleave down-stream caspases (Li, et al., 1997). Figure 2.

In addition to the caspases, other substances regulate the apoptotic process. These are either promoters, such as Bax and Bad, or suppressors of apoptosis, such as p53, Bcl-2, Bcl-X and Bcl-X_L. They interact either with DNA, caspases, the mitochondrial membrane or each other (reviewed in Adams and Cory, 1998).

3. Neurotrophic factors

In the search for substances with putative therapeutic effects in neuronal and retinal degenerative disorders, a lot of attention has been focused on neurotrophic factors. These are polypeptides that are known to regulate survival, differentiation and regeneration of nerve cells. In order not to die by apoptosis, cells of the CNS and retina depend on neurotrophic factors from target cells via synaptic contacts (Rager and Rager, 1978). Neurotrophic factors act on cell-surface receptors, and based on the receptor-system via which they mediate their actions they are classified as either neurotrophins or cytokines. Activation of these receptor systems leads to activation of intracellular second-messenger systems eventually leading to changes in gene-transcription.

The activator protein-1 complex (AP-1), consisting of Fos and Jun, is an example of a transcription factor whose expression is regulated in part by neurotrophic factors. (Angel and Karin, 1991; Pfahl, 1993).

3.1 Neurotrophins

Nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and brain-derived neurotrophic factor (BDNF) are structurally related and are referred to as neurotrophins. They use two receptor systems for their actions. One is the family of receptor tyrosine kinases (Trk); Trk A, Trk B and Trk C, with overlapping specificities for the different neurotrophins. The Trk receptors share similarities with receptor tyrosine kinases used by traditional growth factors such as fibroblast growth factor (FGF). Binding of ligand to these receptors leads to receptor dimerization and activation of the tyrosin kinases and activation of a number of intracellular signalling cascades. For references see (Bothwell, 1995; Klesse and Parada, 1999). In addition, the cytokines bind to the p75 receptor which is a member of the tumour necrosis factor (TNF) receptor family (of which the Fas receptor is also a member). If receptors of both systems are co-expressed in a cell the p75 receptor has a positive modulatory effect on Trk receptor function, whereas on its own the p75 receptor seems to mediate a death signal. For references see (Casaccia-Bonnel, et al., 1998).

3.2 Cytokines

Another group of neurotrophic factors acts via cytokine receptors. Ciliary neurotrophic factor (CNTF) is a member of this group, which also includes cytokines that are active on non-neural cells. The cytokine receptors consist of a conserved extracellular ligand-specific component and several signal-transducing components associated with Janus kinases (JAKs) that become activated upon ligand binding. Among the proteins that are phosphorylated by the JAKs are the signal transducers and activators of transcription (STATs). When activated they form homo- or heterodimers, enter the nucleus and act as transcription factors, thereby coupling cytokine signalling to gene expression. For references see Ihle, 1995; Ip and Yancopoulos, 1996; Ihle, 1996.

4. Retinoic acid

Three of the studies presented in this thesis have focused on another substance with effects on growth, differentiation and apoptosis in the developing nervous system including the retina. This substance is retinoic acid (RA), which is a metabolite of vitamin A (retinol) that functions as a transcription factor regulating gene expression.

RA is synthesized in the mouse neural retina by three enzymes that differ in activity and spatiotemporal expression pattern. In the embryo this results in high RA concentrations in the ventral half, and low concentrations in the dorsal half of the retina. In the adult neural retina the concentration of RA is lower and the spatial distribution is reversed (McCaffery and Dräger, 1993; McCaffery, et al., 1993). However, in the adult, RA synthesis in the RPE exceeds that of the neural retina, resulting in an RA gradient from the RPE with high a concentration to the GCL with a low concentration of RA (McCaffery, et al., 1996).

The receptors for RA belong to the large family of nuclear receptors that also include the steroid- and thyroid hormone receptors, and the vitamin D₃-receptor. The two groups of RA receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), have three subtypes each. The liganded RXRs form either homodimers, or heterodimers with RARs or other nuclear receptors, and bind to retinoic acid response elements (RAREs) in the promoter of RA inducible genes (Chambon, 1996). In addition, RARs and RXRs can interact with the AP-1 complex. This leads to mutual repression of their respective DNA binding activities, consequently leading to decreased transcriptional activity (Yang-Yen, et al., 1991; Schüle, et al., 1991; Salbert, et al., 1993; Fanjul, et al., 1994).

5. Studies of retinal development in culture

There are several advantages with studying retinal cells in culture. The cells are easily accessible for experimentation and can be exposed to various substances by simply adding them to the culture medium. No considerations need to be taken to the substance's ability to cross the blood-retina barrier, as is necessary if drugs are administered in vivo (unless injected intra-vitreally). In addition, effects

of substances from the circulation can be ruled out, as well as putative interactions between circulating substances and the substance to be examined. Development of retinal cells has been extensively studied in cultures of dissociated cells. However, in low-density cultures dissociated retinal cells do not differentiate fully or lose their morphological characteristics (Akagawa and Barnstable, 1986; Araki, et al., 1987; Politi, et al., 1988; Kelley, et al., 1995). In such cultures the identification of individual cell types has to rely on cell-specific markers (Akagawa and Barnstable, 1986). Contacts between cells in the retina facilitate development of the different retinal cell types (Reh, 1992). High-density- and reaggregate cell cultures allow some of these cell-contacts and here the cells differentiate better than in low-density cultures (Akagawa, et al., 1987; Kelley, et al., 1995).

Another type of culture is the explant culture where the cells can be identified based both upon the cell-specific morphology and on their position in the retina. Several different rodent retinal culture systems have been developed, where the tissue is either cultured in pieces (Sparrow, et al., 1990; Germer, et al., 1997; Sheedlo and Turner, 1996), in slices (Feigenspan, et al., 1993) or as a whole organ (Zhao and Barnstable, 1996). In all these culture systems the RPE was absent. However, an important factor for retinal and photoreceptor development is the presence of RPE (Hollyfield and Witkowsky, 1974) or substances produced by the RPE (Sheedlo, et al., 1998). Therefore an organ culture system where the whole retina is explanted and cultured with the RPE attached was developed by Caffé et al in 1989 (Caffé, et al., 1989). This retinal organ culture system was used for the present investigations.

Aims of the study

The overall objective of this study was:

To study mechanisms of retinal development and degeneration in retinal organ culture.

The specific aims were:

1. To characterise photoreceptor development in organ culture.
2. To evaluate the effect on retinal degeneration of combinatorial treatment of BDNF and CNTF vs individual administration.
3. To evaluate the role of the RPE in postnatal retinal development.
4. To examine the effects of RA on postnatal retinal development.
5. To characterise the molecular mechanism of retinal apoptosis in vivo and in vitro.

II. Materials and methods

1. Tissue

Retinas used in culture were taken from newborn mice of the pigmented C3H+/+ or C3Hrd/+ strains at 12-48 hours after birth. For comparison, in vivo retinas from age-matched mice of the same strains were used.

2. The culture system

The animals were decapitated and the heads were immersed in 70% ethanol before the eyes were taken out. In order to facilitate the separation of neural retina and RPE from adjoining mesenchymal cell layers, the eyes were incubated in culture medium without FCS supplemented with 0.025% Proteinase K (Sigma) or 0.033% Proteinase K (ICN Pharmaceuticals Inc) at 37°C. After 15-25 minutes the eyes were moved to culture medium supplemented with 10% FCS (Gibco) in order to stop the enzyme treatment. A cut was made around the iris, and the anterior segment, the lens and the vitreous body were removed. The choroid and sclera were carefully peeled off, leaving the neural retina intact with the RPE attached. For explants without the RPE attached, the RPE was removed together with the choroid and sclera. Four cuts were made from the rim towards the centre of the optic cup and the explant was placed lying flat with the RPE side down on a piece of GN-4 Metrical cellulose filterpaper with pore size 0.8µm (Gelman Sciences) attached to a Monodur PA 56N polyamide grid (AB Derma, Gråbo, Sweden). The explants were placed in culture dishes containing 1.2 - 1.6ml culture medium. The explants were kept in an incubator with 100% humidity and 5% CO₂ in air at 37°C for 6 hours to four weeks. The culture medium was exchanged every other day.

The culture medium used was the chemically defined medium R16 (GIBCO BRL, Gaithersburg, MD (Romijn, 1988) supplemented with 10% fetal calf serum (FCS) (GIBCO BRL). Additions made to the medium for the different studies were: 100 nM or 500 nM all-trans retinoic acid (ATRA) (paper III) or 100 nM or 500 nM 9-cis retinoic acid (9CRA) (paper III, IV and V), 50 ng/ml ciliary neurotrophic factor (CNTF) and brain derived neurotrophic factor (BDNF) either alone or in combination (Paper II), 25µg/ml cyclosporin A (Novartis Sandimmune) (Paper II), 50 µM zVAD-fmk (Bachem AG, Bubendorf, Switzerland) (Paper III), 100 nM DEVD-fmk (Calbiochem-Novabiochem, UK) (Paper II and V).

3. Histological methods

After 2 to 40 days in vitro the explants were fixed in 4% paraformaldehyde and infiltrated with 25% sucrose in Sörensens phosphate buffer or Tyrode solution. The explants were either cryosectioned (8-10 µm), or the neural retina was removed from the underlying RPE and cellulose paper and floated in buffer. Both the sectioned and the floating retinal explants were processed for

immunocytochemistry, and sections were stained with hematoxylin/eosin (H/E) for cell counting. Histochemical and immunocytochemical reactions were examined and photographed with an Axiophot photomicroscope (Zeiss).

3.1 Immunocytochemistry

Immunocytochemistry was performed using the following antibodies: polyclonal anti-opsin (AO) recognizing rods (1:10 000), anti-cone visual pigment antibodies COS-1 (1:50, hybridoma supernatant) and OS-2 (1:10 000, ascites fluid) specific to middle-to-long wave sensitive visual pigment and short-wave sensitive visual pigment respectively (Szél, et al., 1986; Szél, et al., 1988; Röhlich and Szél, 1993), monoclonal anti-protein kinase C recognizing rod bipolar cells (clone MC-5, 1:100; Amersham, Little Chalfont, UK) and monoclonal anti-glial fibrillary acidic protein (GFAP) (clone 6F2, 1:800; DAKO, Glostrup, Denmark) recognizing Müller cells. The bound antibodies were detected with secondary antibodies conjugated to fluorescein isothiocyanate (FITC) (DAKO) or biotin which was reacted with avidin-horseradish peroxidase (avidin-HRP) or peroxidase-anti-peroxidase (PAP) and diaminobenzidine (DAB) (Vector, Burlingame, CA).

3.2 Lectin cytochemistry

Lectin cytochemistry was performed on cryosections with FITC- or rhodamin isothiocyanate (TRITC) conjugates of peanut agglutinin (PNA) and wheat germ agglutinin (WGA) (DAKO) respectively.

3.3 TUNEL assay

The TUNEL assay was performed on cryosections, according to published procedures (Gavrieli, et al., 1992; Perez, et al., 1997) and the bound biotin was reacted with streptavidin conjugated with Cy-3 (Jackson Immuno Research, West Grove, PA), or the TUNEL assay was performed, and positive cells were visualized with fluorescein according to the manual by Boehringer-Mannheim (Boehringer-Mannheim, Mannheim, Germany).

3.4 Caspase assays

Retinas were removed from culture after 6, 18, 19, 36, 48, 54 or 72 hours in culture, or 6 days and 19 hours or 6 days and 48 hours in culture, were briefly rinsed in ice cold 0,9% NaCl, were immediately frozen at -20°C and analyzed for caspase-3 activity within one week. The frozen retinas were placed in 400 µl of a room temperature buffer (50 mM Tris-HCl (pH 7,3) 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0,2% CHAPS, 3 mM NaN₃, 1 mM PMSF, pepstatin (1 µg/ml), leupeptin (2,5 µg/ml), aprotinin (10 µg/ml)), were vortexed, and incubated in the absence or presence of 50 µM z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) (Bachem AG, Bubendorf, Switzerland) at 37°C for 30 minutes and then centrifuged in a Microfuge for 5 minutes.

Samples of supernatants (50 µl) were mixed with 50 µl of respective substrate at a concentration of 50 µM in the above buffer (without CHAPS).

Caspase-3 was determined with DEVD-7-amino-4-methylcoumarin (DEVD-AMC) (Bachem AG) as substrate (final conc 25 μ M). Caspase-8 was determined (excitation 400 nm, emission at 505 nm) with Ac-IETD-AFC (Enzyme systems Products, Livermore, CA) as substrate (final conc 25 μ M). Caspase-9 was determined (excitation 380 nm, emission at 460 nm) with Ac-LEHD-AMC (Peptide Institute Inc., Osaka, Japan) as substrate (final conc 25 μ M). Cleavage of DEVD-AMC was measured either at room temperature using a Perkin-Elmer LS 50B luminescence spectrometer (excitation 380 nm, emission 460 nm) where DEVD-AMC cleavage was linear for 2 hours and fluorescence readings were obtained at 60 minutes, or at 37°C using a Spectramax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, CA) (excitation 380 nm, emission 440 nm) where cleavage was measured within one hour during the linear part of the reaction. Caspase-8 and 9- were determined by measuring cleavage of the respective substrate within one hour during the linear part of the reaction at 37°C using a Spectramax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, CA). Fluorescence readings were compared to standard curves of AMC (casp-3 and casp-9) in the above buffer. Caspase-3 and -9 activities were expressed as pmoles AMC formed (min x mg protein)⁻¹. Caspase-8 activity was expressed as arbitrary fluorescence units (min x mg protein)⁻¹. Protein was measured by using the Pierce BCA protein assay using BSA as the protein standard.

3.5 Electron microscopy

Cultured retinal explants were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer and postfixed in 1% osmium tetroxide. The tissue was embedded in araldite (Durcupan ACM, Fluka, Buchs, Switzerland), and the sections were contrasted with uranyl acetate and lead citrate.

3.6 Cell counts

Sections were examined from the central and peripheral parts of the retinal explants. Photoreceptor counting procedures were performed on haematoxylin/eosin (H/E) stained sections. The number of rows of nuclei in the ONL and INL of the central retina were counted once in each explant.

4. Statistics

All data are presented as means \pm SEM. Cell count data was analyzed using one-way analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (PLSD) posthoc comparisons. The ranked values of opsin content were analyzed using Kruskal-Wallis one-way ANOVA by ranks followed by non-parametric multiple comparisons (Siegel and Castellan, 1988). Statistical analyses were done according to procedures provided by StatView statistical

package (Abacus Concepts, Inc., Berkeley, CA). The difference between groups was regarded as significant if $p < 0.05$.

III. Results and Discussion

1. The organ culture system as a model for retinal development

When retinal development is studied in culture, it is beneficial if the retina can be explanted and cultured as a whole organ with the RPE attached since it is important for normal retinal and photoreceptor development (Hollyfield and Witkovsky, 1974; Raymond and Jackson, 1995; Sheedlo, et al., 1998). As development does not occur uniformly, but proceeds from the centre to the periphery it is also of importance to examine the whole retina. In addition, the topographic separation of the mouse retina into two halves has to be taken into account. During embryonic retinal development there is a dorso-ventral gradient in the synthesis and degradation of RA, in the expression pattern of RA receptors (McCaffery, et al., 1999), and in the expression pattern of growth factors and their receptors (Rissi, et al., 199; Marcus, et al., 1996). Moreover, in the adult there is a dorso-ventral separation of the two cone types so that the majority of S-cones are found in the ventral part and the M-cones exclusively occupy the dorsal part (Szél, et al., 1992). Since the culture system used for the present investigations is an organ culture system where the whole retina with the intact RPE attached is explanted and cultured (Caffé, et al., 1989), it provides an excellent tool for studying retinal development and degeneration.

2. Development of retinal explants in culture

2.1 Lamination of the retina in culture

On postnatal day one or two, at the time of explantation, the retina consists of a ganglion cell layer separated from a thick layer of neuroblastic cells by the inner plexiform layer. During the first week in culture mitotic figures were seen near the ventricular surface of the explant (unpublished observation) and by PN7 all retinal layers were clearly visible. From PN7 and throughout the following three weeks the ONL contained 7-8 rows of nuclei. The INL contained 5-6 cell layers on PN7 and 4-5 on PN21 reflecting the normal developmental thinning of this layer due to apoptosis of Müller and bipolar cells (Young, 1984; Chang, et al., 1993) (paper III). Rosettes were occasionally formed, with a somewhat higher incidence towards the edges of the explants (paper I). During development contact between the RPE and the neural retina is important for normal layering of the retina, and ablation of the RPE in late embryonic development has been found to cause rosette-formation (Raymond and Jackson, 1995). Dissection-induced disturbances in the photoreceptor-RPE contacts can be expected to occur

to a higher degree in the periphery than in the centre of the explant, and this may be the reason for the higher number of rosettes at the edges of the explants. Explants lacking physical contact with the RPE contained a markedly higher number of rosettes throughout the explant compared to those with RPE. These explants also had a wavy appearance and some photoreceptor cells were displaced outside the photoreceptor layer (paper IV) similar to what was found after *in vivo* removal of the RPE (Raymond and Jackson, 1995). In addition, in explants cultured with RPE with occasional patches where the RPE was lost, the RPE-less patches had the typical appearance of an explant without RPE. This suggests that the effects on organization of the retina are not induced by diffusible factors from the RPE, but that a physical contact between the neural retina and the RPE is necessary. This is in accordance with findings on cultured chick retinal cells, where no lamination of reaggregating embryonal retinal cells was induced even if the cells were grown in RPE-conditioned medium or in the presence of, but not in direct contact with, RPE cells (Vollmer, et al., 1984; Vollmer and Layer, 1986; Liu, et al., 1988). However, the absence or presence of RPE was not important for lamination in our culture system, since all the retinal layers were present also in rosettes. Even though the retina is not fully laminated until on PN5-7, our results suggest that lamination of all retinal layers is already induced on PN1 otherwise it would be impaired also in the retinal explants without RPE.

Rosettes are formed in response to different types of retinal injury and their interior may provide a somewhat protected environment for the photoreceptors, possibly by retaining a higher concentration of intraretinally produced factors otherwise diluted in the culture medium. Inside rosettes there was stronger labelling of WGA, PNA, AO and OS-2 (paper I). Photoreceptors differentiated further (Akagawa, et al., 1987) and there was a tendency that opsin expression was slightly advanced in rosettes, compared to normal flat areas of the same explant (unpublished observation).

2.2 Cell death in the retinal explants

During the first three postnatal weeks PCD occurs in all retinal layers in the mouse *in vivo*, and similar apoptosis was found also in the retinal explants. TUNEL positive cells were observed predominantly during the first week in culture. At birth the GCL consists of approximately three layers of cells which in the mature retina have decreased to a single row of cells. *In vivo*, there are ganglion cells dying until PN11 with a peak of cell death at PN2-4 (Young, 1984). In cultured retinas however, apoptotic cells in the GCL occurred at a higher frequency earlier in development most likely due to optic nerve transection. After 18 hours there was a six-fold increase in caspase-3 activity in the explants compared to the activity six hours after explantation. No such increase was observed *in vivo* where a low and steady level of caspase-3 activity was present during the first four postnatal days. In addition, at PN3, after 48 hours in culture, there were numerous TUNEL positive cells in the GCL (paper III and IV), fewer at PN5 and only occasional apoptotic ganglion cells at PN7 (unpublished observations), leaving one row of sparsely distributed cells. The identity of these cells is somewhat uncertain due to the lack of ganglion cell specific markers for mouse. An antibody specific for cholinergic amacrine cells only labelled a fraction

of cells in the GCL implying that not all the cells remaining in the GCL were displaced amacrine cells. The possibility that some ganglion cells do survive in culture, is supported by the finding of a population of ganglion cells that survive axotomy *in vivo* (von Bussman, et al., 1993).

After the peak at 18 hours the caspase-3 activity in the explants gradually decreased so that it was no longer significantly different from the activity in age-matched retinas *in vivo* after 72 hours in culture (paper V). In addition to reflecting apoptosis of ganglion cells due to axotomy, the elevated caspase activity in the explants may also indicate a somewhat higher incidence of apoptosis in the ventricular layer during the first week in culture compared to *in vivo*. At PN7 the ONL of the explants contained approximately 7.5 layer of cells, compared to 10 *in vivo*. There was however, no change in ONL cell number between PN7 and PN21, suggesting that the putative induction of apoptosis in the ONL was caused by the dissection rather than a deficiency in the culture conditions to support long-term survival (paper III).

Despite the large number of studies indicating a significant role for the RPE for photoreceptor development and function, the lack of RPE did not have a significant effect on photoreceptor survival in the culture. Even though there were more TUNEL positive cells in explants lacking RPE on PN3, this did not result in significant thinning of the ONL by PN14 compared to explants cultured with RPE attached.

2.3 Photoreceptor development in culture

Photoreceptors developed outer segments and expressed opsin in the retinal explants with RPE (paper I and III). The outer segments contained stacks of membrane discs, but instead of being organized perpendicular to the outer segment axis they were organized parallel to it. They were also shorter than *in vivo*. Several reports indicate a crucial role for the RPE in outer segment formation and maintenance (Spoerri, et al., 1988; Caffé, et al., 1989; Stiemke, et al., 1994; Raymond and Jackson, 1995; Germer, et al., 1997; Sheedlo, et al., 1998; Mosinger Ogilvie, et al., 1999).

Occasional (rhod)opsin positive cells were detected in some explants on PN3 consistent with the *in vivo* observations (Szél, et al., 1993a), and on PN5 all explants were opsin positive. During the first week the anti-opsin antibody labelled the whole photoreceptor layer, but later it was concentrated to the developing outer segments. After the second postnatal week discrete rod outer segments could no longer be detected, as the fluorescence formed a confluent band. Neither the time that opsin was first expressed, nor the amount expressed during the first postnatal week was affected by the presence of the RPE (paper III).

Cone opsin could be detected with the OS-2 antibody specific for short-wave length sensitive cone-opsin on PN5 in the retinal explants. Surprisingly, no opsin was detected with the COS-1 antibody specific for middle-wave length sensitive cone opsin (paper I). *In vivo* the M-cone starts to express pigment later than the S-cone; on PN11, but in the adult mouse retina it is the dominating type (Szél, et al., 1993a). Despite systematic examination of serial sections of several explants, containing both rosettes and flat parts, and examination of the whole retinal surface with wholemount immunocytochemistry no COS-1 positive cones were

found at any time during the six-week culture period. Although OS-2 positive cones in the explants can be detected at an age comparable to their appearance in vivo (Szél, et al., 1993a) a delayed development of COS-1 positive cones could be caused by the culture procedure. However, a delay of several weeks seems unlikely. Double labelling of the mature explants with the lectin PNA and the OS-2 antibody revealed that all PNA positive elements were also OS-2 positive. Since PNA labels the interphotoreceptor matrix (IPM) ensheathing all cone inner- and outer segments (Blanks and Johnson, 1983), this indicated that all existing cones were S-cones and that the M-cones were completely absent from the explants. In rat the M-cones develop from the S-cones by a shift in spectral sensitivity. Until PN9 all cones are OS-2 positive, but during a transition period many cones express both pigments and gradually the number of OS-2 positive cones drops. After PN20 the ratio of M-cones to S-cones switches so that the M-cones dominate the mature retina (Szél, et al., 1994). If development of the two cone types in mouse occurs in a similar way, it can be speculated that the shift in spectral sensitivity does not occur in the retinal explants resulting in the complete absence of M-cones. Similar to our results are findings from transplantation studies where embryonal rabbit retina is transplanted to the subretinal space of adult rabbits. Also in the adult rabbit retina the M-cone type dominates the cone population. However, although M-cones exist in the transplants, their number remains significantly lower than that of the S-cones. A putative factor inducing M-cone development may still be present in the adult host retina but in insufficient amounts to establish the normal M- to S-cone ratio in the transplant (Juliussen, et al., 1993).

2.4 *rd* photoreceptor degeneration is delayed in culture

In organ culture the cell loss in the *rd* mouse retina is delayed. After three (Mosinger Ogilvie, et al., 1999) or four weeks of age (paper II) the ONL consists of approximately three layers of cells instead of one as in the mature *rd* retina in vivo (Carter-Dawson, et al., 1978). There is also the possibility that the intracellular cGMP level in culture is lower due to leakage out of the cells into the culture medium.

If the retinas were explanted and placed in culture on PN7 instead of on PN2, the degeneration occurred comparable to the degeneration in vivo, resulting in an ONL containing one row of cells after three weeks in culture corresponding to PN28.

3. Effects of growth factors on *rd*- and wild type retinas

CNTF administered to the cultured retinal explants had a slight protective effect on *rd* photoreceptors (paper II). This is in accordance with results from experiments where intravitreal administration of CNTF to *rd* mice rescued photoreceptors so that the ONL on PN21 consisted of one to two rows of photoreceptors instead of one incomplete row (La Vail, et al., 1998).

In our culture system CNTF also caused a decrease in opsin expression, similar to what has been found in rat (Kirsch, et al., 1996; Ezzedine, et al., 1997; Kirsch, et al., 1998). In these studies no increase in the number of photoreceptors was seen, corroborating the results from the present study where there was no effect of

CNTF exposure on the number of photoreceptors in explants from wild-type mice.

The effects of CNTF treatment on photoreceptors were profoundly increased by combining it with BDNF. The treatment with the combined neurotrophic factors resulted in a more efficient *rd* photoreceptor rescue, and the decrease in opsin expression was more apparent. BDNF administered alone did not have any survival promoting effects on photoreceptors in the *rd* retina. This is similar to the finding that NGF and bFGF in combination produced a profound rescue of photoreceptors, whereas neither of these factors administered alone had any survival promoting effects on photoreceptors in the *rd* retina (Caffé, et al., 1993). The availability of transcription factors can be expected to increase if multiple receptor systems are activated by a combination of different neurotrophic factors. Transcription factors are activated when they form homo- or heterodimers, and the increase in biological activity seen by combining different neurotrophic factors can be hypothesized to result from cross-coupling of different receptor systems.

Interestingly, BDNF and CNTF did not decrease opsin expression in patches lacking RPE, but rather caused an increase in its expression.

It can be speculated that CNTF and BDNF may have direct effects on photoreceptors or that the two growth factors may stimulate the expression of inhibitors of rod differentiation in the RPE, in a similar way as is discussed below for RA.

4. Effects of RA on developing retina

Exposure of retinal explants to ATRA or 9CRA resulted in either rod specific apoptosis or an advance in (rod)opsin expression. The result was highly dependent upon the presence or absence of the RPE (paper III).

4.1 Effects of RA on explants with RPE attached

Exposure of retinal explants with RPE attached to 100 and 500 nM ATRA or 9CRA caused a 20-25% decrease in the number of cells in the ONL. No dose-response effect was observed and the effect was present at PN7. After that age no further cell loss was seen. There was no change in cone number or in the number of PNA labelled cone sheaths indicating that RA affected only rod survival. Signs of rod cell-death were seen already on PN3 after two days of RA exposure. Morphological, histological and biochemical data indicated that the rods died by apoptosis. The dying cells had shrunken cell-bodies, condensed chromatin and were TUNEL positive indicative of internucleosomal DNA fragmentation, and they were scattered throughout the ventricular layer. In addition, there was a doubled caspase-3 activity in the 9CRA treated explants compared to control explants after 48 and 54 hours of 9CRA exposure (paper III and V). Since caspases are necessary for the final activation stages of apoptosis (Green, 1998) and since activated caspase-3 is involved in the degradation phase of apoptosis by mediating the internucleosomal DNA cleavage (Enari, et al., 1998), we believe that caspase-3 is involved in the RA-induced rod-selective apoptosis.

In order to investigate whether 9CRA had the ability to induce apoptosis in one week old retinas caspase-3 activity was measured after 19 and 48 hours and the TUNEL assay was performed on explants after 48 hours of 9CRA exposure starting on PN7 (paper IV). However, unlike the observations after the same time of 9CRA exposure starting on PN1 there was no increase in caspase-3 activity and no increase in the number of TUNEL positive cells compared to age-matched control explants. In addition, the number of cell layers in the ONL at PN14 in explants that were cultured for one week in control medium followed by one week in 9CRA containing medium was found not to be different from the number of cell layers in the explants cultured in control medium throughout the two-week culture period. Consequently, the rods were sensitive to the apoptosis inducing effect of 9CRA only during the first postnatal week, possibly as a result of the recently reported gradual down-regulation of retinal RXRs in the postnatal period (McCaffery, et al., 1999).

4.2 Advanced opsin expression in the absence of RPE

Explants lacking RPE that were exposed to 500 nM 9CRA did not have a higher number of TUNEL positive cells on PN3 than control explants with RPE, and there was no difference in caspase-3 activity between these groups (paper III).

On the contrary, opsin expression appeared earlier in 9CRA treated explants without RPE than in all other groups. Figure 4 in paper III shows the ranked values of opsin amount in the different explant groups during the first postnatal week. Sections were stained with the AO antibody and were categorized into four groups depending on the number of opsin positive cells present. A rank of 0 was given to sections completely lacking stained cells, 1 was given to explants with single opsin positive cells, 2 represented sections with several stained cells and 3 was given to explants with a confluent layer of opsin positive cells. At PN3 controls with RPE had an average rank of approximately 0.3. This means that most explants were completely negative, and only a third of the explants contained one positive cell. In controls without RPE and in 9CRA treated explants with RPE there was even less opsin. On the contrary, 9CRA treated explants without RPE had an average rank of 1.5, meaning that all explants (except one) contained opsin. These explants contained either single or several stained cells, or in one case a confluent layer of opsin.

At PN5 and 7 opsin started to be expressed in comparable amounts also in the control groups. However, in the 9CRA treated group with RPE the amount of opsin was still significantly lower on PN7 than in the other groups.

Our results on opsin expression are similar to findings from studies on explants without RPE from embryonal rat where ATRA was found to accelerate opsin expression by one day and to increase the number of cells expressing opsin (Zhao and Barnstable, 1996). Although the mechanism of the RA-induced acceleration of opsin expression is unknown, it could be a direct effect on photoreceptors. It can be speculated that the mammalian opsin promoter may contain a RARE, such as is the case in *Drosophila* (Sun, et al., 1993; Picking, et al., 1996). Another possibility is that RA may down-regulate the expression of an inhibitor of rod differentiation in the RPE. Inhibitors of rod differentiation have been found in rodent retina *in vivo*. They are down-regulated at the time of

photoreceptor differentiation (Bao and Cepko, 1997) and knock out mice lacking them express opsin prematurely in the embryonal period (Tomita, et al., 1996).

In transformed and fetal cells there is evidence of an initial RA mediated differentiation that is followed by apoptotic cell death (Atencia, et al., 1994; Okazawa, et al., 1996; Tong, et al., 1997; Falasca, et al., 1998). To find out if the differentiation promoting effect in the explants without RPE was similarly followed by apoptosis, explants without RPE were cultured for two weeks in 9CRA containing medium and on PN14 the number of cell layers in the ONL was counted (paper IV). However, these explants did not differ from control explants indicating that the apoptosis producing effect of 9CRA was dependent on the presence of RPE. It can be concluded that differentiation and apoptosis of rods in our culture system are mediated via two separate mechanisms and are not occurring sequentially.

4.3 What receptor subtype(s) mediates the RA-induced apoptosis?

ATRA and 9CRA produced similar decreases in cell number in the ONL indicating that the effect was mediated via RAR receptors since the two compounds have similar affinities for this receptor subtype (paper III).

However, explants that were exposed to 9CRA seemed to contain slightly less opsin at PN14 and PN21 as compared to ATRA exposed explants. They also contained a higher number of opsin-labelled cell bodies at PN21, indicating a higher degree of rod degeneration (Nir, et al., 1989). The putatively higher potency of 9CRA indicates that the effect was mediated via the RXR subtype since ATRA has to be isomerized into 9CRA in order to bind to RXRs.

In addition, the fact that 9CRA had lost its apoptosis inducing property in retinal explants on PN7 (paper IV), coincided with the down-regulation of RXR β and RXR γ in the postnatal retina (McCaffery, et al., 1999). This suggests that the apoptosis-producing effect of 9CRA was mediated via RXR β and/or RXR γ .

4.4 How does RA induce apoptosis?

RA may down-regulate the expression of growth factors in the retina. A key event in the cellular response to growth factors is activation of the AP-1 complex (Angel and Karin, 1991). Liganded RARs and RXRs have been found to inhibit AP-1 activity (Yang-Yen, et al., 1991; Schüle, et al., 1991) leading to inhibition of proliferation (Fanjul, et al., 1994; Chen, et al., 1995). RA-induced antagonism of AP-1 activity has also been found to result in down-regulation of growth factor expression per se (Salbert, et al., 1993). Only explants with RPE were sensitive to the apoptosis inducing effect of RA, and it can be speculated that RA, by antagonizing AP-1 activity, cause a decrease in growth factor expression in the RPE. However, the obvious question is then why explants without RPE and consequently without the putative RPE-produced growth factors do not also have an increased rod death. Since RA mediated up-regulation of several pro-apoptotic proteins have been reported (Melino, et al., 1997; Hsu, et al., 1999), it could be speculated that ATRA and 9CRA could induce expression of a pro-apoptotic protein hypothetically present in the RPE. Recent evidence showed that NGF caused apoptosis by acting via p75 receptors in the developing mouse

retina (Frade and Barde, 1999). In this context it is interesting to note that NGF immunoreactivity is found in the RPE (Chakrabarti, et al., 1990). However, it is not known if RA can affect expression of NGF.

Findings on two mouse models of retinal degeneration show similarities with the RA induced rod-selective apoptosis in the present study. In transgenic mice expressing the human Pro23His rhodopsin mutation, RA has been found to accelerate photoreceptor apoptosis (Birnbach and Reh, 1997), and the neural retina of the vitiligo mouse (*Mitf^{vit}* mutant), which has an impaired retinoid metabolism in the RPE and where photoreceptors also die through apoptosis, was recently reported to have a significantly elevated concentration of RA during the retinal degeneration (Duncan, et al., 1999).

5. Molecular mechanism of apoptosis

To elucidate the molecular mechanism by which RA can induce apoptosis in rods we measured the activity of caspase-3, -8 and -9 in 9CRA exposed retinal explants and compared them to the activity in control explants and in vivo retinas (paper V). 6 hours after explantation of the retina there were no differences in the activities of caspase -3, -8 or -9 between control explants, 9CRA treated explants or age-matched in vivo retinas. 18 hours after explantation of the retina there were axotomy-induced increases in the activities of all three caspases in both groups of explants. In control explants the activities gradually decreased and after 72 hours the activity of caspase-3 was not significantly different from the activity in vivo. Also in 9CRA exposed explants the activity of caspases-3 and -9 started to decrease after 18 hours, but after 54 hours they were again elevated so that they were significantly higher than in control explants. The release of cytochrome c is stimulated by all-trans retinoic acid and 13-cis retinoic acid from mitochondria in acute myeloblastic leukaemia rat liver cells (Rigobello, et al., 1999; Zheng, et al., 1999). It is possible that 9CRA had a similar effect in the retinal explants and that the significantly higher caspase-9 activity at 54 hours compared to control explants was a result of a putative 9CRA induced release of apoptogenic factors from mitochondria leading to the activation of caspase-9 and -3.

The temporal pattern of caspase-8 activity was slightly different in 9CRA treated explants than in control explants. Although there were no significant differences in caspase-8 activity between the two explant groups, there was a tendency for the activity in the 9CRA treated explants to decline at a slower rate and to remain at a higher level than in control explants. It may be speculated that a putative 9CRA induced peak of caspase-8 activity could be masked by the high activity at 18 hours.

There is evidence that ATRA can upregulate the expression of Fas ligand (Okamura, et al., 1998) and since both ATRA and 9CRA induce apoptosis in retinal explants it can be speculated that 9CRA induced apoptosis via the Fas receptor, and that the caspase cascade was induced by activation of caspase-8 in response to ligand binding at the Fas receptor (Muzio, 1996).

Recently a mechanism cross-linking the Fas induced and the cytochrome c induced pathways was reported. The cytoplasmic protein Bid is cleaved and activated by caspase-8 (Li, et al., 1998; Luo, et al., 1998; Bossy-Wetzel and Green,

1999). It translocates to the mitochondria and causes the release of apoptogenic proteins and thus transmits the death signal from cell surface receptors to the mitochondria. Such apoptogenic proteins include pro-caspase-3 (Mancini, et al., 1998) -2 and -9 (Susin, et al., 1999a), mitochondrial apoptosis-inducing factor (AIF) (Susin, et al., 1999b) and cytochrome c (Kluck, et al., 1997). When cytochrome c is released it acts as a co-factor in the activation of caspase-9 (Li, et al., 1997). The elevated caspase-9 activity in the 9CRA treated explants at 54 hours may result from such a caspase induced release of cytochrome c rather than from a direct effect on mitochondria. Figure 3.

The mechanism by which the *rd* mutation induces apoptosis in rods is unknown. CsA has a stabilising effects on the mitochondrial membrane preventing release of cytochrome c (Walter, et al., 1998) and mitochondrial swelling (Rigobello, et al., 1999). Despite this, CsA in the concentration used, did not have any effect on photoreceptor survival in the *rd* retina (paper II), suggesting that the apoptotic mechanism was independent of mitochondria, and possibly that the death signal was mediated via Fas activation. However, not even inhibitor of caspase-3, which is the proximal caspase in the apoptotic cascade, resulted in photoreceptor rescue. It can be speculated that this is an effect of cross-talk between the Fas pathway and the mitochondrial pathway leading to (necrotic?) cell death without involvement of caspase-3. These speculations could be further investigated by exposing *rd* retinal explants to a combination of DEVD-fmk and CsA to block both the caspase dependent apoptosis and the mitochondrially induced cell death. However, since only one CsA concentration (25 mg/ml) was used in the present study, a putative effect of higher CsA concentrations on cell survival would also have to be investigated. Figure 3.

6. Can caspase inhibitors prevent retinal degeneration?

Retinal explants exposed to 9CRA from PN1 exhibited a doubled caspase-3 activity after approximately 50 hours and on PN3 they contained a higher number of TUNEL positive cells in the ONL as compared to cultured controls. If these explants were exposed to the unselective caspase blocker zVAD-fmk (50 μ M) (paper III) or the caspase-3 selective blocker DEVD-fmk (100 nM) (paper V) in combination with 9CRA, the 9CRA-induced increase in caspase-3 activity was absent. zVAD-fmk also decreased the number of TUNEL positive cells on PN3, but only in the peripheral parts of the explants. Here TUNEL positive cells were nearly absent both in the ONL and the GCL (unpublished observation). The results point to the possibility of blocking the RA induced apoptosis with caspase inhibitors, as well as the normal developmentally occurring ganglion cell death, similar to what has recently been reported on rescue of ganglion cells after optic nerve transection (Chaudhary, et al., 1999; Kermer, et al., 1998).

Caspase activation is an early event in apoptosis and in order for caspase inhibitors to be efficient they must be administered early. The fact that apoptosis was not blocked in the centre of the GCL in the explants demonstrates this very clearly. The apoptotic process was here most likely beyond the caspase activation stage at the time of zVAD-fmk exposure.

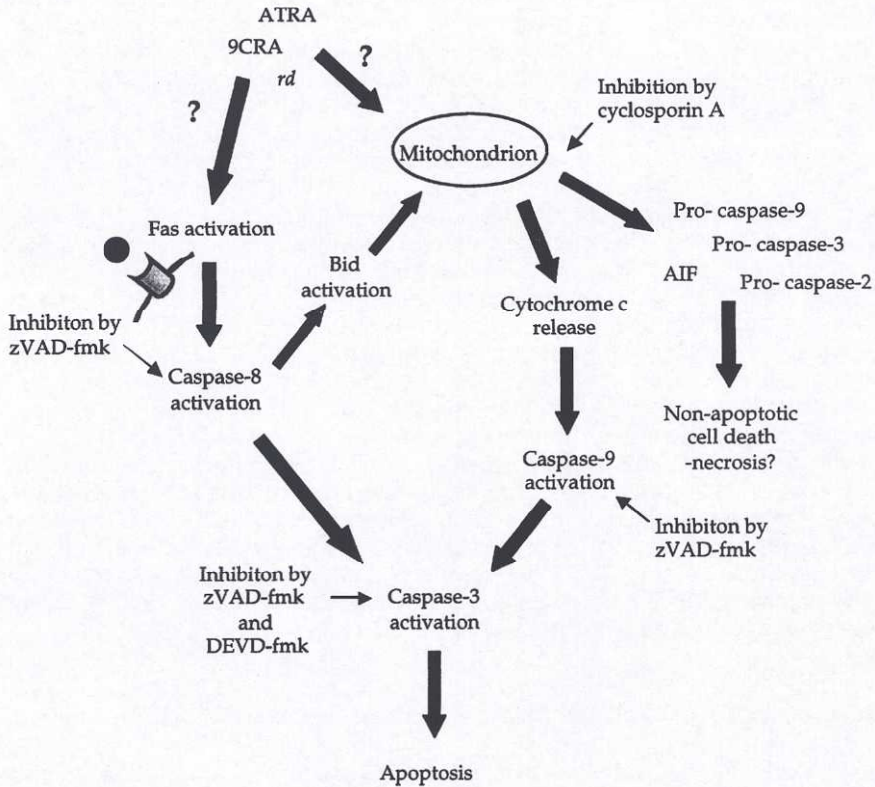


Figure 3. Possible death inducing pathways involved in the ATRA, 9CRA and *rd* induced photoreceptor apoptosis.

In retinal degeneration a protective effect of caspase inhibition has been reported (Davidson and Steller, 1998). Inhibition of retinal cell death in *Drosophila* was accompanied with a significant retention of visual function.

However, the selective caspase-3 blocker DEVD-fmk (100 nM) added to the culture medium did not have any effect on the number of cell layers in the ONL of the *rd* mouse (paper II), caused only a partial rescue of ganglion cells after axotomy in the rat (Kermer, et al., 1998; Chaudhary, et al., 1999), only delayed death of rat fibroblasts exposed to various apoptogenic stimuli (McCarthy, et al., 1997), and only blocked the 9CRA induced increase in caspase-3 activity in the explants (paper V). DEVD-fmk had had no effect on the elevated caspase-3 activity in control explants. It is uncertain if inhibition of caspases can lead to

long-term protection of cells, it may only delay death and change the mechanism of cell death (Hirsch, et al., 1997).

We found baseline caspase-3, -8 and -9 activity in developing mouse retina, similar to the previously reported baseline activity in the adult rat retina (Kermer, et al., 1998). Since apoptosis is a fundamentally important biological process involved in development, regulation of normal cell turnover and protection against tumorigenesis, blocking also this baseline activity may have severe side effects. Therefore the effects of caspase inhibition must be carefully evaluated before it can be used in the treatment of different neuronal and retinal degenerations.

Conclusions

1. In the organ culture system used for the present investigations both photoreceptor types, rods and cones, developed and expressed visual pigments. However, the middle-wave length sensitive cone type did not develop.
2. BDNF and CNTF in combination resulted in a better rescue of photoreceptors in the *rd* retina than these factors administered alone. We speculate that the synergistic effects are achieved by cross-coupling of different receptor systems increasing the availability of transcription factors.
3. In the neonatal mouse, physical contact between the neural retina and the RPE was important for maintaining normal retinal organization. In the absence of RPE rosettes were formed, the retina had a wavy appearance and photoreceptors were occasionally extruded into the "subretinal" space. However, the RPE was not important for lamination of the retinal explant or for the expression of opsin during the first postnatal week.
4. RA can have either an apoptosis inducing effect or a differentiation promoting effect on rods. Exposure of the retina to RA in the presence of RPE results in apoptosis, and in the absence of RPE opsin expression is advanced. We speculate that the apoptosis-inducing effect seen during the first postnatal week is mediated via RXR β and /or RXR γ .
5. Low steady-state levels of caspases- 3, -8 and -9 were present in the neonatal mouse retina *in vivo*, suggesting that these are involved in normal retinal development. Caspase-3 and caspase-9 were elevated in the retinal explants in response to 9CRA exposure suggesting that the 9CRA induced rod-selective apoptosis is caspase-9 and caspase-3 dependent.

IV. Acknowledgements

A lot of people have in one way or the other contributed to the completion of this thesis. I specifically wish to express my gratitude to the following people:

Professor Theo vanVeen, my supervisor, who introduced me to the field of retinal research, for financial, moral and extraordinary support.

Professor Donald Fox, at the College of optometry, University of Houston, for inspiring scientific discussions, thorough manuscript revisions and quick e-mail replies.

Dr Jan-Olof Karlsson, at the department of Anatomy and Cell Biology, Göteborg university, who did all the caspase assays, for fast, fun and fruitful collaboration.

Everybody in our wide-spread research group: Dr Romeo Caffé for teaching me the fine art of organ culturing. Dr Agoston Szél for his endless enthusiasm, and for helping me realize that my first manuscript could be converted into a scientific paper. Inger Holmquist for expert technical assistance, for thinking of, and taking care of everything I had forgotten or was about to forget, and for making the lab such a nice place. Bengt Juliusson, Kristina Mieziowska and Malcolm von Schantz for scientific discussions and good times at ARVO.

Per Sundberg, Staffan Andersson, Torgny Bohlin and Jens Rydell for answering my questions concerning statistics. If, despite their help, anything is wrong with the statistics in this thesis it is entirely my fault.

Barbro "Blomman" Blomgren for expert care-taking of the mice and nice chats with me.

Bengt Svensson for excellent skills and creativeness in toolmaking.

The staff at the Zoology department: Lars-Åke Andersson, Barbro Haraldsson, Magnus Kinding, Ann-Sofi Olsson, Carin Smederöd-Johansson and Agneta Winquist for helping with everything from economy papers to drilling holes in walls.

The participants in the mentoring program at the Faculty of natural sciences, Göteborg university, for fruitful exchange of thoughts, and my "mentor" Björn Wallmark, at AstraZeneca, for sound advice.

"Tjejgänget": Ingela Danielsson, Lena Gustavsson, Pernilla Jonsson, Maria Hellsten, Anna Hjelmgren, Carina Härlin, Susanne Lindh, Kristina Mieziowska, Marianne Saur, Pia Stålhandske, Monica Svesson, Maria Thorell, Anita Tullrot and Anna Zeffer for many good movies and interesting discussions over several cups of coffe.

Everybody at the department of Zoology, for making the working environment so pleasant, and for fun and interesting coffetime discussions.

Madeleine Andersson for nice discussions about science and everything else, for mental support and for sharing my unhappy situation in having to work all summer.

All the people I have met in freefall and all my other friends for making life outside work so much fun.

Jason, for being good late-night company and for typing help.

All those who looked after my cat.

My family for their support and for believing in me. My mother, Ewa, for proof reading this thesis and for patiently answering a million phone calls from me over the last weeks, and my father, Gunnar, for introducing me to the world of science almost 30 years ago, by doing physics experiments in the kitchen. My sister Agneta for answering my numerous and stupid computer questions. Jan, for his constant support and never- (but soon) ending patience (I will never mention my "avhandling" again) and for being so much fun!

The work in this thesis has been supported by grants from Anna Ahrenbergs fond för vetenskapliga mfl ändamål, Adlerbertska forskningsfonden, De Blindas Förening, Göteborgs univeritet, Hierta-Retzius stipendiefond, Kronprinsessan Margaretas Arbetsnämnd för Synskadade, Kungliga och Hvitfeldtska stipendie-stiftelsen, Kungliga Vetenskaps- och Vitterhetssamhället i Göteborg and Lennanders forskningsfond.

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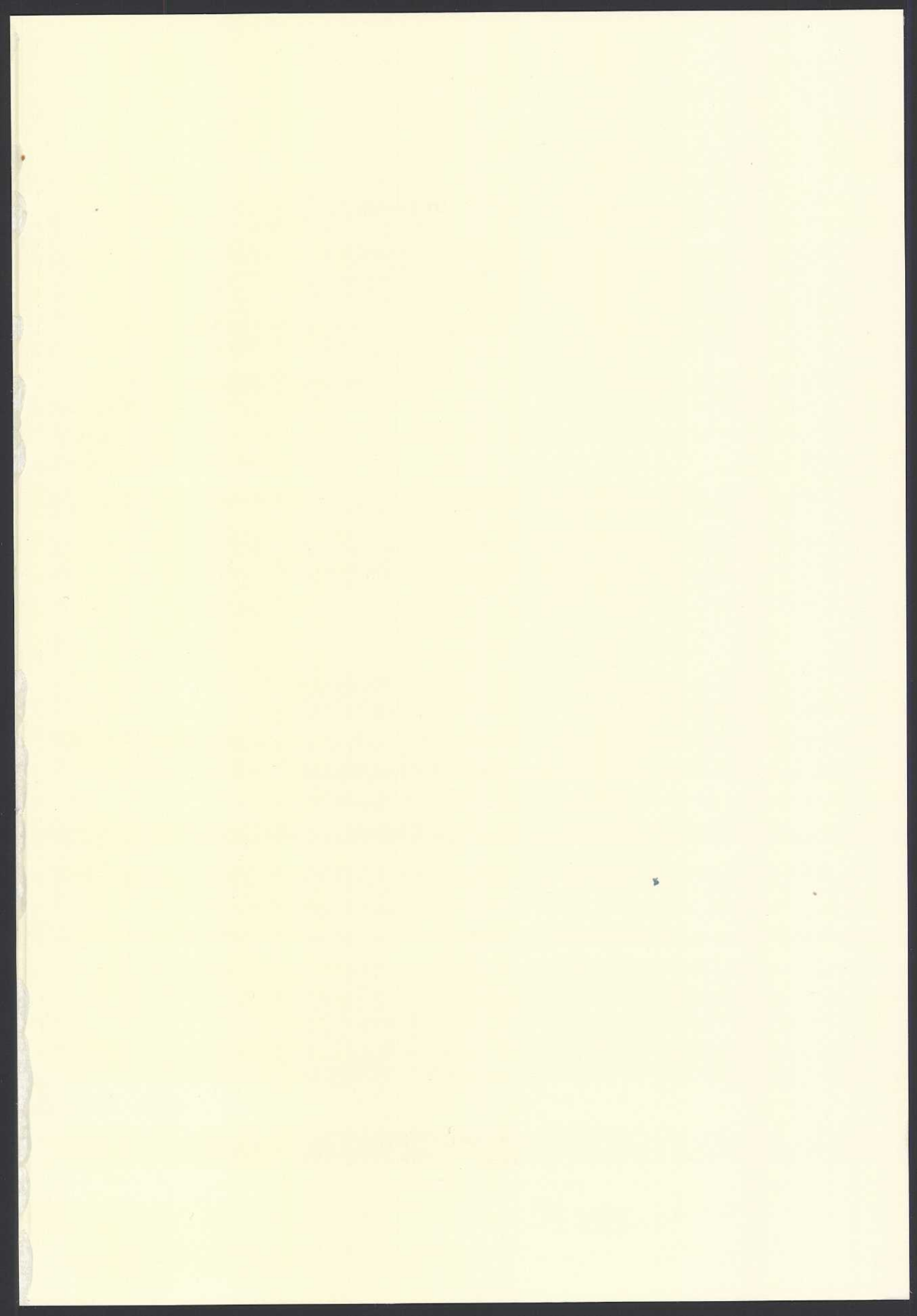
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ISBN 91-628-3723-0