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PROSPECT

Retinal Neuroprotection by Growth Factors: A Mechanistic Perspective

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Abstract For more than a decade it has been known that certain growth factors inhibit apoptosis in genetically determined and experimental models of inner and outer retinal degeneration. The molecular mechanisms underlying these protective effects and the signaling that supports the survival of photoreceptors and retinal ganglion cells in these models have recently come under more in depth investigation. This paper reviews our current understanding of the balance of pro- and antiapoptotic signals that determine cell fate in the retina and how the activation of key signal transduction pathways by specific classes of neurotrophins protects retinal neurons. J. Cell. Biochem. 88: 57–75, 2003. © 2002 Wiley-Liss, Inc.

Key words: apoptosis; growth factors; glaucoma; neuroprotection; photoreceptors; retina; retinal degeneration; retinal ganglion calls; signal transduction

Genetically determined retinal degenerations (RDs) are a heterogeneous group of outer retinal diseases caused by mutations in genes of the phototransduction cascade, genes encoding structural proteins integral to photoreceptor (PR) outer segment assembly and integrity, critical retinal pigment epithelial cell functions, ocular differentiation, and systemic metabolism. The underlying pathophysiology of RDs can be divided into several distinct groups comprising: (i) defects in disc morphogenesis and protein routing; (ii) phototransduction cascade mutations that create the equivalent of chronic photic activation; (iii) metabolic overload and toxicity; (iv) loss of trophic support; and (v) developmental defects. Over the past decade, the genetic mutations that cause many of these degenerations have been well characterized, and numerous experimental animal models have been developed (knockout, knockin, light-induced, and transgenic) that have permitted the initial dissection of the molecular mechanisms underlying the death of PR cells in these diseases.

Glaucoma is a progressive optic neuropathy characterized by the loss of retinal ganglion cells (RGCs) from the inner retina. Risk factors for glaucoma include elevated intraocular pressure, genetic predisposition, and race, among others but to date, the known genetic mutations underlying the development of glaucoma remain incompletely understood. RGC death has been extensively studied in vitro and in vivo using primary and established RGC neuronal cell lines and animal models of optic nerve injury. Studies of the role of trophic factors in RGC survival following optic nerve injury parallel a large body of work on the role of neurotrophic factors in the survival of central nervous system (CNS) and peripheral nervous system (PNS) neurons. Much of the following analysis of the pathways affecting the survival of PRs and RGCs will take place in the context of what has been learned about the mechanisms by which neurotrophic factors protect neurons in the CNS.

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The goal of this study is to review our current understanding of how the activation of several key signal transduction pathways by different classes of neurotrophins may result in the protection of retinal neurons and to review the molecular mechanisms that underlie the survival of retinal cells. The discussion will permit us to use current models to draw certain parallels between the survival of CNS neurons and RGCs and to identify specific distinctions between protection of these cells and PR neuroprotection and survival.

PATHOPHYSIOLOGY OF RETINAL DEGENERATIONS

It is axiomatic that the final common pathway of PR and RGC neuronal degeneration in the retina is apoptosis [Chang et al., 1993; Lolley et al., 1994; Portera-Cailliau et al., 1994; Tso et al., 1994; Adler, 1999]. What is less clear is how disparate genetic defects ultimately drive the cell down the apoptotic pathway. For example, why do rod outer segment (ROS) structural defects and open membrane channels both commit the PR cell to a premature demise? Why are retinal neurons protected from the effects of genetically programmed degeneration by certain growth factors, but not others, and what is the specific mechanism of action by which neurotrophic cytokines protect diseased or injured retinal neurons from apoptosis? To answer these questions, we will begin with what is known about how retinal and non-retinal neurons die following the loss of neurotrophic support.

Retinal Ganglion Cell Degeneration

The programmed cell death (PCD) of central nervous system neurons and glia is a prominent feature of brain development and is controlled by the availability of target-specific neurotrophic factors [Oppenheim, 1991, review]. Critical dependence upon neurotrophic factors as well as the loss of neurotrophic and neuropoetic support during development underlies the high level of PCD seen in the CNS, and this dependence is now believed to play a similar role in acquired neuropathological conditions such as Alzheimer's and Parkinson's disease [Thompson, 1995].

Trophic factor deprivation (TFD) by serum, K^+, and neurotrophin depletion has been used to model neuronal apoptosis in vitro. TFD has been demonstrated in primary and established CNS and PNS neuronal cell lines, and it has proven to be a powerful method for understanding the cellular responses to neurotrophic signaling and activation of PCD within the cell [Estus et al., 1994; Armstrong et al., 1997; Philpott et al., 1997; Harris et al., 2002; Putcha et al., 2002]. TFD-induced apoptosis has been demonstrated in an immortalized rat RGC cell line [Krishnamoorthy et al., 2001], and shares many characteristics with TFD in CNS neurons.

RGC survival is also modeled by experimental optic nerve injury in vivo [Wein and Levin, 2002, review]. Transection (axotomy) or a crush injury of the optic nerve induces reproducible retrograde axonal degeneration and cell death of RGCs in small animals. RGC survival, functional electroretinography, and secondary effects on the retina can all be examined using this model, and the efficacy of intraocular and systemic neuroprotective agents and gene therapy can be determined in vivo. Finally, the mechanisms effecting PCD and the neuroprotection (NP) signaling pathways in RGCs have been elucidated by transgenic manipulation and inhibition of the signal transduction intermediaries using this model; they are presented in detail below.

Photoreceptor Degeneration

To a large degree, the links between the known pathophysiology of the various types of genetically determined photoreceptor degenerations and the PCD seen in these conditions remains obscure. The same mutant genotype commonly gives rise to different clinical phenotypes [Phelan and Bok, 2000]. However, it is possible to speculate about the mechanisms of induced damage in the retina from what we do know. These speculations may be borne out by recent molecular evidence of the activation of intracellular signaling pathways in the experimental models discussed.

Abnormal disc morphogenesis is seen in RDs associated with rhodopsin, peripherin/*rds*, cGMP phosphodiesterase, Rim1, and other mutations [Pierce, 2001, review]. Protein misrouting to synaptic terminals and abnormal vesicle formation are also seen in rhodopsin mutations [Colley et al., 1995]. Abnormal ROS and inner segment architecture results in shortened ROS and disrupted interactions with the microvilli of the retinal pigment epithelium (RPE). The intimate connections between the

ROS and the RPE are critical for the RPE to provide the necessary trophic functions to maintain the health of the PRs, such as recycling of vitamin A, phagocytosis of shed ROS (the primary defect in the Royal College of Surgeons (RCS) rat), and neurotrophic factors. Disruption of the normal ROS/RPE architecture may diminish trophic support in the outer retina. There is normally a high oxygen gradient across the length of the ROS. Disruption of oxygen metabolism from loss of cGMP-gated cation channels in the ROS has been speculated to reduce the load on the Na^+/K^+ ATPase pumps and to reduce oxygen consumption resulting in increased levels of O_2 and reactive oxygen species at the level of the PR cell bodies [Travis, 1998]. Damage from various reactive oxygen species and lipid peroxidation may induce PCD in this model.

Transduction cascade mutations, such as the autosomal recessive mutation in the cyclic GMP phosphodiesterase gene, cause rapid retinal degeneration in the rd^-/rd^- mouse and result in the accumulation of toxic levels of intracellular cGMP [Lolley et al., 1977]. Elevated cGMP may lead to increased conductance of cGMP-gated cation channels, increasing influx of Na^+ and Ca^{++} ions. This could cause a metabolic overload and stimulate Ca^{++}-mediated apoptosis. The high metabolic activity and large numbers of mitochondria necessary to sustain oxidative metabolism in the PR have also been speculated to contribute to a higher risk of Ca^{++}-induced apoptosis [Travis, 1998].

Rhodopsin mutations are pure rod cell defects; yet in retinitis pigmentosa (RP) the mutation eventually results in the degeneration of cone cells as well. This non-cell-autonomous PR degeneration in RP suggests that secondary degeneration of cones may be caused by liberation of a diffusible apoptosis-inducing factor from degenerating rods [Seigel and Liu, 1997] or perhaps by TFD from an unknown rod cell trophic factor. A similar degenerative effect on rod cells has been demonstrated in cone deficient transgenic mice [Ying et al., 2000]

RPE Dysfunction

The health of the neurosensory retina is critically dependent upon the RPE for certain functions, including phagocytosis of diurnally shed PR outer segments, recycling of vitamin A metabolites, and, probably, trophic factor production. A mutation in the receptor tyrosine kinase Mertk locus results in the defect in phagocytosis seen in the RCS rat and causes a rapid retinal degeneration thought to result from accumulation of toxic PR membrane debris in the subretinal space [D'Cruz et al., 2000]. Accumulations of debris from incompletely digested PR membranes have also been postulated to be the source of drusen in patients with age-related macular degeneration [Mata et al., 2000]. The mechanism of injury is unknown but may be caused by damage from membrane peroxidation, which could induce stress-related apoptotic pathways. Toxic damage to the RPE resulting from the accumulation of vitamin A metabolites may also be a factor in the *RPE65* mutation, a cause of Leber's congenital amaurosis [Redmond et al., 1998].

Another function of the RPE is the production of trophic hormones, like basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-1), that have paracrine and autocrine activity in the RPE and may play a role as survival factors for the overlying neurosensory retina [Schweigerer et al., 1987; Waldbillig et al., 1991; Martin et al., 1992; Takagi et al., 1994; Gupta et al., 1997]. Pigment epithelial derived factor from the RPE was also recently shown to have protective effects in retinal ischemia [Ogata et al., 2001]. Atrophy of the RPE cell layer in vivo, with loss of trophic support may induce a physiologic effect in PRs analogous to TFD-induced apoptosis in CNS neurons and RGCs.

Chronic Activation of the Phototransduction Cascade

Chronic activation of the phototransduction cascade, from either genetic mutation or experimental manipulation using constant light, can induce PR degeneration. Mutations in genes regulating the activated state of rhodopsin, such as arrestin and rhodopsin kinase, induce mild forms of RD in cyclic light conditions in animal models. Retinal degeneration is more severe when the animals are exposed to constant light, and is prevented entirely if they are raised in the dark [Chen et al., 1999a,b]. The "equivalent-light" induction of apoptosis [Fain and Lisman, 1993; Lisman and Fain, 1995] in these models may be caused by several factors including, hypermetabolic states, abnormal calcium homeostasis, and oxidative stress and lipid peroxidation [Travis, 1998; Fain and Lisman, 1999].

APOPTOSIS IN RETINAL NEURONS

The induction of apoptosis in retinal neurons appears to occur via the major PCD pathway described for neurons of the CNS. This includes, in part, activation of specific caspases, caspase-9 and caspase-3, activation of the c-jun N-terminal kinase (JNK) pathway, and the proapoptotic Bcl-family proteins.

Caspases

The cysteine-containing, aspartate-specific proteases (caspases) are key initiators and effectors of apoptosis in neurons. Intrinsic apoptosis signals induce the release of cytochrome c, which complexes with Apaf-1 and procaspase-9, leading to activation of PCD through the effector caspases. Extrinsic apoptotic signals are mediated through death receptors of the tissue necrosis factor (TNF) superfamily like Fas, which triggers activation of the initiator caspase-8 [Cohen, 1997].

The apoptosis effector protein caspase-3 plays a role in developmental PCD in the brain [Kuida et al., 1996], and its activation also plays a role in K^+/serum withdrawal-induced apoptosis [Armstrong et al., 1997]. Caspase-3 activation by the initiator caspase-9 is also a key factor that induces apoptosis in RGCs after optic nerve transection, and likely results from release of cytochrome c by the mitochondria [Kermer et al., 1998]. Inhibitors of the caspase-9 significantly increase RGC survival in the axotomy model [Kermer et al., 2000].

Caspase-3 is also activated in outer retinal degeneration in the transgenic rat expressing the S334ter rhodopsin mutation [Liu et al., 1999]. Inhibition of caspase-3 activity in this model inhibits PR degeneration, suggesting that a caspase-3-dependent mechanism may play a role in genetically determined PR degeneration. Other caspases appear to play a role in inducing PCD in retinal neurons as well. Caspase-2 is expressed in the RGC layer following ischemic injury in the rat and is suppressed by intraocular injection of brain-derived neurotrophic factor (BDNF). In the constant light-induced model of PR degeneration, caspase-1 appears to play a more significant role in PCD. Caspase-1 was found in the outer nuclear layer [Katai and Yoshimura, 1999; Grimm et al., 2000], and PR cell death was not associated with an increase in the expression of caspase-3.

Although the caspase-3 inhibitor z-VAD-fmk (Kamiya Biomedical, Seattle, WA) protects transgenic rats with the S334ter opsin mutation in vivo, inhibition of caspase-3 with z-VAD-fmk did not protect PRs harboring the rd^-/rd^- mouse cGMP phosphodiesterase mutation in vitro [Caffe et al., 2001]. Thus, the relative neuroprotective effects of caspase-3 inhibition may be mutation-specific, or there may be fundamental differences in the mechanisms of caspase-induced apoptosis in retinal cells in vitro and those in vivo.

JNK Pathway and Key Transcription Factors

The JNK pathway plays a major role in TFD-mediated apoptosis in CNS neurons. The c-jun protein is activated by phosphorylation by the JNK kinases [Pulverer et al., 1991] and forms homo- and heterodimers with c-fos and other nuclear proteins, which together comprise the AP-1 transcription factor (TF). The AP-1 TF binds nuclear response elements and mediates transcriptional regulation. The JNK pathway is activated in neurons undergoing TFD and has also been shown to be involved in apoptosis in neuronal PC12 cells [Xia et al., 1995]. TFD induces sustained activation of the JNK pathway with phosphorylation and activation of c-jun [Estus et al., 1994], and this sustained activity appears to be required to induce apoptosis.

TFD also causes the translocation of the proapoptotic protein Bax from the cytosol to the mitochondrial membrane and release of cytochrome c with subsequent activation of caspases [Putcha et al., 1999; Harris et al., 2002]. PCD-arrested neurons undergo several metabolic changes, including decreased protein synthesis, RNA synthesis, and glucose uptake, and are metabolically compromised [Deshmukh et al., 1996]. Thus, survival factors that stop apoptosis may leave the surviving cells in a weakened state and susceptible to subsequent apoptosis from other signals. This may account for some of the differences seen in the efficacy of growth factors (GFs) as survival factors.

The central role of c-jun in TFD-induced apoptosis is demonstrated by the ability of blocking antibodies and dominant negative c-jun constructs to inhibit apoptosis when injected directly into neurons [Estus et al., 1994]. Blocking the JNK pathway using the mixed lineage kinase (an upstream c-jun kinase) inhibitor CEP-1347 (Cephalon Inc, West Chester,

PA) inhibits TFD-induced neuronal cell death in sensory, sympathetic, and motor neurons [Maroney et al., 1999; Harris et al., 2002] and mimics the NP effects of neurotrophins. Sustained activation of the JNK pathway is required for cell death from TFD and is required for the associated metabolic dysfunction. In sympathetic neurons, phosphatidylinositol 3-kinase (PI3-K) activation is protective even in the setting of c-jun activation from TFD, but it does not change c-jun expression. Similar results are seen with BDNF protection of RGCs following ischemia [Kurokawa et al., 1999]. Inhibition of PI3-K activation using LY294002 (Cell Signaling Technology, Beverly, MA) or by a dominant negative PI3-K does not cause cell death in the presence of trophic factors like nerve growth factor (NGF). Thus, neuronal protection by trophic factors like NGF and BDNF occur via multiple protective pathways, including PI3-K, and the protective features of PI3-K activation must occur independently from c-jun or downstream of c-jun activation.

As noted above, the constant light model of RD induced PCD in part by activating AP-1. The c-fos, c-jun, and caspase-1 proteins are upregulated in response to light-induced damage [Grimm et al., 2000]; however, proapoptotic genes such as Bad, Bax, caspase-3 were not upregulated, nor were the antiapoptotic genes Bcl-2 and Bcl-X_L. Interestingly, c-fos$^{-/-}$ mice are resistant to constant-light-induced PR cell death [Hafezi et al., 1997; Wenzel et al., 2000]; therefore, c-fos and, by extension, the AP-1 transcription factor must play a critical role in inducing PCD in the constant-light model. Genetic and chemical induction of apoptosis is not inhibited in c-fos$^{-/-}$ mice [Wenzel et al., 2000], suggesting that c-fos activation, as a key intermediary in apoptosis may be a light-model-specific phenomenon. The forkhead transcription factor FKHRL1, and Bad also mediate TFD-mediated apoptosis in CNS neurons. TFD leads to FKHRL1 dephosphosphorylation, causing its translocation to the nucleus and target gene activation.

Proapoptotic Bcl-Family Proteins

In neurons, as in other cells, the balance between survival and cell death is determined by the relative level of activated pro- and antiapoptotic signaling proteins, a so-called apoptotic thermostat. Proapoptotic proteins like Bad and Bax induce mitochondrial permeability, which leads to cytochrome c release and cell death [Yang et al., 1995; Pastorino et al., 1996]. Antiapoptotic proteins like Bcl and Bcl-X_L inhibit this association and shift the balance of cell homeostasis away from apoptosis.

The susceptibility of RGCs and PRs to apoptotic signals likely depends upon the state of balance between these signals at the time of the insult. Growth factors, survival factors, and other extracellular signaling molecules that activate the PI3-K and MEK/ERK survival pathways (see below) would tend to tip the balance in favor of survival. These survival cascades act to inhibit the caspase activation pathways and proapoptotic proteins and result in a cellular state that would favor survival. Specific inhibitors of the proapoptotic proteins, as well as upregulators of the antiapoptotic Bcl-family proteins would be expected to have a similar effect in promoting cell survival.

Supporting Actors in Retinal Apoptosis

In addition to the proteins of the caspase activation pathway and JNK-mediated transcriptional activation, several other molecules have been shown to induce apoptosis in retinal neurons or play a supporting role in the process. TNFα and its receptor are upregulated in the retina and in the optic nerve of patients with glaucoma and have been posited to play a role in RD in human glaucoma [Yuan and Neufeld, 2000; Tezel et al., 2001]. The presence of the TNFα receptor in RGCs also renders them sensitive to TNFα-induced apoptosis in tissue culture [Tezel and Wax, 2000]. The role of TNFα in RD is not entirely clear, however, because some evidence suggests that it may also have protective effects, by activating PI3-K and inducing activation of NF-κB [Choi et al., 2000].

Neurotransmitter release may also play a role in potentiating neuronal damage in the retina by excitotoxicity. Glutamate is only mildly toxic to the retina when Müller cell glutamate transporters are functional and evidence suggests that glutamate transport can regulate the neurodegenerative effects of glutamate in the rat retina [Izumi et al., 2002], implying a possible role for neurotransmitter blockade in mediating excitotoxic RD. Similarly, N-methyl-D-aspartate receptor antagonists inhibit PCD in an optic nerve crush model [Yoles et al., 1997] and may be protective in an experimental model of glaucoma by altering the regulation of neurotransmitter release and reducing RGC

injury [Hare et al., 2001]. Glutamate-induced reactive oxygen species-mediated apoptosis in cerebellar granule cells is inhibited by BDNF [Skaper et al., 1998]. This protective effect was reduced by the MEK/ERK pathway inhibitor PD98059 (Sigma, St. Louis, MO), indicating that this pathway is involved in protection against neurotransmitter-induced apoptosis in primary neurons.

GROWTH FACTORS IN RETINAL NEUROPROTECTION

The growth factors that have been shown to provide the most neuroprotection to retinal neurons in vivo and in vitro are ligands for two major families of membrane-bound receptor tyrosine kinases (RTKs), the fibroblast growth factor receptor and the Trk neurotrophin receptor family [LaVail et al., 1992]. These include basic FGF, the neurotrophic cytokines NGF and BDNF, and the neuropoetic cytokine ciliary neurotrophic factor (CNTF).

Fibroblast Growth Factors

Retinal degeneration is delayed (but not prevented) by intravitreal injection of aFGF and bFGF proteins and viral-mediated bFGF gene transfer in animal models of light-induced retinal injury and in several animal models of genetic RD (Fig. 1) [Faktorovich et al., 1990; LaVail et al., 1991; LaVail et al., 1992; Perry et al., 1995; McLaren and Inana, 1997; Akimoto et al., 1999; Uteza et al., 1999; Lau et al., 2000]. Fibroblast growth factor gene therapy also improves functional electroretinography [Ali et al., 2000]. Endogenous bFGF is clearly involved in the repertoire of retinal cell responses to injury and is upregulated in response to diverse retinal injuries, including those resulting from constant light [Gao and Hollyfield, 1996; Nir et al., 1999], laser photocoagulation [Xiao et al., 1998], mechanical puncture [Faktorovich et al., 1992], and optic nerve crush [Bush and Williams, 1991].

Optic nerve crush protects PRs from subsequent photic damage [Bush and Williams, 1991] and is associated with upregulation of the expression of bFGF in PRs in rodent retina and in astrocytes in the optic tract [Kostyk et al., 1994]. Other cells or long-range intercellular signaling must mediate NP in this model, because PRs and RGCs do not synapse directly. Expression of bFGF is also modulated by the

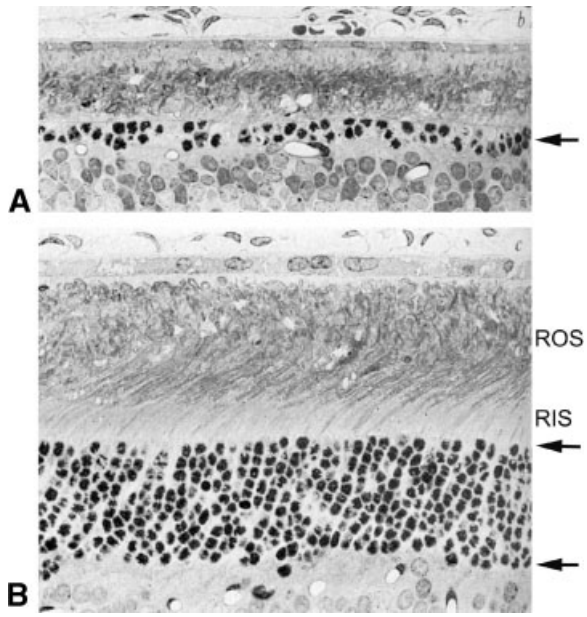

Fig. 1. Plastic-embedded sections of Royal College of Surgeons (RCS) rat retinae. **A**: Postnatal day 53 (P53) retina from a control animal. The outer nuclear layer is reduced to 1–2 rows in thickness (arrow). The photoreceptor inner segments (RIS) and outer segments (ROS) are absent and the outer segment zone is composed of membrane debris. **B**: Section from a P53 retina, in a region of optimum survival, 1 month after subretinal injection of bFGF. The outer nuclear layer is 8–10-cell thick (arrows) and the RIS and ROS are well preserved and unchanged from the time of injection. The control eye in the same animal was fully degenerated. Reprinted by permission from *Nature,* Faktorovitch EG, Steinberg RH, Yasumura D, Matthes MT, LaVail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. Nature 347:83–86. Copyright 1990, Macmillan Publishers Ltd.

RPE and it acts as an outer retinal survival factor in vivo [Hackett et al., 1997] and as a survival factor for purified PRs in vitro [Fontaine et al., 1998]. Basic FGF transcripts are seen in the RPE, the retinal inner nuclear layer, PR inner segments [Wen et al., 1998], and Müller cells [Cao et al., 1997]. FGF receptors are also present in the retina [Tcheng et al., 1994] and bFGF is bound by ROS [Fayein et al., 1990], demonstrating the capacity for PRs to respond directly to FGF survival signaling.

Expression of bFGF may also play a larger role in providing constitutive trophic support to the retinal PRs. Inhibition of bFGF signaling in normal retina by a dominant negative bFGF receptor has been shown to induce retinal degeneration in a transgenic model [Campochiaro et al., 1996]. Thus, bFGF may provide critical ongoing trophic support to the outer retina, not

just NP under conditions of stress or genetic degeneration.

Brain-Derived Neurotrophic Factor and Nerve Growth Factor

All of the known cellular effects of neurotrophic cytokines stem from ligand binding to membrane-bound RTKs, because mutations of tyrosine kinase activity render neurotrophins biologically inactive. The BDNF receptor, TrkB, is expressed in RGCs and dopaminergic amacrine cells in the vertebrate retina [Maisonpierre et al., 1990; Jelsma et al., 1993; Cellerino and Kohler, 1997] and by the RPE [Bennett et al., 1999]. TrkB receptor immunoreactivity is also seen in cones but not rods.

There is ample evidence to show that BDNF provides autocrine and paracrine trophic support to RGCs. BDNF supports the survival of cultured embryonic rat RGCs [Johnson et al., 1986] and both BDNF and NT-4 (another TrkB ligand) protect immortalized rat RGCs from TFD-induced PCD [Krishnamoorthy et al., 2001]. Upregulation of Trk receptor gene expression appears to be a normal response of RGCs to axotomy [Cui et al., 2002] and BDNF enhances the survival of RGCs after optic nerve injury [Mansour-Robaey et al., 1994]. RGCs likely require a combination of growth factors in vitro, including insulin (or IGF-2), CNTF, BDNF (or neurotrophins NT-4/5), and intracellular cAMP, for survival [Meyer-Franke et al., 1995]. Transgenic expression of BDNF prolongs the survival of retinal ganglion cells after experimental axotomy [Di Polo et al., 1998], and axon-mediated gene transfer of a Trk receptor into RGC via retrograde transport from axonal terminal arbors delays axotomy-induced apoptosis [Garcia and Sharma, 1998]. TrkB gene transfer into RGCs together with administration of exogenous BDNF also increases RGC survival [Cheng et al., 2002]. NGF is also a survival factor for RGCs following optic nerve transection. Repeated intraocular injections of NGF protect RGCs from degeneration following optic nerve transection for up to 7 weeks [Carmignoto et al., 1989].

Ciliary Neurotrophic Factor

Expression of the neuropoietic cytokine CNTF (or intraocular injection) delays photoreceptor degeneration in several types of genetic degeneration and ischemic injury [Unoki and LaVail, 1994; Cayouette and Gravel, 1997; Bok et al., 2002]. Although CNTF transgene expression rescues photoreceptor cells in small animal models, it may not preserve normal retinal function [Liang et al., 2001]. CNTFα receptors are mainly expressed in neurons and glia and are found in the inner retina but do not appear to be present in PRs.

Endogenous CNTF is upregulated in response to retinal injury [Faktorovich et al., 1992; Wen et al., 1995] and constant light [Wen et al., 1995]. In addition, preconditioning the retina with sublethal stress resulted in prolonged activation of bFGF and CNTF [Liu et al., 1998]. CNTF and BDNF provide more effective NP of rd^{-}/rd^{-} photoreceptors than either cytokine alone, so there may be an additive or synergistic effect of these and other survival factors in the retina [Caffe et al., 1993, 2001]. As noted above, neither receptor is expressed by the PR cell, although both are expressed by RPE and Müller cells. This raises the question of whether the NP signal is transduced by other cells in the retina. CNTF protects the Müller cells. Are the Müller cells (and perhaps the RPE) secondarily protecting the rods and cones?

NEUROPROTECTIVE SIGNALING PATHWAYS

The intracellular signaling activated by diverse neurotrophins, such as NGF, CNTF, and FGF, utilize overlapping transduction cascades. Ligand binding to the RTKs is characterized by intermolecular autophosphorylation. The stimulated kinase domain phosphorylates tyrosine residues that provide docking sites for downstream target molecules in the signal transduction cascade. Different receptor molecules give rise to distinct signaling outputs, but these are often mediated through common intermediaries of downstream signaling pathways. In addition, RTKs can be activated by more than one ligand, but with different affinities and potentially different signaling outputs. Thus, two ligands may differentially activate the same signaling cascade through the same, or different receptors. The interconnections between neurotrophin receptor signaling will be discussed in detail below.

PI3-K/Akt Signaling Pathway

The PI3-K signaling pathway is activated by ligand binding to several RTKs, including the FGF and Trk receptor families. In vitro studies show that NGF-mediated survival (via the TrkA

receptor) uses the PI3-K pathway, whereas TrkB receptor signaling by BDNF and NT-4 occurs through both PI3-K and alternative pathways. NGF induces sustained activation of the PI3-K downstream mediator Akt [Coffer et al., 1998], and suppresses the JNK activation that induces apoptosis upon TFD [Virdee and Tolkovsky, 1996]. Activation of PI3-K mediates numerous cellular functions, including proliferation, chemotaxis, metabolic homeostasis, and importantly, inhibition of apoptosis [see Katso et al., 2001, review]. The anti-apoptotic effects of PI3-K cascade are mediated through phosphorylation of the key downstream effector substrate Akt (protein kinase B), a serine/threonine kinase [Dudek et al., 1997; Kauffmann-Zeh et al., 1997] (Fig. 2).

Akt promotes cell survival in several different ways: (i) it phosphorylates and inhibits the proapoptotic protein Bad; (ii) it inactivates IKK-α, the inhibitor of the survival factor nuclear factor kappa-B (NF-κB); (iii) it inhibits the proapoptotic forkhead transcription factor FKHLR1; and (iv) it directly inhibits the activation of PCD initiator caspase-9 [Datta et al., 1997; Katso et al., 2001]. Direct activation of PI3-K is sufficient to permit neuronal cell survival in the absence of NGF and prevents cell death from TFD-induced NGF withdrawal. PI3-K/Akt activation is sufficient to permit the survival of sympathetic neurons in the absence of NGF [Philpott et al., 1997]. Akt activation appears to be critical to the survival of oligodendrocytes treated with TNF-α [Pastorino et al., 1999; Takano et al., 2000] because overexpression of a dominant negative Akt mutant inhibited the protective effects of NGF in this model. Akt can also be activated through other RTK survival pathways, like IGF-1, which has been shown to mediate neuronal cell survival [Dudek et al., 1997; Zheng et al., 2002]. RGC survival mediated by the IGF-1 receptor is PI3-K pathway dependent [Kermer et al., 2000]. PI3-K activation appears to be an important (perhaps

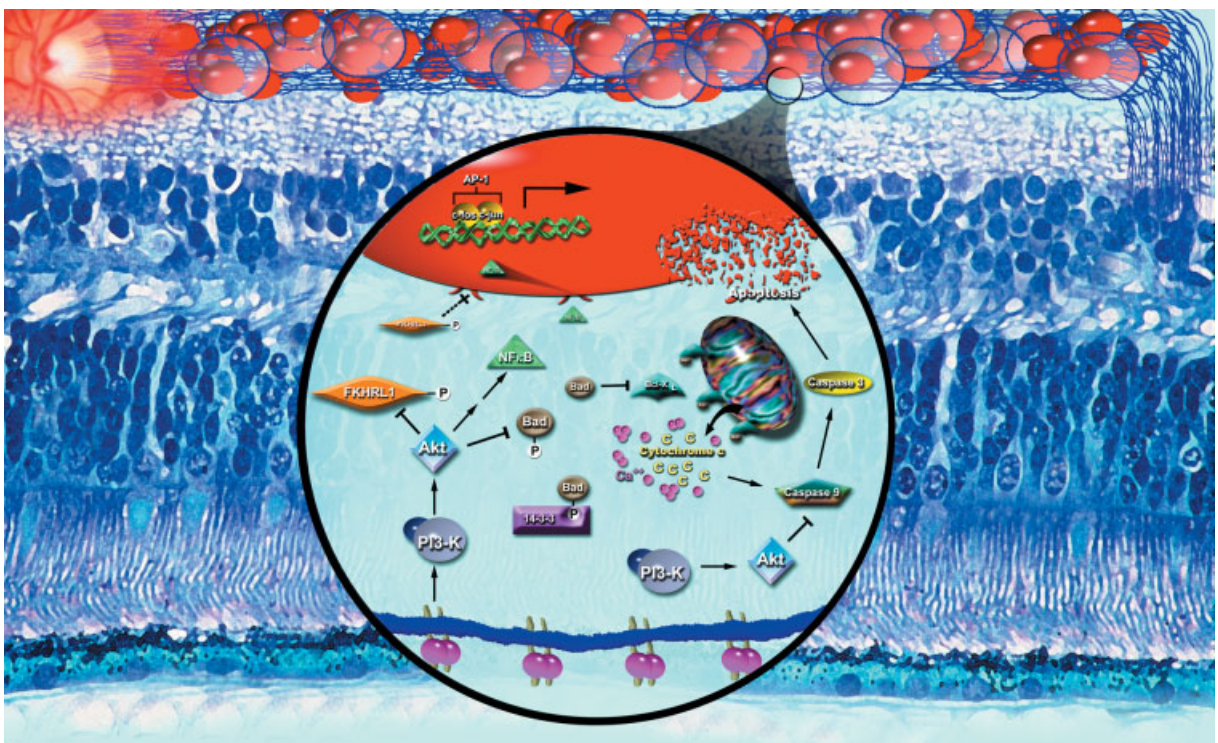

Fig. 2. Schematic overview of the phosphatidylinositol 3-kinase (PI3-K) signal transduction cascade. The anti-apoptotic effects of PI3-K cascade are mediated through phosphorylation of the key downstream effector substrate Akt. Akt promotes cell survival by phosphorylating and inhibiting the proapoptotic protein Bad, by inactivating the inhibitor of the survival factor nuclear factor kappa-B (NFκB), by phosphorylating and inhibiting the proapoptotic forkhead transcription factor FKHLR1, and by inhibiting the activation of caspase-9. The AP-1 transcription factor, c-fos/c-jun, binds nuclear response elements and mediates transcriptional regulation of apoptotic events. Phosphorylated Bad is sequestered in the cytosol by 14-3-3 family proteins, preventing its binding to antiapoptotic Bcl family proteins like Bcl-X_L. Inhibition of the antiapoptotic Bcl-proteins permits release of cytochrome c and calcium (Ca^{++}) from the mitochondria and induces apoptosis.

critical) factor for the survival of some neurons. Wortmannin (an inhibitor of PI3-K, Sigma, St. Louis, MO) induces death in PC12 cells, even in the presence of NGF [Yao and Cooper, 1995]. PI3-K activation is also the part of the intrinsic repertoire of neuronal responses to injury in the retina [Hayashi et al., 1996].

RTK binding by neurotrophins also activates PI3-K-independent survival pathways (e.g., MEK/ERK, see below) since, in the presence of NGF, inhibition of PI3-K does not prevent NGF-mediated neuronal survival [Philpott et al., 1997]. This was also shown for neurons where the JNK inhibitor CEP-1347 protects neurons but does not act via the PI3-K pathway. Therefore, neurons can survive in the absence of PI3-K signaling, provided alternative pathways are available [Harris et al., 2002].

BDNF-mediated neuronal survival in vitro is through sustained coactivation of the PI3-K and mitogen-activated protein kinase (MEK/ERK) pathways and is inhibited by K-252a (a Trk inhibitor, Kyowa Hakko Kogyo Co., Tokyo, Japan) [Yamada et al., 1997]. BDNF protects cortical neurons from TFD-induced apoptosis [Hetman et al., 1999]. The PI3-K pathway is the major survival pathway in TFD; however, the ERK pathway is the major one for BDNF protection from camptothecin-induced apoptosis. Thus, different survival pathways may predominate depending on the nature of the proapoptotic signal. BDNF protects adult RGCs in vivo [Klocker et al., 2000] and activates both PI3K and MEK/ERK pathways to suppress the activation of effector caspase-3, but PI3-K pathway inhibition does not attenuate BDNF protection of RGCs through the MEK/ERK pathway.

Forkhead transcription factors are key mediators of Akt signaling in the survival response. FKHRL1 phosphorylation by Akt retains the transcription factor in the cytoplasm (by association with 14-3-3 proteins) and inhibits apoptosis [Brunet et al., 1999]. Downregulation of forkhead transcription factors may be more important to neuronal survival than downregulation of proapoptotic genes or upregulation of NF-κB-dependent signaling [Marshall, 2000].

The 14-3-3 proteins recognize serine-phosphorylated targets and work in concert with various signal transduction cascades like PI3-K to modify the intracellular location of recognized proteins by sequestering them in specific cell compartments. For example, Akt phosphorylates FKHRL1 and Bad, and by doing so, Akt inactivates each target protein. The phosphorylated target is then bound by a 14-3-3 protein and sequestered in the cytoplasm, preventing transcriptional activation and dimer formation with Bcl-X_L, respectively. Thus, the 14-3-3 proteins play a supporting role in antiapoptotic signaling via a variety of signal transduction events that may be relevant to cell survival.

MEK/ERK Signaling Pathway

The mitogen-activated protein kinase cascade (MEK/ERK) is a highly conserved, signaling pathway that integrates many extracellular signals, primarily through ligand binding to the RTKs. Activation of the extracellular signal-related kinase kinase (MEK) and phosphorylation of its substrate, extracellular signal-related kinase (ERK), is dependent upon RTK-mediated Ras activation through Sos, Grb2, and other adaptor proteins [Kolch, 2000] (Fig. 3). The MEK/ERK pathway controls the expression of genes regulating cell proliferation, differentiation, and apoptosis and crosstalks with other signal transduction pathways, such as PI3-K. The MEK/ERK pathway is activated not only by RTK binding, but also by reactive oxygen species, and it has anti-apoptotic effects by opposing the proapoptotic effects of JNK and stress-activated p38 MAP kinase.

Neurotrophins induce the sustained activation of the MEK/ERK pathway. Ras activation supports the survival of some neurons following TFD [Borasio et al., 1993; Nobes and Tolkovsky, 1995]. Conversely, inhibition of the Ras/MEK/ERK pathway by PD98059 does not induce sympathetic neuron cell death [Virdee and Tolkovsky, 1996], consistent with alternative survival pathways in neurons.

TrkB gene expression is downregulated in RGCs after axotomy and correlated with a lack of protection by BDNF after axotomy, even though BDNF gene expression is upregulated [Gao et al., 1997]. TrkB receptor gene transfer into RGCs, together with administration of exogenous BDNF, does increase RGC survival by a MEK/ERK-mediated mechanism [Cheng et al., 2002]. Thus, increased cytokine expression alone may not be sufficient to protect injured retinal neurons.

Activation of the MEK/ERK pathway is seen in both PRs and in Müller cells, and phosphorylated ERK is protective in a constant-light

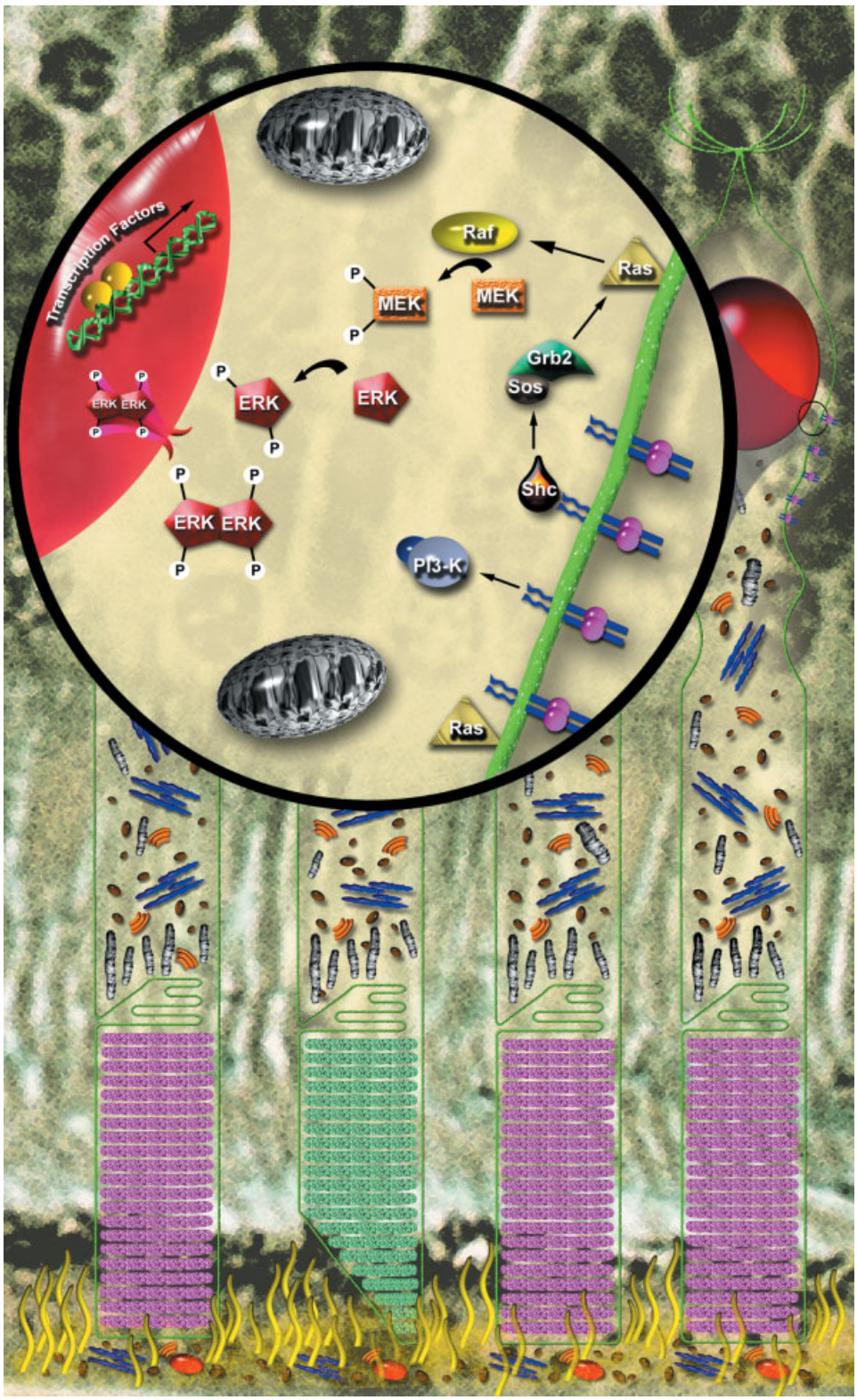

Fig. 3. Schematic overview of the mitogen-activated protein kinase (MEK/ERK) signal transduction cascade. Neurotrophins induce the sustained activation of the MEK/ERK pathway via Ras activation mediated through adaptor proteins such as Shc, Sos, and Grb2. Activation induces sequential phosphorylation of extracellular signal-related kinase kinase (MEK) and its substrate, extracellular signal-related kinase (ERK). Phosphorylated ERK dimerizes and translocates to the nucleus where it functions as a transcriptional activator, promoting proliferative, and survival gene expression. Crosstalk also occurs with the PI3-K pathway.

model of RD. These findings suggest that ERK activation may mediate PR cell survival [Liu et al., 1998], perhaps through activity of the Müller cell [Wahlin et al., 2000]. An injury stimulated autocrine/paracrine loop that stimulates ERK activation may maintain high levels of antiapoptotic proteins like Bcl-2 [Desire et al., 2000], and be protective. Additional evidence suggests that NP by neurotrophins may show some cell specificity. Sustained MEK/ERK activation is not required for BDNF-induced cerebellar granule cell survival [Gunn-Moore et al., 1997]. CNTF-related cytokines and RTKs both activate ERK, but CNTF activation of ERK is delayed and inhibited by protein kinase inhibitor H7, whereas RTK activation of ERK is rapid and not inhibited [Boulton et al., 1994]. So, the neurotrophin activation of the same survival pathway through different RTKs may have a different biological effect, and differences in receptor, temporal activation, duration, and intensity of the signal, and crosstalk between pathways may confer different phenotypic responses to a similar neurotrophic signal.

FGF-mediated survival of retinal neurons in chicks is mediated through ERK activation and increased expression of Bcl-2 and Bcl-X_L. Inhibition of bFGF signaling enhances TFD-induced apoptosis. FGF delays PR degeneration in rats in vivo [Faktorovich et al., 1990; LaVail et al., 1992] and stimulates survival of PRs in vitro. PR survival appears to be dependent upon ERK activation via MEK but MEK-independent pathways also exist for inner retinal neurons and glia [Kinkl et al., 2001]. Pretreatment of PRs with the MEK inhibitor U0126 (Cell Signaling Technology, Beverly, MA) abolished bFGF-induced ERK phosphorylation and inhibited survival, and U0126 reduced, but did not abolish, ERK phosphorylation in the inner retina. However, these data were not confirmed for PRs in vivo, and it has been suggested that signaling is mediated indirectly through Müller cells or inner retinal neurons [Wahlin et al., 2000].

CNTF signal transduction is mediated through its interaction with its receptor, CNTFRα, and the transmembrane signaling molecules gp130 and leukemia inhibitor factor receptor-β. CNTF ligand binding activates both the Jak/STAT (signal transducer and activator of transcription) and the Ras/MEK/ERK pathways, which transduce the signal [Boulton et al., 1994; Heinrich et al., 1998, review] (Fig. 3). CNTF does not induce sustained activation of the MEK/ERK pathway. In addition, CNTF activates ERK in some cells, but not others, suggesting that pathways leading to ERK can be differentially activated in response to the same signals depending on the cell type and so elicit different cell type-specific phenotypic responses. Differences in the level of activation among other signaling intermediaries may also account for different activities.

Finally, there appears to be a fundamental difference in the response of neurons to the protective effects of neurotrophins following ischemic damage. MEK1 protein kinase inhibition protects against neuronal damage resulting from ischemic brain injury [Alessandrini et al., 1999; Namura et al., 2001]. MEK inhibitors appear to protect against ischemic reperfusion injury in part by blocking glutamate toxicity [Stanciu et al., 2000]. The mechanism may be mediated by enhanced c-fos expression or by inhibition of glutamate neurotransmitter release and reduced excitotoxicity. Thus, the retina may not benefit from neurotrophin signaling that activates the MEK/ERK cascade, following ischemic injury.

Bcl Family Proteins

The Bcl family of antiapoptotic proteins is found in the cytoplasm and outer mitochondrial membrane. They regulate the transmembrane potential and control the release of apoptosis-inducing cytochrome c into the cytoplasm. They are ubiquitously expressed, including in cells of the retina. The protective effects of the PI3-K/Akt and MEK/ERK signaling pathways are mediated, in part, by upregulation of the pro-survival members of the Bcl family, Bcl-2 and Bcl-X_L [Pugazhenthi et al., 1999, 2000]. As shown above, increased expression of Bcl-2 and Bcl-X_L inhibits TFD- and axotomy-induced apoptosis.

Evidence suggests that a homeostatic balance exists between pro- and antiapoptotic factors in the cell. Upregulation of antiapoptotic Bcl proteins is capable of providing NP to retinal neurons, even in the presence of PI3-K and MEK/ERK inhibition. Overexpression of Bcl-2 in transgenic mice protects neonatal motor neurons from degeneration after axonal injury in vivo, inhibits axotomy-induced apoptosis in RGCs in vivo, and preserves long-term RGC visual responses [Dubois-Dauphin et al., 1994; Porciatti et al., 1996]. Bcl-2 overexpression

delays PR degeneration by apoptosis from constant [Chen et al., 1996] and high-intensity light [Joseph and Li, 1996]. Transfer of the Bcl-2 gene to the retina delays retinal degeneration in several genetic models including, rd^{-}/rd^{-} [Bennett et al., 1998], rds^{-}/rds^{-} [Nir et al., 2000], and $Pdeg^{tm1}/Pdeg^{tm1}$ mice [Tsang et al., 1997].

Other antiapoptotic proteins may play a similar role in protecting PRs from PCD. Expression of the baculovirus caspase inhibitor protein p35 [Clem et al., 1991] protects PRs from degeneration and preserves PR function in *Drosophila* rhodopsin mutants $ninE^{RH27}$ and $rdgC^{306}$. This suggests that the evolutionarily conserved caspases mediate rhodopsin mutation-induced PR degeneration across species [Davidson and Steller, 1998].

There is some conflicting data in the literature about the efficacy of Bcl family genes in retinal neuroprotection. For example, overexpression of Bcl-2 can clearly inhibit neuronal cell death [Allsopp et al., 1993; Mah et al., 1993] and delay photoreceptor degeneration, however, Bcl-2 transferred to RGCs in rats increased their susceptibility to the cytotoxic effects of a glutamate agonist as well as to axotomy-induced optic nerve injury [Garcia and Sharma, 1998; Simon et al., 1999]. Light-induced apoptosis is accelerated in transgenic mouse retinas overexpressing human EAT/mcl-1, an anti-apoptotic gene related to Bcl-2 [Shinoda et al., 2001]. These data suggest that merely overexpressing Bcl-like genes may not always be protective.

Other Mediators of Neuronal Survival

Heat shock proteins (Hsps) appear to play a role in supporting the survival of retinal neurons. Induction of Hsp 72 protects RGCs in a rat model of glaucoma [Park et al., 2001], and Hsp 70 inhibits JNK activation and apoptosis. Conversely, inhibition of Hsp 27 can induce apoptosis via caspase-8 activation in the retina [Tezel and Wax, 1999].

Activated Hsps blunt the PCD response in the constant light model of retinal degeneration and can induce tolerance to lethal stress [Jolly and Morimoto, 2000]. Exposure of retinal cells to sublethal stress conditions, sufficient to induce the expression of Hsps protects against a subsequent light intensity challenge that by itself is lethal. Thus, the homeostatic balance between cell death and survival likely depends both upon the specific nature and intensity of the stress and the "state" of the cell. It is a combination of the activity of individual components of the survival signaling pathways and the relative contributions of other effector molecules, like Hsps, at the time of the stress that will ultimately determine the fate of the cell.

Shc A is a docking protein involved in intracellular signaling and acts as a scaffold for the assembly of signaling proteins in the MEK/ERK and other activated RTK pathways. Shc A has been shown to sensitize cells to small increases in epidermal and platelet-derived growth factors and is necessary to sensitize the cell to MEK/ERK activation when these growth factors are in low concentration [Lai and Pawson, 2000]. In certain circumstances, the ability to respond to external cues including survival signals, may be in part determined by the level of Shc A expression that acts to coordinate the activity of core signaling intermediaries. Linking core signaling pathways using different sets of adaptor proteins with activated receptors may be the method by which intracellular modulation of complex and overlapping extracellular signals occurs [Marshall, 2000].

SIGNALING PATHWAY AND CELLULAR CROSSTALK

There is significant overlap of the intracellular signaling cascades activated in response to FGF, BDNF, CNTF, and NGF ligand binding. As we have seen, the PI3-K and MEK/ERK cascades are the primary interpreters of extracellular neurotrophin signaling. In turn, these key intermediaries effect specific molecular responses that support neuronal survival, including (i) inhibition of caspases, Bad, and transcription factors, like FKHRL1; (ii) activation of prosurvival signals; and (iii) activation of adaptor proteins that mediate signaling. Using shared components, the main signaling pathways must integrate different extracellular stimuli into a defined cellular response and at the same time ensure that these related pathways are in some manner insulated from each other.

There is evidence of differential activation of the RTK receptors by various members of the families of receptor-specific ligands, suggesting that neurotrophins acting at the same receptor may effect distinct signaling responses in the cell [Minichiello et al., 1998]. These effects may be mediated by the duration of activation, by interactions with other pathways, and by the

homeostatic and developmental state of the cell. For example, BDNF supports the survival of cultured embryonic rat RGCs, but the effect is only transient in postnatal RGCs. [Johnson et al., 1986].

Another example is the Jak/STAT pathway, which mediates the effects of CNTF. Different receptors activate different patterns of Jak kinases, and can activate different combinations in different cell types [Stahl et al., 1994]. They may also recruit distinct sets of secondary substrates, such as adaptor and docking proteins, which could confer additional specificity of action through common intermediaries.

For protection to be afforded directly to neuronal cells, the cells must express receptors for the neurotrophin mediating the physiologic effect. Otherwise, the effect must be transduced through intermediaries that express the receptors. Activation of ERK by intravitreal injection of BDNF, CNTF, and bFGF causes an increase in c-fos immunoreactivity in Müller, amacrine, and RG cells, but not in PRs [Wahlin et al., 2000]. Similar results were seen in retinal explants and suggested that protection of PRs by these neurotrophins is mediated through intercellular signaling by the Müller cell. The intimate association between the ROS and the microvilli of the RPE suggests that intercellular signaling may also occur between PRs and the RPE. Coordinated trophic signaling directly to PRs and RGC neurons as well as intercellular communication must cooperate to mediate the effects of neurotrophic signaling in the retina.

SUMMARY

The role of survival factors in mediating neuronal apoptosis, and the intracellular molecular signaling that occurs in response to RTK receptor activation is now well established in the ophthalmic and neuroscience literature. The responses of RGCs and PRs to TFD-induced apoptosis models and experimental injury parallel those seen in CNS and PNS neurons. The apoptotic pathways mediating PCD in PRs are preserved across species and are driven primarily by activation of caspase and proapoptotic Bcl-family proteins. Transcriptional activation through the JNK pathway may also play a specific role in light-induced neuronal injury.

Neuronal fate appears to be determined by the relative levels of activated pro- and antiapoptotic proteins, and regulated in large part by RTK signaling. Neurotrophins acting through the RTKs activate both the PI3-K and MEK/ERK cascades in retinal neurons. Dissection of the cascades using kinase-specific inhibitors [Davies et al., 2000] suggests that the PI3-K pathway is probably the major survival pathway in RGCs in the TFD model of PCD, as well as after axotomy. In contrast, the MEK/ERK pathway is critical to the survival of PRs. The PI3-K pathway activation provides a strong protective effect through sustained activation of the downstream intermediary Akt. The protective effects of Akt activation are mediated primarily through phosphorylation of Bad and the FKHRL1 transcription factor, but other prosurvival effects also occur. PI3-K pathway activation by other RTK ligands, like IGF-1, have been shown to have NP effects in vitro [Dudek et al., 1997; Zheng et al., 2002] and may provide synergistic survival signaling to retinal neurons at risk for apoptosis. These data suggest that using gene therapy to overexpress key signaling intermediaries, like Akt, or antiapoptotic genes, like Bcl-2, may provide a more directed or complementary approach to neurotrophin therapy to protect the retina, and have potentially fewer adverse effects. The protective effects of Hsps in enhancing neuronal survival suggest that this may also be a fruitful area of additional investigation that could be synergistic with neurotrophin-based strategies.

The ideal goal of somatic neurotrophin therapy for RDs is to enable delivery of drugs or genes that can provide sustained trophic support to retina neurons, irrespective of the genetic mutation present. This goal may be difficult to achieve. To date, neurotrophins have only been shown to delay and not prevent RD. Genetic models may also show differences in efficacy depending on the mutation and underlying pathophysiology of the degeneration. In addition, the opposite effect of MEK/ERK pathway activation on RGC survival in axotomy and ischemic injury demonstrates that different experimental models of neuronal injury can give opposing results. If we want to apply therapeutic NP to human diseases, the models we use must more closely match the human disease processes. Additional work needs to be done utilizing ocular hypertensive models of RGC injury to determine whether the results obtained from growth factor NP in axotomy models can be extrapolated to the most common mechanism of RGC injury in patients with

glaucoma. Similarly, mutation-specific treatment strategies may need to be developed for the phototransduction cascade mutations causing retinitis pigmentosa.

For neurotrophic factors to be protective, the neuron must have adequate receptors or sufficient activation of intracellular intermediaries to facilitate transduction of the extracellular signal. PR-target cell signaling may also be mediated through the actions of Müller cells and the RPE, which can respond directly to neurotrophins. An alternative NP strategy in the retina may be to upregulate or induce de novo RTK gene expression. Increasing sensitivity to existing extracellular signals may also be possible by overexpressing signaling pathway adaptor intermediaries like Shc A.

New methods, like microarray technology, will help to further our understanding of the molecular responses of neurons to genetic, TFD, and experimentally induced injury and may point to new survival effector pathways. New protein transfer reagents (Gene Therapy Systems, San Diego, CA; Targeting Systems, Santee, CA; Active Motif, Carlsbad, CA; Pierce, Rockford, IL) can be used to deliver antibodies directly into retinal neurons in vitro, and perhaps in vivo. This method can inhibit the activity of specific signaling pathway intermediaries and determine the role of these molecules in mediating neuronal survival in genetic and experimental models and may also identify new points of integration and crosstalk between survival pathways.

The studies described above demonstrate the high degree of complexity in the cellular responses to neurotrophin signal integration. They suggest that the activation of critical signaling proteins may be responsive to coordinated inputs from more than one signaling pathway and that varying the amplitude and duration of pathway activation may confer different biological responses through the same receptor mechanism. Neurons may have survival pathway-specific preferences, but they appear to be responsive to alternative survival pathways if necessary. Although, we still do not fully understand the molecular mechanisms driving PCD in photoreceptor mutants, the role of the RTK signaling pathways in controlling apoptotic signals is well established. The previous work in this field clearly demonstrates the complexity of the overlapping and complementary transduction cascades at work in maintaining the balance of homeostasis regulating the life and death of neuronal cells in the retina. Understanding the inter-relationships between these pathways and the key components that drive the state of balance in one direction or the other is the challenge before us. It is only with a deeper understanding of the fundamental mechanisms at work within the cell that we will be able to apply growth factor pharmacology and gene therapy in a meaningful way to human retinal diseases.

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