[Frontiers in Bioscience 8, s1140-1156, September 1, 2003]

GLAUCOMA: OCULAR ALZHEIMER'S DISEASE?

Stuart J. McKinnon

Departments of Ophthalmology and Cellular and Structural Biology, University of Texas Health Science Center at San Antonio 7703 Floyd Curl Drive, MC 6230, San Antonio, Texas 78229-3900, USA

TABLE OF CONTENTS

1. ABSTRACT

Glaucoma is a chronic neurodegeneration of the optic nerve and one of the leading causes of vision loss in the world among the aging. Retinal ganglion cells (RGCs) have been shown to die by apoptosis, or programmed cell death. Central to apoptosis is the activation of specific proteases, termed caspases. Caspases are activated in chronic neurodegenerations such as Alzheimer's disease (AD) as well as in RGCs after optic nerve transection. In rat glaucoma models we have shown that caspase-3, a major effector of the apoptotic cascade, is activated in RGCs and cleaves amyloid precursor protein (APP) to produce neurotoxic fragments that include amyloid-beta. Caspase-8, which initiates apoptosis after activation of receptors of the tumor necrosis factor (TNF) superfamily, is also activated in RGCs. This suggests a new hypothesis for RGC death in glaucoma involving chronic amyloid-beta neurotoxicity, mimicking AD at the molecular level. With loss of the protective effect of APP and upregulation of toxic APP fragments, RGCs die from chronic caspase activation, loss of synaptic homeostasis, amyloid-beta cytotoxicity and excitotoxicity. The benefits are that treatments for AD could be used to treat glaucoma, and strategies developed to treat glaucoma could treat other neurodegenerations.

2. INTRODUCTION

Glaucoma is one of the leading causes of vision loss in the world, particularly among the elderly (1-3). Glaucoma is an optic neuropathy characterized by RGC death, axon loss and an excavated appearance to the optic nerve head (4). Glaucoma is usually associated with elevated intraocular pressure (IOP), but a subset of "normal tension glaucoma" patients develops damage without ever manifesting high IOPs. Clearly, IOP-independent mechanisms of RGC death are present in glaucoma. RGC death mechanisms in experimental animal models of glaucoma and human glaucoma have been shown to involve programmed cell death or apoptosis (5-8). Specific triggers of apoptosis have been implicated in glaucoma, including blockage of axonal transport (9-11), glutamate excitotoxicity (12), antibodies to heat shock proteins (13), ischemia (14), and vasoactive regulators such as endothelin (15) and nitric oxide (16). Regardless of trigger, the

apoptotic cascade produces cell suicide by invoking a series of cellular events that has been conserved throughout evolution.

2.1. Apoptosis

Apoptosis, or programmed cell death (PCD), constitutes a genetically coded "suicide" program that is activated when cells are no longer needed, or have been seriously damaged. Apoptosis is a basic death mechanism common to many cells. Inappropriate activation of apoptosis has been implicated in a variety of neurodegenerative disorders such as amyotrophic lateral sclerosis and Parkinson's disease (17), and ischemic conditions such as stroke (18). Apoptosis is defined in morphological terms, as cells demonstrate characteristic histologic and electron microscopic changes such as decrease of cytoplasmic and nuclear volumes, degradation of nuclear DNA by endonucleases that causes condensation of chromatin ("apoptotic bodies"), and blebbing of plasma membrane (19). Apoptosis can occur in cells by distinctly different pathways. One pathway involves cell membrane-bound receptors belonging to the tumor necrosis factor (TNF) superfamily of receptors and activation of caspase-8 (20). Another pathway involves disruption of electron transport and energy metabolism at the level of the mitochondria, involving caspase-9. Reactive oxygen species are formed with mitochondrial redox changes, resulting in cell death (21).

2.2. Caspases

A class of aspartate-specific proteases of the interleukin-1beta-converting enzyme family, or caspases, has been shown to be important in the cellular implementation of apoptosis. Caspases are so named as proteases that cleave on the carboxyl side of aspartate residues, with specificity determined by a flanking sequence of four amino acids. Caspases such as caspase-3 are translated as inactive precursors, and activation involves proteolytic processing between sub-domains, leading to the association of large and small subunits to form a heterodimer. Activated caspases kill cells not by indiscriminate protein digestion, but by selective destruction of DNA repair enzymes such as PARP (polyADP-ribose polymerase - a nuclear protein involved in regulation of energy stores) (22), nuclear lamina and intermediate filaments. Caspases also cleave an inhibitory complex (ICAD) of a caspase-activated endonuclease (CAD), which proceeds to fragment chromosomal DNA, giving the characteristic laddering pattern seen on electrophoretic gels (20). The retinoblastoma tumor suppressor protein RB1 is cleaved by caspase-3 during apoptosis (23). RB1 is proposed to be a critical substrate in cell cycle regulation (24) and in apoptosis involving activation of the TNF-alpha pathway (25).

The activation of caspase-8 is considered an initiating step in the apoptosis cascade (26). Apoptosis initiated by caspase-8 involves cell membrane-bound receptors of the tumor necrosis factor superfamily, including TNFR, Trail, Fas, and the low-affinity neurotrophin receptor p75NTR. Ligands such as TNF-alpha and FasL bind to TNF receptors, causing receptor trimerization. A so-called "death domain" in the cytoplasmic portion of each receptor recruits adaptor proteins such as FADD. An effector domain of FADD binds to and activates caspase-8, forming an active signaling complex that is known to activate caspase-3 and to induce cell death (20). In a cell culture model, retinal cell apoptosis is initiated through the "extrinsic" pathway involving membrane-bound death receptors and caspase-8 activation (27).

Due to its central role in neuronal death, caspase activation has been the focus of intensive research in chronic neurodegenerations. In a model of RGC death, intraocular peptide caspase inhibitors reduced apoptosis after optic nerve transection in rats. In Huntington's disease, caspases cleave polyglutamine proteins (huntingtin, atrophin-1, ataxin-3, androgen receptor) (21, 28). In the MPTP model of Parkinson's disease, peptide caspase inhibitors protect cultured cerebellar granule neurons (29). In Alzheimer's disease, presenilin-1 and -2 mutants (which cause severe early-onset autosomal dominant Alzheimer's) are cleaved by caspases (30, 31). Although not considered a neurologic disorder, in diabetes pancreatic islet cells display caspase activation with elevated APP and amyloid-beta levels (32, 33). These findings point to the important central theme of caspase activation in chronic neurodegeneration, and the interest in developing caspase inhibitors.

2.3. Apoptosis and glaucoma

We know that apoptosis is important in RGCs during development. In primates approximately half of the ganglion cells fail to make central connections within the lateral geniculate nucleus, and die by apoptosis. Studies of ganglion cell death in adult animals began by using models of optic nerve transection and optic nerve crush injury, both that cause nearly complete ganglion cell depletion. Adult rats were subjected to optic nerve transection or crush injury, and displayed an initial abrupt loss of ganglion cells, followed by a later more protracted phase of ganglion cell loss (34). Further analysis of the ganglion cells subjected to optic nerve transection demonstrated the typical morphological changes seen in apoptosis, consisting of chromatin condensation, the formation of apoptotic bodies, and DNA fragmentation (35, 36). Rat eyes subjected to 4 months of IOP elevation displayed DNA fragmentation and reduction in mitochondrial membrane potential of RGCs consistent with apoptosis (37). RGCs in rabbits and monkeys subjected to conditions of experimental glaucoma and optic nerve transection died by apoptosis (5, 7). Apoptotic RGCs have been present in significantly greater numbers of human retinas with glaucoma, when compared to age-matched control retinas (6). These studies directly implicate apoptosis in ganglion cell death due to glaucoma.

Apoptosis has been implicated in the mechanism of IOP elevation as well, by altering trabecular meshwork function and disrupting aqueous humor outflow. Apoptosis modulators such as caspase-1, Fas, Bcl-2, Bcl-X_L, and Bax were detected in human trabecular meshwork, and apoptosis could be induced in these cells via the Fas/FasL pathway (38). Endothelial leukocyte adhesion marker-1

(ELAM-1) is a cell-adhesion molecule involved in fluid transport. ELAM-1 expression is regulated by NF-KappaB, a transcription factor activated by multiple signaling pathways that promotes cellular survival (39). ELAM-1 levels are increased in the trabecular meshwork of human glaucoma patients, and may represent a protective response to apoptotic insults in the aqueous outflow pathways (40).

2.4. Caspase substrates in Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative condition characterized by the insidious onset of dementia in older age. Neuropathological findings consist of neurofibrillary tangles and deposition of amyloid in neuritic plaques concentrated in the hippocampal and parahippocampal areas (41). Amyloid deposition occurs through the abnormal proteolytic processing of the integral membrane protein amyloid precursor protein (APP), yielding an abnormal accumulation of amyloid-beta peptide consisting of 40 or 42 amino acids (42). Recent work has shown that hippocampal neurons in AD die by the mechanism of apoptosis, and amyloid-beta appears to play a central role (43, 44). Neuronal cell cultures undergoing apoptosis have been shown to increase the rate of amyloid-beta production by 3- to 4-fold (45). Additionally, mutations in APP lead to inherited forms of AD, known as familial Alzheimer's disease (FAD). APP mutations responsible for human FAD have been used to generate over-expression of amyloid-beta and cause apoptotic neuronal death (46). Various transgenic mouse models of AD have been generated to study this neuronal pathophysiology. Mice expressing the Swedish mutation (tg2576; K670N/ M671L) show a 5- to 6-fold increase of human APP expression (when compared to wild-type littermates) with an over-production of amyloid-beta$_{1-40}$ (47). The "Indiana" mutation (V717F) introduces a caspase cleavage site into APP and causes elevated amyloid-beta production and earlier onset of dementia (48, 49).

Multiple substrates for caspases have been identified that are involved in chronic neurodegenerations (50). The processing of APP and its involvement in apoptosis seen in AD has led to the investigation of the role of caspases, and caspase-3 cleavage sites have recently been recognized in the APP protein sequence (51, 52). Cleavage of the C-terminal cytoplasmic tail of APP by caspase-3 yields neurotoxic peptide fragments that upregulate amyloid-beta production in dying hippocampal neurons. Caspase-3 activity has also been found to colocalize in senile plaques along with APP cleavage products and amyloid-beta (52). The adapter proteins Fe65 and X-11 normally bind to the C-terminal NPTY clathrin-coated pit internalization sequence of APP and maintain high APP and low amyloid-beta levels (53). Caspase activation disrupts the adapter protein-APP interactions, leading to a loss of clathrin-coated vesicular endocytosis, disruption of anterograde transport, and an increase in amyloid-beta levels. Caspase-8 activation and neuronal apoptosis has been induced by amyloid-beta in cultured hippocampal neurons. Amyloid-beta induced apoptosis was inhibited by the synthetic peptide caspase inhibitor IETD-fmk, and by virally expressed dominant-negative FADD (54). CrmA, an inhibitor of caspase-8, was found to protect primary hippocampal cultures from amyloid-beta-induced apoptosis (55). Interestingly, APP is highly expressed in the RGC, and undergoes rapid anterograde transport in the optic nerve (56), where it plays a role in calcium regulation and synaptic homeostasis (57-59).

Recent work has shown that local over-expression of APP causes neuronal degeneration and caspase activation. When APP was over-expressed in rat hippocampus using adenovirus vectors, neurons underwent severe degeneration in a few days. The degenerating neurons accumulated different epitopes of APP, and amyloid-beta immunoreactive materials were not detected in the extracellular space. Electron microscopic examinations demonstrated that degenerating neurons had shrunken perikarya along with synaptic abnormalities (60). When an APP-expressing adenovirus was injected into the dorsal hippocampal region, neurons underwent severe degeneration from 3 to 7 days after viral inoculation. Most degenerating neurons were immunopositive with both APP and activated caspase-3. In the neighborhood of the degenerating neurons, microglia were activated and macrophages appeared to phagocytose the caspase-3-immunopositive degenerating neurons (61). Adenovirus over-expression of wild-type APP in neurally differentiated embryonal carcinoma NT2 cells caused severe degeneration and accumulation of full-length APP and amyloid-beta immunoreactive peptides, and caspase-3 activation. Western blot analysis revealed that activated caspase-3 subunits were generated in APP-accumulating neurons. Addition of the caspase-3 inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde significantly reduced the severity of degeneration exhibited by APP-overexpressing neurons. Immunocytochemical analyses revealed that some APP-accumulating neurons contained activated caspase-3 subunits and exhibited the characteristics of apoptosis, such as chromatin condensation and DNA fragmentation. Activation of caspase-3 was also observed *in vivo* in rat hippocampal neurons infected with the APP-expressing adenovirus (62). These results suggest that intracellular accumulation of wild-type APP causes neuronal degeneration *in vivo*, and that wild-type APP is an intrinsic activator of caspase-3-mediated death machinery in postmitotic neurons.

APP undergoes fast anterograde axonal transport mediated by the kinesin family of plus-end motor proteins. Kinesins consist of a tetramer of two kinesin heavy chains (Khc) and two kinesin light chains (Klc) (63). APP forms a complex with kinesin by binding tetra-trico peptide repeat (TPR) domains of the Klc subunit (64). An axonal membrane compartment has recently been identified that contains APP, BACE, (the beta-secretase of APP) (65) and presenilin-1 (PS1, the gamma-secretase of APP) (66), and proteolysis of APP occurs in this compartment in axons. This suggests that processing of APP to amyloid-beta by secretases can occur in an axonal membrane compartment transported by kinesin-I (67). Association of APP with microtubules and axonal transport of APP is greatly decreased in transgenic mice expressing mutant Klc1 (68). Studies have localized APP to the endoplasmic reticulum, a

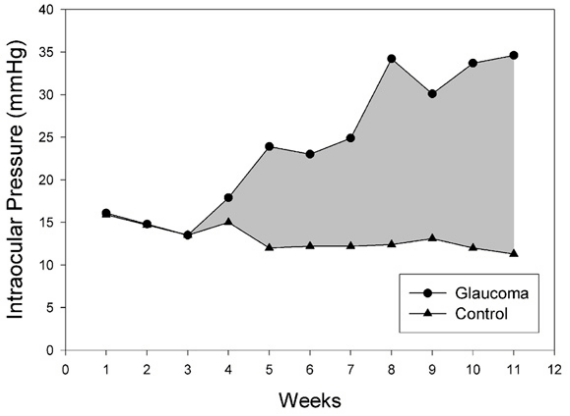

Figure 1. Intraocular pressure (IOP) was measured weekly for a glaucoma (circle) and control (triangle) rat eye pair. The gray area between the hypertensive and control eye plots constitutes the IOP integral, in units of mmHg-days. The IOP integral is a quantitative measure of exposure to chronically elevated IOP in the treated eye, and is 798 mmHg-days in this example.

cellular organelle involved in axonal transport and calcium homeostasis (69, 70). The ER is also the site of presenilins, a class of proteins involved in APP transport and processing, membrane trafficking, and calcium regulation (71, 72). The normal functions of presenilins are currently under investigation, but mutations in the presenilin gene produce proteins that are cleaved by caspases (51). These mutations are responsible for the majority of early-onset Alzheimer's disease, and are transmitted in an autosomal dominant pattern with 100% penetrance. Presenilin-1 mutant knockout mice show synaptic alterations, mitochondrial dysfunction and altered calcium homeostasis (73, 74). Recently, a murine caspase, caspase-12, has been shown to localize to the ER. Caspase-12 was activated in response to stress to the ER, and participates in caspase-mediated cleavage of APP, upregulation of amyloid-beta, and disruption of intracellular calcium homeostasis (75). This paper suggests why Alzheimer's disease develops slowly over many years, despite the fact that apoptosis is classically described to occur within 24-48 hours. The slow accumulation of amyloid-beta could reach a critical level, triggering neuronal apoptosis through a "self-perpetuating circle of APP cleavage, amyloid-beta production and caspase-dependent apoptosis" (76).

3. METHODS

3.1. Chronic ocular hypertensive rat glaucoma model

To produce chronic ocular hypertension in rats, we perform limbal injections of hypertonic saline by the method of Morrison (77), which induce sclerosis of venous outflow channels and obstruction of aqueous humor outflow. Adult Norway brown rats were anesthetized with ketamine (75 mg/kg) and acepromazine (1.5 mg/kg), and a small incision was made in the superior limbal conjunctiva. A pulled glass micropipette attached by PE-50 tubing to a tuberculin syringe was inserted into a vein near the cornea and about 0.1 ml of 2.0 N saline was injected into the limbal venous system. The injection was then repeated two weeks later. Each retina was examined following saline injection by dilated planar ophthalmoscopy to rule out retinal ischemia. Erythromycin ointment was applied to the operated eye. The un-operated eye served as a normotensive control. All animal procedures were approved by our Institutional Animal Care and Use Committee and conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research.

3.2. Intraocular pressure (IOP) measurement and analysis

IOP was measured in both eyes under topical anesthesia pre-operatively and on a weekly basis post-operatively using a calibrated Tonopen XL (Medtronic Solan, Jacksonville, FL). Immediately after induction of sedation, both eyes were anesthetized with a drop of proparacaine hydrochloride 1% (Bausch and Lomb, Tampa, FL). IOPs at each time point were recorded as the average of ten consecutive Tonopen measurements for each eye. Measures of IOP exposure for each animal were calculated by performing separate integrations of the IOP over the days of exposure for the treated and control eye. The control eye integral value was then subtracted from the treated eye integral, yielding the "IOP integral", expressed in units of mmHg–days (Figure 1). Animals were excluded from the analysis on the basis of no measurable IOP exposure. Exclusion criteria were: 1) a negative IOP-integral difference; 2) no IOP elevation in treated eye > 3 mm over control eye ever during the 12-week period; or 3) six or more consecutive IOPs in the treated eye lower than the control eye.

3.3. Optic nerve axon counts

After sacrifice, eyes were dissected and optic nerve heads removed and fixed in cold EM-grade 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 hours. The nerves were rinsed in cacodylate buffer (pH 7.4), postfixed in 2% osmium tetroxide in cacodylate buffer, dehydrated in alcohol and embedded in epoxy resin. One-micron thick cross-sections of the optic nerves were stained with toluidine blue, and approximately 40% of the total axons were counted after RGB thresholding and size and form factor exclusions, using the KS400 imaging system (Carl Zeiss, Thornwood, NY). Axon counts were reported as percentages, i.e. the ratios of axon counts between treated and paired control eyes.

3.4. Immunohistochemistry

Longitudinal retinal sections that include retina and optic nerve of each eye measuring seven microns in thickness were collected onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were immunolabeled by a modified streptavidin-biotin peroxidase technique (78). After methanol fixation, endogenous tissue peroxidase activity was quenched with 3% H_20_2. The sections were blocked with 2% normal serum in PBS, followed by blocking with ABC avidin blocker and biotin blocker (Vector Laboratories, Burlingame, CA). The sections were incubated overnight at 4^{o}C with either mouse monoclonal antibody to N-terminal and full-length APP (22c11,

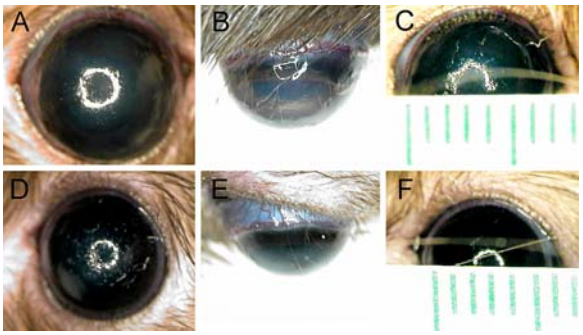

Figure 2. External photographs of a glaucoma (A, B, C) and control (D, E, F) eye pair. The glaucoma eye displayed enlargement of the globe (buphthalmos), deepening of the anterior chamber, and an approximate 2 mm increase in corneal diameter. A, D) Frontal views. B, E) Side views. C, F) Corneal diameters were measured with a millimeter ruler.

Chemicon, Temecula, CA; 10 microgram/ml), or mouse monoclonal antibody to amino acids 17-24 of amyloid-beta (4G8, Senetek, Napa, CA; 2 microgram/ml). Following washing, the sections were incubated with anti-mouse biotin conjugated IgG (1:500 dilution, Kierkegaard and Perry, Gaithersburg, MD), washed again and incubated with peroxidase labeled streptavidin (1:500 dilution, Kierkegaard and Perry). After incubation in 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO), sections were mounted in Kaiser's glycerol jelly and imaged by Nomarski optics (Zeiss Axioskop, Carl Zeiss Inc, Thornwood, NY). Antibody controls included non-immune serum and exclusion of primary antibody.

3.5. Immunogold electron microscopy

Rat eyes subjected to unilateral chronic IOP elevation were perfusion fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. After fixation, small strips of retina and optic nerve were embedded in LR Gold resin (London Resin Co., Berkshire, Great Britain) at 4 ^{o}C (79). Ultra-thin sections (approximately 60 nm thickness) were cut on an ultramicrotome (LKB Ultratome V; Leica, Paris, France) using a diamond knife (Diatome, Port Washington, PA), and mounted on Formvar-coated (Ernest Fullam Inc., Latham, NY) nickel grids (Ted Pella, Inc., Reading, CA). Immunogold EM-labeling using antibody to full-length APP (22c11, 10 microgram/ml) was performed using a three-step procedure. After primary incubation with 22c11, sections were incubated with mouse anti-rabbit IgG H+L (50 microgram/ml; Jackson ImmunoResearch Laboratories Inc., West Grove , PA), followed by a third-stage goat anti-mouse IgG conjugated to 10 nm gold particles (1:10 dilution; Amersham Pharmacia). Controls included sections omitting the primary antibodies, pre-immune serum, and control peptides. Grids were examined in a transmission EM (Joel JEM-1200EX, Tokyo, Japan), photographed, and digitally scanned.

3.6. ELISA

A sensitive method for quantitation of intracellular amyloid-beta employs enzyme-linked immunosorbent assay, or ELISA. Because rodent amyloid-beta differs from human amyloid-beta at three amino acid positions, specific capture and detection antibodies have been developed that were used for measuring amyloid-beta levels in mice and rat tissues. In collaboration with Dr. Steven Younkin (Director of Research, Mayo Clinic, Jacksonville), we assayed retinal proteins from paired ocular hypertensive and control rat eyes to determine changes in levels of amyloid-beta$_{1-40}$ and amyloid-beta$_{1-42}$. Retina and optic nerves from paired ocular hypertensive and control rat eyes (n = 5 rats) were first lysed in 70% formic acid to ensure adequate amyloid-beta extraction. Sandwich ELISA was performed using monoclonal antibodies specific for different species of amyloid-beta. BNT-77 (specific for the first 10 amino acids of amyloid-beta) was used as a capturing antibody, and horseradish peroxidase-conjugated BA-27 specific for amyloid-beta$_{1-40}$, and BC-05 specific for amyloid-beta$_{1-42}$ were used as detection antibodies. To calibrate the sensitivity of the ELISA for detecting amyloid-beta, synthetic amyloid-beta$_{1-40}$ and amyloid-beta$_{1-42}$ peptides (Bachem Bioscience Inc., King of Prussia, PA) were used to generate standard curves. The BNT77, BA-27, and BC-05 mAbs (Takeda Industries, Tsukuba, Japan) were prepared and characterized as described previously (80, 81).

4. RAT MODEL OF CHRONIC OCULAR HYPERTENSION

In order to study the molecular events that are important in glaucomatous optic neuropathy in greater detail, we have utilized rodent models of RGC degeneration. Various rodent models of glaucoma exist, including rat and mouse optic nerve crush and transection, acute IOP elevation (ischemia), aqueous humor outflow obstruction (rat chronic IOP elevation models), glutamate excitotoxicity (intravitreal injection), and genetic dba2J mice (pigment dispersion glaucoma) (82). We have employed an ocular hypertensive model of rat glaucoma for our initial studies. We investigated optic nerve damage and IOP profiles using the outflow obstruction model of Morrison (77). Ocular hypertension was induced in one eye of adult brown Norway rats (n = 69) by limbal injection of hypertonic saline, which causes sclerosis of aqueous humor outflow vasculature and subsequent IOP rise. One striking feature in ocular hypertensive eyes is marked enlargement of the globe (Figure 2), particularly at high IOP exposures. A representative 10% increase in corneal diameter corresponds to an increase of about 15% in globe volume. The hypertonic saline injection model also produces marked loss of RGC axons and posterior deformation of the optic nerve head ("cupping") that is readily apparent on direct visual examination of rat optic nerves (Figure 3). The appearance of rat optic nerve cupping strongly resembles the cupping seen in human glaucoma patients.

Integrations of IOP over time give a quantitative estimate of exposure to elevated IOP in a treated eye when compared to the paired control eye. These were performed for each animal in the hypertonic saline injection group manifesting elevated IOP. The IOP integral (see Methods) between treated and control pairs was positively correlated

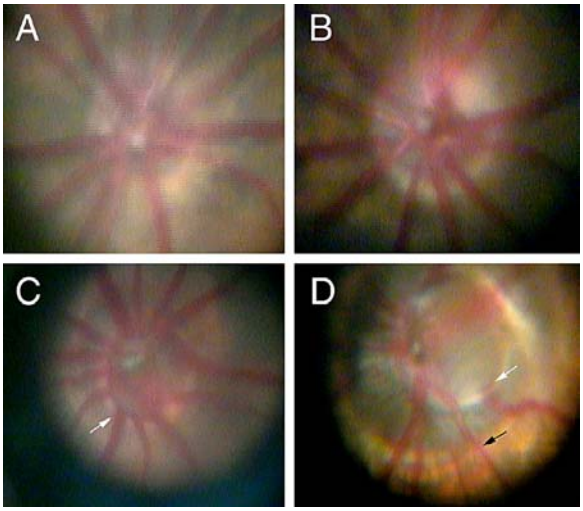

Figure 3. Rat optic nerve heads digitally imaged by planar ophthalmoscopy using a coverslip and an operating microscope. A) A control nerve had normal vasculature and a planar configuration. B) A nerve subjected to mild IOP exposure (178 mmHg-days) showed no change in configuration. C) A nerve subjected to moderate IOP exposure (460 mmHg-days) displayed marked optic nerve head cupping similar to that seen in human glaucoma. Note the blood vessel (white arrow) that disappears as it dives over the rim of the cup. D) A nerve subjected to severe IOP exposure (536 mmHg-days) not only showed cupping (white arrow), but also showed a second zone of posterior scleral deformation (black arrow) due to scleral expansion.

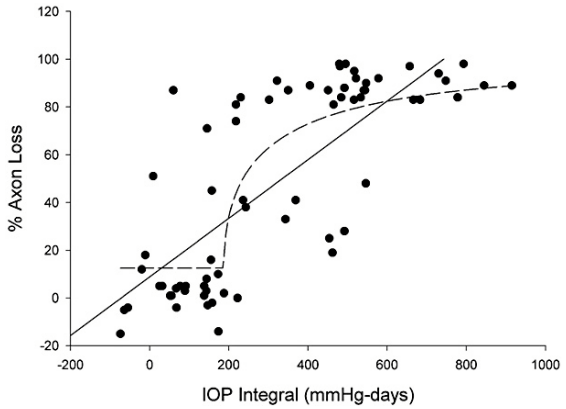

Figure 4. Optic nerve damage, expressed as % axon loss, linearly correlates (solid line) with IOP exposure as measured by the IOP integral (in mmHg-days). Complete axon loss occurs at or below 800 mmHg-days. A fit to a sigmoidal curve (dotted line) reveals a threshold for damage at approximately 200 mmHg-days of IOP exposure, and axon loss that is most rapid at the earliest stages.

with the degree of axon loss (Figure 4; R^2=0.59, P = 0.0001). The IOP integral values revealed a linear dose-response effect (intercept = 8.8, slope = 0.123) of pressure exposure on optic nerve axon loss, although individual variability was seen. Interestingly, a better correlation was obtained (R^2=0.66, P = 0.0001) when the data was fit to a sigmoidal curve using a 5-parameter Weibull function (SigmaPlot, SPSS Inc., Chicago, IL). Parallels with human glaucoma can also be made from these correlations. A threshold for damage was noted at an integral value of approximately 200 mmHg-days, which for an approximately one-hundred day experiment, corresponds to an average of 2 mmHg IOP elevation in the treated vs. control eye. After this threshold is met in this model, axon loss may occur most rapidly during the earliest stages of exposure to elevated IOP, suggesting earlier and more aggressive IOP-lowering treatment in human glaucoma patients. Total axon loss occurs at approximately 800 mmHg-days, which corresponds to an average of 8 mmHg IOP elevation in the treated vs. control eye over the same period. The range of IOP elevations are similar to those seen clinically in glaucoma patients.

4.1. Retinal gene expression

The Ribonuclease Protection Assay (RPA) is an extremely sensitive method for the detection and quantification of RNA species in a mixture of total cellular RNA. In this RPA experiment labeled RNA probe sets were obtained that were complementary to target apoptosis-related mRNAs of interest (RiboQuant, Pharmingen, San Diego, CA). The rat apoptosis probe set allowed simultaneous quantification of several apoptosis-related mRNAs in a single sample of rat retina RNA. Rat retina RPAs were performed using total RNA isolated from control rats, rats subjected to optic nerve transection (at time points 10 minutes, 1 hour, 6 hours, 24 hours, 5 days post-transection), and experimental glaucoma. We showed that bcl-x_L mRNA was expressed in all retinas at a much higher level than bcl-2. Also, caspase-3 mRNA was expressed at a level comparable to bcl-x_L, and higher than caspase-2 and caspase-1. Densitometry of individual mRNAs in the rat retina RPA showed that caspase-3 was expressed constitutively in normal retina, and was quickly upregulated after transection, reaching a maximum at 6 hours after transection. In glaucomatous retina, the levels of caspase-3 mRNA were also upregulated. Surprisingly, caspase-3 mRNA levels in glaucoma were higher than the 6-hour maximum of expression after transection (83). It is clear that apoptosis genes are upregulated under conditions of stress to the retina, and that caspases play an important role in mechanisms of RGC death. It also appears that the gene expression profile is different after chronic ocular hypertension when compared to acute trauma such as axotomy. Extrapolating results derived from acute optic nerve trauma models to glaucoma should therefore be done with caution.

4.2. Caspase activation

In human and experimental animal models of glaucoma, apoptosis has been noted in RGCs. Activation of the apoptosis initiator caspase-8 has been previously noted in RGC cultures exposed to heat-shock proteins (27). Work done in our lab in the rat glaucoma model has documented the early activation of caspase-8 in RGCs exposed to elevated IOP (83). Cryopreserved retinas sectioned from ocular hypertensive and control rats were immunolabeled using antibodies directed against pro-caspase-8 (SK 441, Smith-Kline/Beecham; 1:800 dilution), and activated caspase-8 (SK440, Smith-Kline/Beecham: 1:750 dilution).

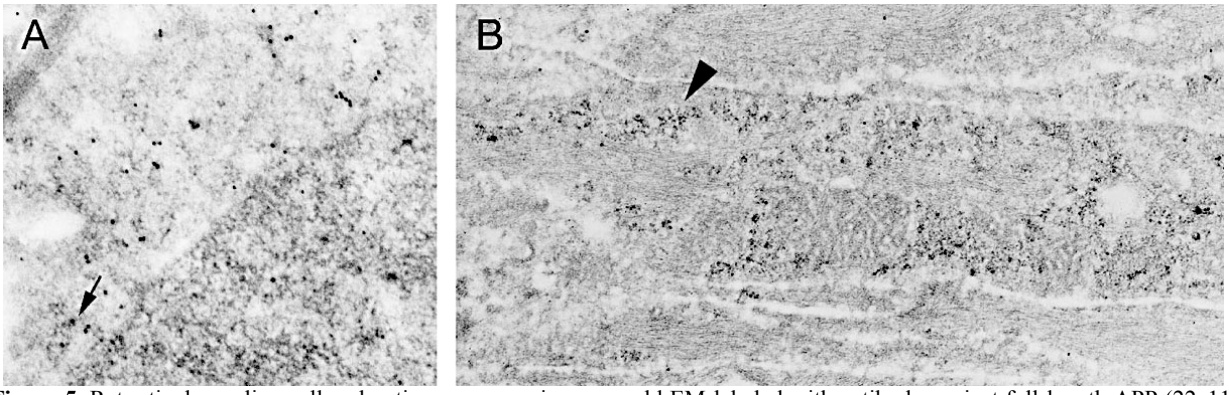

Figure 5. Rat retinal ganglion cell and optic nerve axon immunogold EM-labeled with antibody against full-length APP (22c11; 10 nm gold particles). A) APP is localized to perinuclear Golgi (arrow) and B) axonal endoplasmic reticulum (arrowhead) of RGCs. Magnification = 21,300x

A masked reading of slides showed statistically significant elevation of immunolabeling (chi^2, $p<0.0001$) of activated caspase-8 in glaucoma eyes (22/27 or 81%) when compared to control eyes (1/27 or 4%). Immunolabeling of pro-caspase-8 was positive in both glaucoma and control RGCs (p=NS) (83). Another caspase protease that occupies a central effector role in apoptosis is caspase-3. To better understand RGC death mechanisms, we have studied the role of caspase-3 activation in various glaucoma models. The animal glaucoma model consisted of induction of ocular hypertension in one eye of rats by limbal vein injection of hypertonic saline (n=8). The untreated contralateral eyes served as controls. The treated eyes had an average IOP exposure over 12 weeks of 254 mmHg-days, slightly above the observed 200 mmHg-days threshold for damage. Rat retinas were cryopreserved in OCT compound, and seven-micron thick sections were mounted for immunostaining. Antibodies used were directed against activated caspase-3 (CM1, IDUN Corp., San Diego, CA; 1:3000 dilution). Immunohistochemical labeling was detected with a modified streptavidin-biotin peroxidase technique. Immunolabeling against activated caspase-3 was detected in RGCs of glaucoma rat eyes, but not in normal control eyes. A masked reading of slides showed statistically significant elevation of activated caspase-3 immunolabeling (chi^2, $p<0.004$) in ocular hypertensive eyes (23/27 or 85%) when compared to control eyes (11/27 or 41%) (83). Western blot analysis was performed on retinal protein obtained from brown Norway rats with unilateral experimental glaucoma. The blot was probed with CM1 antibody directed against the p20 subunit of activated caspase-3 (CM1; 1:1333). Average densitometry was compared between glaucoma and control eyes (n=8 each). The p20 subunit was present to a higher degree in glaucoma eyes when compared to the contralateral control eyes. The ratio of the p20 to p32 bands was calculated to correct for unequal protein loading. Caspase-3 activation was significantly higher ($P = 0.002$) in the glaucoma group when compared to the control eye group. As a confirmatory test, combined caspase-3/7 activation was quantified by colorimetric assay. Ocular hypertensive retinas had significantly higher ($P = 0.009$) caspase-3/7 activity than did control retinas (83).

4.3. Abnormal processing of amyloid precursor protein

We have shown that caspases, specifically caspase-8 and caspase-3, are activated in an ocular hypertensive model of rat glaucoma. As caspase activation and abnormal processing of APP have been implicated in chronic neurodegenerations such as Alzheimer's disease, we feel that abnormal processing of APP by caspases may be a significant contributing factor in the pathophysiology of glaucoma. Our research efforts have been directed characterizing the role of abnormal APP processing in glaucoma. APP is cleaved by several proteases. The most studied, the secretases, are involved in the release of a fragment of APP, the amyloidogenic protein amyloid-beta. Proteolysis by gamma-secretase is the last processing step resulting in release of amyloid-beta. Cleavage occurs after residue 40 of amyloid-$beta_{1-40}$, and also after residue 42 of amyloid-$beta_{1-42}$. Both isoforms are toxic to neurons and are associated with Alzheimer's disease (44). Amyloid-$beta_{1-40}$ production has been localized to the trans-Golgi network (TGN); amyloid-$beta_{1-42}$ production has been localized to endoplasmic reticulum (ER) (84). Immunoelectron microscopy is an immunohistochemical technique that allows sub-cellular localization of proteins using antibody detection systems linked to conjugated gold particles. Immunoelectron microscopy has localized APP to endoplasmic reticulum (ER) and Golgi apparatus (71). In order to determine sub-cellular expression of APP in RGCs, an antibody to full-length APP (22c11) was employed in a three-step labeling procedure to label RGC cell bodies and optic nerve axons (Figure 5). On the left, a retinal ganglion cell shows immunogold labeling of APP (small black particles, 10 nm size) in the perinuclear Golgi. On the right, an optic nerve axon is shown with immunogold particles are lined up along the length of the axon, localizing to ER. These findings confirm the location of APP in RGCs to be consistent with other neurons in the central nervous system.

Prior studies have demonstrated that over-expression of APP in neurons can trigger cellular changes including mitochondrial disruption (85), synaptic degeneration (60) and caspase activation (61, 62). Structural changes at the optic nerve head are a hallmark of

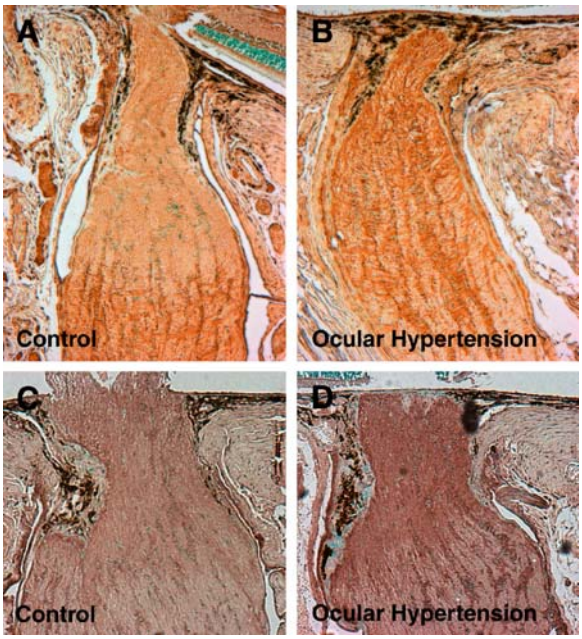

Figure 6. at optic nerve heads labeled with 22c11 antibody recognizing full-length APP and 4G8 antibody recognizing amyloid-beta. A, B) APP localizes to the retro-laminar optic nerve in control nerves, and shifts to the laminar optic nerve in hypertensive nerves. C, D) Amyloid-beta labeling in the laminar nerve head is light in control nerves, and is upregulated in hypertensive nerves. Magnification = 10x.

glaucoma, and blockage of both anterograde and retrograde axonal transport has been documented (86). If blockage of anterograde transport of APP occurs at the optic nerve head in glaucoma, the local build-up of APP could represent a potential trigger for caspase activation in RGCs. We sought to determine if anterograde blockage of axonal transport in the rat ocular hypertension model was associated with local accumulation of APP at the optic nerve head. By using an N-terminal mouse monoclonal antibody to APP that recognizes full-length APP (22c11, Chemicon, Temecula, CA), we performed immunohistochemical labeling of APP of paraffin-embedded optic nerve heads taken from a rat treated in one eye to produce ocular hypertension. Immunohistochemical labeling was detected with a modified streptavidin-biotin peroxidase technique. The hypertensive eye had a high degree of pressure exposure over 12 weeks (777 mmHg-days) and marked optic nerve axon loss (compared to the control eye) of 84%. In the control eye (Figure 6A) APP staining is very light in the pre- and post-laminar areas. Heavy APP staining is seen inferiorly in the control optic nerve distal to the lamina cribrosa. In the hypertensive eye (Figure 6B), APP immunolabeling is heavy in the pre-laminar, laminar and post-laminar nerve head. Distal optic nerve labeling was noted to be light. Amyloid-beta immunolabeling using 4G8 antibody was also seen to be light in the pre-laminar and laminar control nerve (Figure 6C), while in the hypertensive eye (Figure 6D), pre-laminar and laminar labeling of amyloid-beta is prominent. These results show qualitative evidence of a shift of APP expression from distal optic nerve to proximal lamina cribrosa in the hypertensive rat optic nerve, indicating anterograde build-up of APP in the optic nerve head. The upregulation of amyloid-beta in the proximal nerve head in rat ocular hypertension also indicates that abnormal proteolysis of APP produces toxic fragments such as amyloid-beta.

Recent work has shown that APP serves as a substrate for caspases, in particular caspase-3 (52, 87). As we have demonstrated above, caspase-3 is activated in the rat ocular hypertension model of glaucoma. In collaboration with Dr. Donald Nicholson (Merck-Frosst, Montreal, Canada), we obtained an antibody that specifically recognizes the C-terminal caspase-3 cleavage product of APP (deltaC-APP). We sought to determine if immunohistochemical evidence of APP cleavage by caspase-3 could be detected in rat RGCs in the ocular hypertension model. Rat retinas were cryopreserved in OCT compound, and seven-micron thick sections were mounted for immunostaining. Antibodies used were directed against the caspase-3 cleavage product of APP (deltaC-APP at 1:600 dilution). Immunohistochemical labeling was detected with a modified streptavidin-biotin peroxidase technique. Immunohistochemistry using the antibody against deltaC-APP showed little labeling in control RGCs and marked labeling in hypertensive RGCs. DeltaC-APP immunolabeling closely matched that seen with anti-activated caspase-3 in all retinas examined. Masked readings showed statistically significant elevation of deltaC-APP immunolabeling ($\chi 2$, $p<0.013$) in ocular hypertensive retinas (14/27 eyes or 52%) when compared to control retinas (4/27 eyes or 15%) (83). In addition, this experiment served as a confirmation of caspase-3 activation in RGCs in rat ocular hypertension. Given that caspase-3 activation with subsequent cleavage of APP has been associated with apoptosis of hippocampal neurons in brains of Alzheimer's disease (52), it is likely that exposure of RGCs to similar neurotoxic C-terminal fragments of APP contributes to RGC apoptosis in glaucoma.

In order to further delineate the immunohistochemical evidence of caspase-3 processing of APP in rat ocular hypertension, we performed Western blot analysis on retinal protein obtained from five Norway brown rats with unilateral ocular hypertension. The average pressure exposure for the hypertensive eyes over a 12-week period was 104 mmHg-days (range 7 - 230). This represents a mild IOP exposure (at or below the observed 200 mmHg-days threshold for axon loss), when molecular events prior to RGC death are expected to occur. The blot was probed with antibodies directed against specific epitopes of the APP molecule, using antibodies 22c11 (0.5 microgram/ml) recognizing full-length APP and 4G8 recognizing amyloid-beta peptide (0.2 microgram/ml). Concentrations of lysates were quantified by the Bradford method and equal amounts (60 micrograms) of protein were loaded per lane. The levels of full-length APP were reduced in the hypertensive retinas when compared to the control retinas. Densitometry of these 115 kDa bands confirmed a statistically significant decrease in full-length APP in the hypertensive retinas when compared to the control retinas ($p<0.02$, paired t-test). The reduction of full-length APP provides direct evidence of abnormal

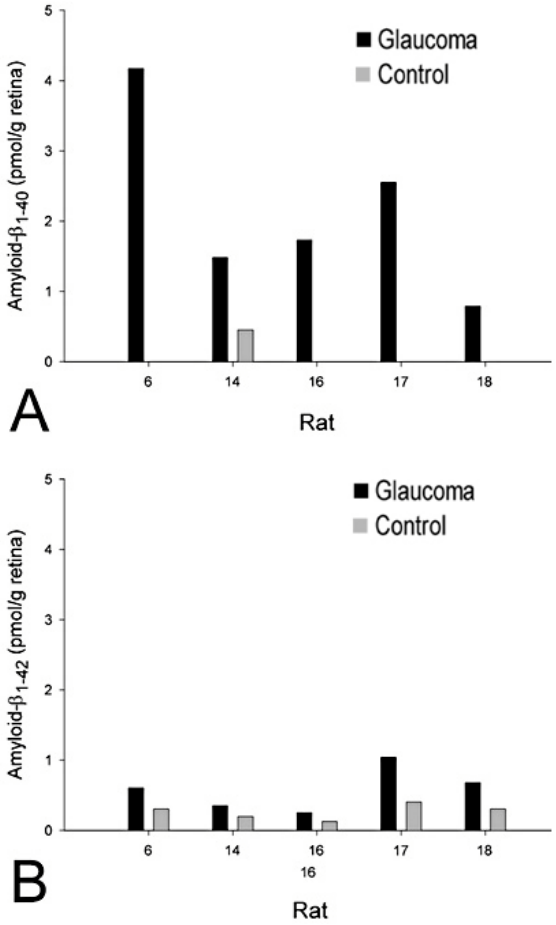

Figure 7. Amyloid-beta ELISA of rat retinal protein taken from five pairs of glaucoma and control eyes. A) Amyloid-beta$_{1-40}$, and B) amyloid-beta$_{1-42}$ levels were elevated in retinas from eyes exposed to chronically elevated IOP. There is no detectable amyloid-beta$_{1-40}$ in 4 out of 5 controls. Amyloid-beta$_{1-40}$ elevation is approximately double that of amyloid-beta$_{1-42}$ in the glaucoma retinas.

processing of APP in rat ocular hypertension. When the same immunoblot was re-probed with 4G8, proteolytic fragments of APP containing amyloid-beta epitopes were identified in ocular hypertensive and control retinas. Densitometry of these 22 kDa bands quantified a statistically significant increase in amyloid-beta epitopes in hypertensive retinas when compared to control retinas ($P = 0.0001$, paired t-test). Using the 4G8 antibody we have thus demonstrated upregulation of amyloid-beta-containing fragments in rat ocular hypertension, and have provided further direct evidence that APP is abnormally processed in glaucoma.

Cryopreserved retinas sectioned from rat ocular hypertension and control eyes were then immunolabeled using an antibody directed against amyloid-beta epitopes (R1282, kindly furnished by Dennis Selkoe, Brigham & Women's; 1:250 dilution). Control RGCs showed little labeling; ocular hypertensive retinas showed strong RGC labeling. A masked reading of slides showed striking and statistically significant elevation (chi^2, $p<0.0001$) of amyloid-beta immunolabeling in ocular hypertensive eyes (22/23 or 96%) when compared to control eyes (0/23 or 0%) (83). We then used ELISA to measure both amyloid-beta$_{1-40}$ and amyloid-beta$_{1-42}$ levels in total retinal protein extracted from retinas of five rats with unilateral elevation of intraocular pressure. Amyloid-beta$_{1-40}$ was significantly elevated ($P = 0.023$) in ocular hypertensive retinas (Figure 7A), going from undetectable levels in four out of five control eyes. The only control retina that had measurable amyloid-beta$_{1-40}$ was rat 14, with the highest level of IOP exposure (359 mmHg-days), well above the 200 mmHg-days threshold others (88) and we have independently observed for RGC axon loss. Amyloid-beta$_{1-42}$ undergoes an approximate doubling in ocular hypertensive retinas when compared to controls (Figure 7B), but the elevations are below that seen with amyloid-beta$_{1-40}$.

Exposure of cortical neurons to 1 microgram/ml of monoclonal antibody 22c11 (Chemicon), which binds to the N-terminal extracellular domain of APP, has been shown to induce morphological changes including neurite degeneration, nuclear condensation, and DNA cleavage consistent with apoptosis (89). We have employed a similar strategy to determine whether rat RGCs can be induced to undergo apoptosis *in vivo*. Before use, 22c11 stock (50 microgram/ml) was purified using Micro Bio-spin chromatography columns (BioRad, Hercules, CA) Given an estimated vitreous volume in the rat of 40 microliters, 2 microliters of a 20 microgram/ml solution was injected into the vitreous space (taking care to avoid the lens), giving a final intravitreal concentration of 1 microgram/ml. A similar control group received intravitreal injections of an equal amount of IgG. The retinas were assayed at three different timepoints (2 days, 5 days, and 12 days post-injection) for DNA fragmentation using TUNEL (90). Examination of retinas taken at day 12 post-injection showed no DNA fragmentation by TUNEL in IgG-treated controls, while numerous TUNEL-positive RGCs were seen in the 22c11-treated retinas (Figure 8). Negative control retinas omitting TdT displayed no labeling, and positive control retinas pre-treated with DNase displayed extensive TUNEL positivity in RGCs. These preliminary qualitative results indicate that disruption of APP processing using intravitreal injection of a monoclonal antibody to the extracellular domain of APP causes DNA fragmentation consistent with apoptosis.

5. DELAYED APOPTOSIS IN RAT GLAUCOMA

Alpha-fodrin is an important neuronal cytoskeletal protein and a target for caspase-3 degradation during the initiation phase of apoptosis, contributing to structural rearrangements including membrane blebbing (91). Alpha-fodrin and caspase-3 colocalize with neurofibrillary tangles (NFTs) and amyloid-containing plaques in AD brains (92, 93). Recent research has shown extensive accumulation of abnormal deposits of caspase-3 cleaved alpha-fodrin in a large number of viable AD neurons (92-94). This suggests that caspase activation may not immediately lead to cell death, which indicates an extended apoptotic-like degenerative process that is profoundly different from the classical view of rapid

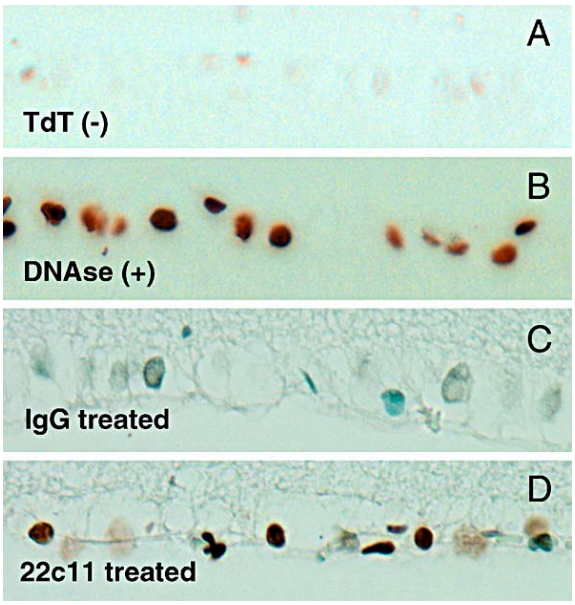

Figure 8. Rat RGCs exposed to intravitreal injection of 22c11 antibody were assayed using TUNEL. A) The TdT (-) control showed no DNA fragmentation in RGCs. B) The DNAse (+) control showed TUNEL labeling in RGCs. C) The IgG-treated RGCs showed no DNA fragmentation. D) After 12 days, the 22c11-treated RGCs showed numerous TUNEL (+) RGCs. Magnification = 20x.

apoptosis (92). Given these findings, we determined that caspase-3 cleaves alpha-fodrin in a similar fashion as that detected in AD neuronal tissue (95). We induced chronic ocular hypertension in five adult brown Norway rats, and monitored IOP exposure for five weeks. Retinal protein from both eyes of each rat was immunoblotted with a mouse monoclonal antibody recognizing full-length alpha-fodrin (Chemicon International, Inc., Temecula, CA, 1:2000 in TBST). Densitometry (ImageQuant, Molecular Dynamics, Piscataway, NJ) was performed and tested for significance using the paired Student's t-test to determine differences in caspase-3 specific alpha-fodrin cleavage between ocular hypertensive and control retinas. Densitometry confirmed a statistically significant increase in caspase-3 cleaved alpha-fodrin in ocular hypertensive retinas compared to paired controls ($P = 0.01$, paired t-test). We assumed that the basal level of caspase-3 activity in each control eye was similar. We then normalized caspase-3 activity for each COH retina by taking the ratio of densitometries of the COH vs. control retinas and determined if caspase-3 activity, as measured by alpha-fodrin cleavage, correlated with IOP exposure. Linear regression showed a high degree of correlation between caspase-3 activity and IOP exposure ($R^2 = 0.915$, $P = 0.011$) To show this relationship, we took the ratio of COH versus paired control bands for each eye. Caspase-3 activity seems to increase linearly with low IOP exposures that are not expected to cause RGC loss.

5.1. Caspase inhibition in rat glaucoma using AAV-mediated gene therapy

Adeno-associated viral (AAV) vectors have proven to be more effective at delivery of genes to the retina, due to the virus's ability to stably integrate DNA into the genome and lack of host immune response to the virus. For example, long-term expression of the marker lacZ was seen in the optic nerve 1 year after AAV-mediated intravitreal gene delivery (96). Given that caspases are activated in rat ocular hypertension, we determined that AAV-directed retinal ganglion cell expression of baculovirus repeat-containing 4 (BIRC4, also known as XIAP), a potent inhibitor of the apoptosis effector caspase-3, prevented optic nerve axon loss in the chronic ocular hypertensive rat glaucoma model (97). 57 adult Norway brown rats (225-250 gm) were randomized into three groups, and received a masked unilateral intravitreal injection of 2 microliters of one of three constructs: 1) AAV linked to the chicken beta-actin (CBA) promoter and the coding region of BIRC4 (AAV:CBA:BIRC4; n=18), 2) control reporter construct AAV:CBA:GFP (n=19), or 3) balanced salt solution (BSS, n=20). After one month to allow ganglion cell expression, glaucoma was induced in the treated eye by the method of limbal injection of hypertonic saline (2.0 M). IOP was recorded weekly for 12 weeks with a calibrated Tonopen. After sedation, treated and control eyes were enucleated, and optic nerves cross-sections stained with toluidine blue. Axon counts were obtained using the KS400 imaging system (Zeiss). After exclusion of animals with prolonged hypotony or no documented IOP rise, axon counts and percent axon survival between treated and control eyes for the three treatment groups were averaged and compared (Table 1). The percentage of axons surviving (glaucoma vs. control) in the BIRC4 group was significantly higher than both the GFP and BSS groups. Expression of GFP protein in the retina did not appear to have a deleterious effect on optic nerve axons in hypertensive rat eyes.

6. CONCLUSIONS AND PERSPECTIVES

6.1. Similarities between glaucoma and Alzheimer's disease

Glaucoma and Alzheimer's disease are chronic neurodegenerative conditions, and similarities in neuropathology have been noted. Optic nerves from AD patients show loss of large magnocellular RGCs, the cell type that dies earliest in glaucoma (98). Neurofilament triplet proteins that are components of pathological neurofibrillary tangles also show localization to large RGCs (99, 100). Excitotoxic triggers such as elevated glutamate (12, 101) and nitric oxide synthase upregulation with reactive oxygen species formation (16) have been implicated in glaucoma, as they have in Alzheimer's disease (102). Synaptic dysfunction in AD is associated with deficient glutamate transport function (103, 104) and caspase activity (105), leading to increased susceptibility to excitotoxic injuries. Most recently, a study of nursing home patients in Germany found a statistically significant increase in the prevalence of glaucoma (as defined by characteristic optic nerve or visual field changes) in patients diagnosed with AD (29/112 or 25.9%) compared with a matched control group without AD (6/116 or 5.2%) (106). A separate study measured automated visual fields from mildly affected AD patients (107). Significant loss was most pronounced in the inferonasal and inferotemporal arcuate regions, in a pattern that is very similar to

Table 1. Optic nerve axon counts and survivals for experimental groups in the BIRC4 gene therapy glaucoma experiment

Axon Counts (mean ± SD)	**BIRC4**	**GFP**	**BSS**	**Comparison of % Axon Survivals**	**One-way ANOVA**
Treated	44,982 ± 36,066	18,750 ± 22,509	18,823 ± 16,962	BIRC4 vs. GFP	P<0.05
Control	86,746 ± 12,229	86,863 ± 15,685	85,117 ± 8,176	BIRC4 vs. BSS	P<0.05
% Survival	49.7 ± 37.4	22.3 ± 27.4	21.8 ± 18.6	GFP vs. BSS	P<0.93
Number	12	11	14		

glaucomatous visual field loss. Visual field loss has recently been shown to occur at a greater rate in AD patients when compared to open-angle glaucoma patients (108). Allelic forms of apoE (i.e. the epsilon4 allele), lipoproteins important in lipid metabolism, are known to be major risk factors for AD (109). In human glaucoma patients, a single-nucleotide polymorphism (SNP) in the promoter region of apoE has been shown to amplify the primary open-angle (POAG) phenotype of increased optic nerve cup-to-disk ratio and visual field loss. Increased IOP and resistance to IOP-lowering medication was noted in patients with the interaction of another apoE promoter SNP with a SNP in the promoter of myocilin, a protein noted to be upregulated in trabecular meshwork after exposure to steroids and mutated in juvenile OAG (110). These findings point to the exciting possibility of a functional link in basic apoptotic cell death mechanisms between glaucoma and Alzheimer's disease.

Caspase activation with cleavage of APP has been shown to upregulate amyloid-beta production in cultured hippocampal neurons and in brains of Alzheimer's patients (52). Glaucoma is a chronic neurodegeneration involving apoptosis and we have detected abnormal APP processing, upregulation of amyloid-beta, and the caspase-3-mediated C-terminal cleavage product of APP in RGCs in an ocular hypertensive rat model of glaucoma. We have also demonstrated the activation of caspase-8 in our rat glaucoma model, and it appears to be an early event in the process (83). Our findings of caspase-8 activation directly implicate the extrinsic pathway, involving TNF receptor superfamily members, in RGC apoptosis due to exposure to elevated IOP. This family includes TNFR1 (p55), Fas, and the low-affinity neurotrophin receptor (p75NTR). Other groups have found increased expression of TNFR1 and ligand TNF-alpha in retinas and optic nerves from human glaucoma patients, when compared to age-matched controls (111, 112). Mutations in a recently identified gene, optineurin, were found in 16.7% of families with hereditary POAG, including individuals with normal intraocular pressure. The optineurin gene codes for a protein of unknown function that is implicated in the TNF-alpha signaling pathway (113). Also of interest is p75NTR, which acts a "dependence" receptor. Dependence receptors such as p75NTR, DCC (*deleted in colorectal cancer*) and the androgen receptor inhibit apoptosis when bound by their obligate ligand, but induce apoptosis when the receptor is unbound (114). Nerve growth factor (NGR) normally binds to p75NTR, but amyloid-beta has been shown to bind to p75NTR and induce apoptosis *in vitro*. In glaucoma, amyloid-beta may displace NGF from p75NTR, initiating the apoptotic cascade and resulting in RGC death.

The "intrinsic" pathway of apoptosis involves disruption of electron transport and energy metabolism at the level of the mitochondria. Changes in mitochondrial membrane potential cause cytochrome-c release. Cytochrome c, Apaf-1 and procaspase-9 assemble to form a complex, causing activation of caspase-9. We have not observed caspase-9 activity in RGCs in our rat glaucoma model, nor are there any published reports of caspase-9 activation in glaucoma, so the involvement of the intrinsic pathway in glaucoma has yet to be demonstrated. An alternative caspase pathway that operates in parallel with caspase-9 is one involving caspase-2. Caspase-2 is interesting in that it shares sequence homology with initiator caspases caspase-9 and -1, but also has cleavage specificity similar to effector caspases-3 and -7 (115). When endogenous inhibitor of apoptosis proteins (IAPs) such as BIRC4 suppress caspase-9 activity, caspase-2 is required for neuronal death induced by growth factor deprivation (116). Caspase-2 is localized to the Golgi (117) and mediates neuronal apoptosis induced by amyloid-beta. In a study by Troy (118), neurons derived from caspase-2 knockout mice were totally resistant to amyloid-beta-induced toxicity. They also showed that nitric oxide inhibitors did not protect wild-type neurons from amyloid-beta$_{1–42}$ toxicity, whereas nitric oxide generators were completely protective. It has been postulated that caspase activity is inhibited by nitrosylation (119). Caspase-2 has been localized to RGCs and amacrine cells after transient retinal ischemia (120). Our RPA data (see Section 4.1) show that caspase-2 mRNA expression is present in rat retinas and is upregulated in chronic ocular hypertension (83), although not to the same extent as caspase-3. Given that caspase-2 is necessary in amyloid-beta-induced neurotoxicity, we are currently investigating the role of caspase-2 in glaucomatous RGC apoptosis.

To our knowledge, fibrillar amyloid-beta has not been detected in the retinas of human glaucoma patients. A high ratio of amyloid-beta$_{1-42}$ to amyloid-beta$_{1-40}$ predisposes toward amyloid plaque formation (121), but our results (see above) show that this ratio is low. This may explain why plaques have not been observed in retinas from glaucoma patients. Because fibrillar amyloid-beta can kill neurons *in vitro*, it is assumed that fibrils initiate the neurodegeneration in AD. Previous work by Jen showed that single intravitreal injections of fibrillar amyloid-beta$_{1-40}$ or amyloid-beta$_{1-42}$ produced cell death primarily in photoreceptors, with only scattered cell death seen in RGCs after 4 days (122). They further noted that after 5 months, intravitreal exposure of retinas to amyloid-beta$_{1-42}$ produced a significant reduction in large RGCs with a concomitant significant increase in medium and small RGCs, with

marked Muller cell and microglial activation (123). It appears that a chronic exposure of RGCs to amyloid species is necessary to cause RGC death, and we believe that the chronic nature of glaucoma parallels this observation closely. Interestingly, in AD there is only a weak correlation between fibrillar amyloid-beta load and neurological dysfunction (124). An emerging and different view, however, is that fibrils are not the only toxic form of amyloid-beta relevant to AD. Small oligomeric forms of amyloid-beta known as amyloid-beta-derived diffusible ligands or "ADDLs" also have potent neurotoxic activity, and appear to better explain the amyloidogenic hypothesis in AD (125). Given that caspase-3 activation with subsequent cleavage of APP has been associated with apoptosis of hippocampal neurons in brains of Alzheimer's disease (52), it is likely that exposure of RGCs to similar soluble fragments of APP contributes to RGC apoptosis in glaucoma. Supporting this hypothesis is that amyloid-beta appears in an intracellular location in rat RGCs subjected to elevated IOP (83), implying that amyloid-beta exists in a soluble form in glaucoma.

6.2. Vascular pathology in glaucoma and Alzheimer's disease

Vascular deposits of amyloid-beta in AD are known to contribute to cerebral amyloid angiopathy (CAA), which results in degeneration of vascular endothelial and smooth muscle cells (126, 127) and hemorrhagic stroke (128). Amyloid-beta1-40 is reported to be more abundant in vasculature of AD patients, whereas amyloid-beta1-42 is more abundant in senile plaques (129). Ultrastructural studies of mutant APP mouse brain vasculature by electron microscopy (EM) and immunogold EM using antibodies directed against amyloid-beta1-40 and amyloid-beta1-42 showed higher levels of amyloid-beta1-40 deposited in vessel walls than amyloid-beta1-42. Amyloid-beta deposition was prominent in pial arterioles of leptomeninges, suggesting a particular vulnerability of this type of blood vessel to apoptosis (130). Of note is that pial vessels of this type also supply the laminar optic nerve. Vascular amyloid-beta deposition has also been shown to negatively affect the blood-brain barrier (131). These data support the hypothesis that amyloid-beta plays an important role in the damage to endothelial cells observed in AD brains (132). C-terminal fragments of APP of 22 kDa in length have also been noted to be upregulated in cerebral vasculature in AD and Down's syndrome brains (133, 134). These findings raise the intriguing possibility that vascular amyloid deposition may occur in glaucoma. We believe that elevated levels of amyloid-beta1-40 and other APP fragments cause a form of CAA that damages retinal and optic nerve head blood vessels, contributing to the pathology of glaucoma. This may also provide a mechanism for optic disk "splinter hemorrhages" sometimes noted in glaucoma patients. We intend to further study the expression of APP and in the microvasculature of the retina and optic nerve head in the rat ocular hypertension model of glaucoma.

Our findings also parallel the pathology seen in other chronic neurodegenerations such as AD, in which neurons undergo chronic caspase activation, slow build-up of cleavage products, and a delayed course of apoptosis (135). The accumulation of caspase-3 cleaved alpha-fodrin in what appear to be viable RGCs suggests that caspase activation may not immediately lead to RGC cell death. This indicates that RGCs undergo an extended, slow, apoptotic-like degenerative process that is profoundly different from the rapid, classical apoptotic pathway found in many cell lines (92). The advantage of this theory is of importance in finding successful approaches for neuroprotection of RGCs. If caspase activation in glaucoma leads to protracted rather than rapid apoptosis of RGCs, a much longer therapeutic window exists for achieving inhibition of various steps of apoptosis with caspase inhibitors. Indeed, in a chronic ocular hypertensive rat model of glaucoma, we have documented activation of caspase-8 and caspase-3, both of which are inhibited by BIRC4. Expression of BIRC4 before induction of ocular hypertension significantly protected approximately 50% of optic nerve axons. Adeno-associated virus, with its excellent safety profile and stable integration, is a promising vector for gene delivery to retinal ganglion cells. Gene therapy by intravitreal delivery of caspase inhibitors has demonstrated potential as a neuroprotective therapy for glaucoma.

Our data suggest a novel hypothesis for RGC death in glaucoma involving chronic amyloid-beta neurotoxicity, in a manner that mimics AD at the molecular level. With the loss of the protective effect of APP and the upregulation of toxic APP fragments that include amyloid-beta, RGCs ultimately die from chronic caspase activation, loss of synaptic homeostasis, amyloid-beta cytotoxicity and other excitotoxic events. The potential benefits from this work are that treatments contemplated for AD could be used to treat glaucoma. Conversely, novel neuroprotective strategies developed to treat glaucoma could be used to treat other chronic neurodegenerations as well.

7. ACKNOWLEDGEMENTS

The author would like to thank the following associates for their invaluable contributions: William W. Hauswirth, Ph.D., Eric LaCasse, Ph.D., Donna M. Lehman, Ph.D., Qiuhong Li, Ph.D., Peter Liston, Ph.D., Lisa A. Kerrigan-Baumrind, Robert G. Korneluk, M.D., Mary Ellen Pease, Harry A. Quigley, M.D., Nancy L. Ransom, George S. Robertson, Ph.D., Herbert A. Reitsamer, M.D., N. Grace Tahzib, M.D., Steven G. Younkin, M.D., Ph.D., and Donald J. Zack, M.D., Ph.D.

This work was supported by grants from American Health Assistance Foundation-National Glaucoma Research, National Eye Institute (EY00361), American Glaucoma Society, Foundation Fighting Blindness, Macula Vision Research Foundation, Research to Prevent Blindness, Howard Hughes Medical Institute, San Antonio Area Foundation, Nathan Shock Aging Research and Education Council, and Aegera Therapeutics Corporation. We thank Martine St. Jean of Aegera Oncology for technical assistance.

8. REFERENCES

1. Leske M.C.: The epidemiology of open angle glaucoma. *Am J Epidemiol* 118, 116-191 (1983)

2. Tielsch J.M., A. Sommer & J. Katz: Racial variations in the prevalence of primary open-angle glaucoma: the baltimore eye survey. *JAMA* 266, 369-374 (1991)

3. Quigley H.A.: Number of people with glaucoma worldwide. *Br J Ophthalmol* 80, 389-393 (1996)

4. Quigley H.A. & W.R. Green: The histology of human glaucoma cupping and optic nerve damage: clinicopathologic correlation in 21 eyes. *Ophthalmol* 86, 1803-1830 (1979)

5. Garcia-Valenzuela E., S. Shareef, J. Walsh & S.C. Sharma: Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp Eye Res* 61, 33-44 (1995)

6. Kerrigan L.A., D.J. Zack, H.A. Quigley, S.D. Smith & M.E. Pease: TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch Ophthalmol* 115, 1031-1035 (1997)

7. Quigley H.A., R.W. Nickells, L.A. Kerrigan, M.E. Pease, D.J. Thibault & D.J. Zack: Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci* 36, 774-786 (1995)

8. Nickells R.W.: Apoptosis of retinal ganglion cells in glaucoma: an update of the molecular pathways involved in cell death. *Surv Ophthalmol* 43 Suppl 1, S151-161 (1999)

9. Quigley H.A. & D.R. Anderson: Distribution of axonal transport blockade by acute intraocular pressure elevation in the primate optic nerve head. *Invest Ophthalmol Vis Sci* 16, 640-644 (1977)

10. Quigley H.A., J. Guy & D.R. Anderson: Blockage of rapid axonal transport. Effect of intraocular pressure elevation in primate optic nerve. *Arch Ophthalmol* 97, 525-531 (1979)

11. Quigley H.A., R.W. Flower, E.M. Addicks & D.S. McLeod: The mechanism of optic nerve damage in experimental acute intraocular pressure elevation. *Invest Ophthalmol Vis Sci* 19, 505-517 (1980)

12. Dreyer E.B., D. Zurakowski, R.A. Schumer, S.M. Podos & S.A. Lipton: Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. *Arch Ophthalmol* 114, 299-305 (1996)

13. Tezel G., G.M. Seigel & M.B. Wax: Autoantibodies to small heat shock proteins in glaucoma. *Invest Ophthalmol Vis Sci* 39, 2277-2287 (1998)

14. Osborne N.N., M. Ugarte, M. Chao, G. Chidlow, J.H. Bae, J.P. Wood & M.S. Nash: Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv Ophthalmol* 43 Suppl 1, S102-128 (1999)

15. Yorio T., R. Krishnamoorthy & G. Prasanna: Endothelin: is it a contributor to glaucoma pathophysiology? *J Glaucoma* 11, 259-270 (2002)

16. Neufeld A.H., A. Sawada & B. Becker: Inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma. *Proc Natl Acad Sci U S A* 96, 9944-9948 (1999)

17. Wong P., D. Taillefer, J. Lakins, J. Pinneault, G. Chader & M. Tenniswood: Molecular characterization of human TRPM-2/Clusterin, a gene associated with sperm maturation, apoptosis, and neurodegeneration. *Eur J Biochem* 221, 917-925 (1994)

18. Rosenbaum D.M., J. Kalberg & J.A. Kessler: Superoxide dismutase ameliorates neuronal death from hypoxia in culture. *Stroke* 25, 857-863 (1994)

19. Mirabelli F., A. Salis, V. Marinoni, G. Finardi, G. Bellomo, H. Thor & S. Orrenius: Menadione-induced bleb formation in hepatocytes is associated with the oxidation of thiol groups in actin. *Arch Biochem Biophys* 264, 261-269 (1988)

20. Enari M., H. Hug & S. Nagata: Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* 375, 78-81 (1995)

21. Marks N. & M.J. Berg: Recent advances on neuronal caspases in development and neurodegeneration. *Neurochem Int* 35, 195-220 (1999)

22. Tewari M., L.T. Quan, O.R. K, S. Desnoyers, Z. Zeng, D.R. Beidler, G.G. Poirier, G.S. Salvesen & V.M. Dixit: Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81, 801-809 (1995)

23. An B. & Q.P. Dou: Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. *Cancer Res* 56, 438-442 (1996)

24. Hensey C. & J. Gautier: A developmental timer that regulates apoptosis at the onset of gastrulation. *Mech Dev* 69, 183-195 (1997)

25. Tan X. & J.Y. Wang: The caspase-RB connection in cell death. *Trends Cell Biol* 8, 116-120 (1998)

26. Muzio M., A.M. Chinnaiyan, F.C. Kischkel, O.R. K, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, M. Mann, P.H. Krammer, M.E. Peter & V.M. Dixit: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* 85, 817-827 (1996)

27. Tezel G. & M.B. Wax: Inhibition of caspase activity in retinal cell apoptosis induced by various stimuli in vitro. *Invest Ophthalmol Vis Sci* 40, 2660-2667 (1999)

28. Petersen A., K. Mani & P. Brundin: Recent advances on the pathogenesis of Huntington's disease. *Exp Neurol* 157, 1-18 (1999)

29. Morrison B.M., P.R. Hof & J.H. Morrison: Determinants of neuronal vulnerability in neurodegenerative diseases. *Ann Neurol* 44, S32-44 (1998)

30. Loetscher H., U. Deuschle, M. Brockhaus, D. Reinhardt, P. Nelboeck, J. Mous, J. Grunberg, C. Haass & H. Jacobsen: Presenilins are processed by caspase-type proteases. *J Biol Chem* 272, 20655-20659 (1997)

31. Kim T.W., W.H. Pettingell, Y.K. Jung, D.M. Kovacs & R.E. Tanzi: Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. *Science* 277, 373-376 (1997)

32. Badman M.K., K.I. Shennan, J.L. Jermany, K. Docherty & A. Clark: Processing of pro-islet amyloid polypeptide (proIAPP) by the prohormone convertase PC2. *FEBS Lett* 378, 227-231 (1996)

33. Yagui K., T. Yamaguchi, A. Kanatsuka, F. Shimada, C.I. Huang, Y. Tokuyama, H. Ohsawa, K. Yamamura, J. Miyazaki, A. Mikata, S. Yoshida & E. Makino: Formation of islet amyloid fibrils in beta-secretory granules of transgenic mice expressing human islet amyloid polypeptide/amylin. *Eur J Endocrinol* 132, 487-496 (1995)

34. Villegas-Perez M.P., M. Vidal-Sanz, M. Rasminsky, G.M. Bray & A.J. Aguayo: Rapid and protracted phases of retinal ganglion cell loss follow axotomy in the optic nerve of adult rats. *J Neurobiol* 24, 23-36 (1993)

35. Berkelaar M., D.B. Clarke, Y.C. Wang, G.M. Bray & A.J. Aguayo: Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J Neurosci* 14, 4368-4374 (1994)

36. Garcia-Valenzuela E., W. Gorczyca, Z. Darzynkiewicz & S.C. Sharma: Apoptosis in adult retinal ganglion cells after axotomy. *J Neurobiol* 25, 431-438 (1994)

37. Mittag T.W., J. Danias, G. Pohorenec, H.M. Yuan, E. Burakgazi, R. Chalmers-Redman, S.M. Podos & W.G. Tatton: Retinal damage after 3 to 4 months of elevated intraocular pressure in a rat glaucoma model. *Invest Ophthalmol Vis Sci* 41, 3451-3459 (2000)

38. Agarwal R., M. Talati, W. Lambert, A.F. Clark, S.E. Wilson, N. Agarwal & R.J. Wordinger: Fas-activated apoptosis and apoptosis mediators in human trabecular meshwork cells. *Exp Eye Res* 68, 583-590 (1999)

39. Koulich E., T. Nguyen, K. Johnson, C. Giardina & S. D'Mello: NF-kappaB is involved in the survival of cerebellar granule neurons: association of IkappaBbeta phosphorylation with cell survival. *J Neurochem* 76, 1188-1198 (2001)

40. Wang N., S.K. Chintala, M.E. Fini & J.S. Schuman: Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. *Nat Med* 7, 304-309 (2001)

41. Katzman R.: Alzheimer's disease. *N Engl J Med* 314, 964-973 (1986)

42. Kang J., H.G. Lemaire, A. Unterbeck, J.M. Salbaum, C.L. Masters, K.H. Grzeschik, G. Multhaup, K. Beyreuther & B. Muller-Hill: The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325, 733-736 (1987)

43. Smale G., N.R. Nichols, D.R. Brady, C.E. Finch & W.E. Horton, Jr.: Evidence for apoptotic cell death in Alzheimer's disease. *Exp Neurol* 133, 225-230 (1995)

44. Mattson M.P.: Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiol Rev* 77, 1081-1132 (1997)

45. LeBlanc A.: Increased production of 4 kDa amyloid beta peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. *J Neurosci* 15, 7837-7846 (1995)

46. LaFerla F.M., B.T. Tinkle, C.J. Bieberich, C.C. Haudenschild & G. Jay: The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nat Genet* 9, 21-30 (1995)

47. Hsiao K.K., D.R. Borchelt, K. Olson, R. Johannsdottir, C. Kitt, W. Yunis, S. Xu, C. Eckman, S. Younkin, D. Price, C. Iadecola, H.B. Clark & G. Carlson: Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer amyloid precursor proteins. *Neuron* 15, 1203-1218 (1995)

48. Citron M., T. Oltersdorf, C. Haass, L. McConlogue, A.Y. Hung, P. Seubert, C. Vigo-Pelfrey, I. Lieberburg & D.J. Selkoe: Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* 360, 672-674 (1992)

49. Holcomb L., M.N. Gordon, E. McGowan, X. Yu, S. Benkovic, P. Jantzen, K. Wright, I. Saad, R. Mueller, D. Morgan, S. Sanders, C. Zehr, K. O'Campo, J. Hardy, C.M. Prada, C. Eckman, S. Younkin, K. Hsiao & K. Duff: Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat Med* 4, 97-100 (1998)

50. Mattson M.P. & W. Duan: "Apoptotic" biochemical cascades in synaptic compartments: roles in adaptive plasticity and neurodegenerative disorders. *J Neurosci Res* 58, 152-166 (1999)

51. Vito P., T. Ghayur & L. D'Adamio: Generation of anti-apoptotic presenilin-2 polypeptides by alternative transcription, proteolysis, and caspase-3 cleavage. *J Biol Chem* 272, 28315-28320 (1997)

52. Gervais F.G., D. Xu, G.S. Robertson, J.P. Vaillancourt, Y. Zhu, J. Huang, A. LeBlanc, D. Smith, M. Rigby, M.S. Shearman, E.E. Clarke, H. Zheng, L.H. Van Der Ploeg, S.C. Ruffolo, N.A. Thornberry, S. Xanthoudakis, R.J. Zamboni, S. Roy & D.W. Nicholson: Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid- beta precursor protein and amyloidogenic A beta peptide formation. *Cell* 97, 395-406 (1999)

53. Guenette S.Y., J. Chen, A. Ferland, C. Haass, A. Capell & R.E. Tanzi: hFE65L influences amyloid precursor protein maturation and secretion. *J Neurochem* 73, 985-993 (1999)

54. Ivins K.J., P.L. Thornton, T.T. Rohn & C.W. Cotman: Neuronal apoptosis induced by beta-amyloid is mediated by caspase-8. *Neurobiol Dis* 6, 440-449 (1999)

55. Ivins K.J., J.K. Ivins, J.P. Sharp & C.W. Cotman: Multiple pathways of apoptosis in PC12 cells. CrmA inhibits apoptosis induced by beta-amyloid. *J Biol Chem* 274, 2107-2112 (1999)

56. Morin P.J., C.R. Abraham, A. Amaratunga, R.J. Johnson, G. Huber, J.H. Sandell & R.E. Fine: Amyloid precursor protein is synthesized by retinal ganglion cells, rapidly transported to the optic nerve plasma membrane and nerve terminals, and metabolized. *J Neurochem* 61, 464-473 (1993)

57. Moya K.L., L.I. Benowitz, G.E. Schneider & B. Allinquant: The amyloid precursor protein is developmentally regulated and correlated with synaptogenesis. *Dev Biol* 161, 597-603 (1994)

58. Masliah E.: Mechanisms of synaptic dysfunction in Alzheimer's disease. *Histol Histopathol* 10, 509-519 (1995)

59. Morimoto T., I. Ohsawa, C. Takamura, M. Ishiguro, Y. Nakamura & S. Kohsaka: Novel domain-specific actions of amyloid precursor protein on developing synapses. *J Neurosci* 18, 9386-9393 (1998)

60. Nishimura I., T. Uetsuki, S.U. Dani, Y. Ohsawa, I. Saito, H. Okamura, Y. Uchiyama & K. Yoshikawa:

Degeneration in vivo of rat hippocampal neurons by wild-type Alzheimer amyloid precursor protein overexpressed by adenovirus-mediated gene transfer. *J Neurosci* 18, 2387-2398 (1998)

61. Masumura M., R. Hata, I. Nishimura, T. Uetsuki, T. Sawada & K. Yoshikawa: Caspase-3 activation and inflammatory responses in rat hippocampus inoculated with a recombinant adenovirus expressing the alzheimer amyloid precursor protein. *Brain Res Mol Brain Res* 80, 219-227 (2000)

62. Uetsuki T., K. Takemoto, I. Nishimura, M. Okamoto, M. Niinobe, T. Momoi, M. Miura & K. Yoshikawa: Activation of neuronal caspase-3 by intracellular accumulation of wild- type Alzheimer amyloid precursor protein. *J Neurosci* 19, 6955-6964 (1999)

63. Johnson C.S., D. Buster & J.M. Scholey: Light chains of sea urchin kinesin identified by immunoadsorption. *Cell Motil Cytoskeleton* 16, 204-213 (1990)

64. Kamal A., G.B. Stokin, Z. Yang, C.H. Xia & L.S. Goldstein: Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron* 28, 449-459 (2002)

65. Cai H., Y. Wang, D. McCarthy, H. Wen, D.R. Borchelt, D.L. Price & P.C. Wong: BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. *Nat Neurosci* 4, 233-234 (2001)

66. Ray W.J., M. Yao, J. Mumm, E.H. Schroeter, P. Saftig, M. Wolfe, D.J. Selkoe, R. Kopan & A.M. Goate: Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. *J Biol Chem* 274, 36801-36807 (1999)

67. Kamal A., A. Almenar-Queralt, J.F. LeBlanc, E.A. Roberts & L.S. Goldstein: Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. *Nature* 414, 643-648 (2001)

68. Rahman A., A. Kamal, E.A. Roberts & L.S. Goldstein: Defective kinesin heavy chain behavior in mouse kinesin light chain mutants. *J Cell Biol* 146, 1277-1288 (1999)

69. Kouchi Z., T. Kinouchi, H. Sorimachi, S. Ishiura & K. Suzuki: The deletion of the C-terminal tail and addition of an endoplasmic reticulum targeting signal to Alzheimer's amyloid precursor protein change its localization, secretion, and intracellular proteolysis. *Eur J Biochem* 258, 291-300 (1998)

70. Mucke L., E. Masliah, W.B. Johnson, M.D. Ruppe, M. Alford, E.M. Rockenstein, S. Forss-Petter, M. Pietropaolo, M. Mallory & C.R. Abraham: Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. *Brain Res* 666, 151-167 (1994)

71. Mattson M.P., Q. Guo, K. Furukawa & W.A. Pedersen: Presenilins, the endoplasmic reticulum, and neuronal apoptosis in Alzheimer's disease. *J Neurochem* 70, 1-14 (1998)

72. Keller J.N., Q. Guo, F.W. Holtsberg, A.J. Bruce-Keller & M.P. Mattson: Increased sensitivity to mitochondrial toxin-induced apoptosis in neural cells expressing mutant presenilin-1 is linked to perturbed calcium homeostasis and enhanced oxyradical production. *J Neurosci* 18, 4439-4450 (1998)

73. Begley J.G., W. Duan, S. Chan, K. Duff & M.P. Mattson: Altered calcium homeostasis and mitochondrial dysfunction in cortical synaptic compartments of presenilin-1 mutant mice. *J Neurochem* 72, 1030-1039 (1999)

74. Wong T.P., T. Debeir, K. Duff & A.C. Cuello: Reorganization of cholinergic terminals in the cerebral cortex and hippocampus in transgenic mice carrying mutated presenilin-1 and amyloid precursor protein transgenes. *J Neurosci* 19, 2706-2716 (1999)

75. Yuan J.: Transducing signals of life and death. *Curr Opin Cell Biol* 9, 247-251 (1997)

76. Mehmet H.: Caspases find a new place to hide. *Nature* 403, 29-30 (2000)

77. Morrison J.C., C.G. Moore, L.M. Deppmeier, B.G. Gold, C.K. Meshul & E.C. Johnson: A rat model of chronic pressure-induced optic nerve damage. *Exp Eye Res* 64, 85-96 (1997)

78. Lutty G., K. Ikeda, C. Chandler & D.S. McLeod: Immunohistochemical localization of transforming growth factor-beta in human photoreceptors. *Curr Eye Res* 10, 61-74 (1991)

79. Nir I., N. Agarwal, G. Sagie & D.S. Papermaster: Opsin distribution and synthesis in degenerating photoreceptors of rd mutant mice. *Exp Eye Res* 49, 403-421 (1989)

80. Suzuki N., T.T. Cheung, X.D. Cai, A. Odaka, L. Otvos, Jr., C. Eckman, T.E. Golde & S.G. Younkin: An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science* 264, 1336-1340 (1994)

81. Suzuki N., T. Iwatsubo, A. Odaka, Y. Ishibashi, C. Kitada & Y. Ihara: High tissue content of soluble beta 1-40 is linked to cerebral amyloid angiopathy. *Am J Pathol* 145, 452-460 (1994)

82. John S.W., R.S. Smith, O.V. Savinova, N.L. Hawes, B. Chang, D. Turnbull, M. Davisson, T.H. Roderick & J.R. Heckenlively: Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice. *Invest Ophthalmol Vis Sci* 39, 951-962 (1998)

83. McKinnon S.J., D.M. Lehman, L.A. Kerrigan-Baumrind, C.A. Merges, M.E. Pease, D.F. Kerrigan, N.L. Ransom, N.G. Tahzib, H.A. Reitsamer, H. Levkovitch-Verbin, H.A. Quigley & D.J. Zack: Caspase activation and amyloid precursor protein cleavage in rat ocular hypertension. *Invest. Ophthalmol. Vis. Sci.* 43, 1077-1087 (2002)

84. Hartmann T., S.C. Bieger, B. Bruhl, P.J. Tienari, N. Ida, D. Allsop, G.W. Roberts, C.L. Masters, C.G. Dotti, K. Unsicker & K. Beyreuther: Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat Med* 3, 1016-1020 (1997)

85. Askanas V., J. McFerrin, S. Baque, R.B. Alvarez, E. Sarkozi & W.K. Engel: Transfer of beta-amyloid precursor protein gene using adenovirus vector causes mitochondrial

abnormalities in cultured normal human muscle. *Proc Natl Acad Sci U S A* 93, 1314-1319 (1996)

86. Minckler D.S. & G.L. Spaeth: Optic nerve damage in glaucoma. *Surv Ophthalmol* 26, 128-148 (1981)

87. Barnes N.Y., L. Li, K. Yoshikawa, L.M. Schwartz, R.W. Oppenheim & C.E. Milligan: Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons. *J Neurosci* 18, 5869-5880 (1998)

88. Chauhan B.C., J. Pan, M.L. Archibald, T.L. LeVatte, M.E. Kelly & F. Tremblay: Effect of intraocular pressure on optic disc topography, electroretinography, and axonal loss in a chronic pressure-induced rat model of optic nerve damage. *Invest Ophthalmol Vis Sci* 43, 2969-2976 (2002)

89. Rohn T.T., K.J. Ivins, B.A. Bahr, C.W. Cotman & D.H. Cribbs: A monoclonal antibody to amyloid precursor protein induces neuronal apoptosis. *J Neurochem* 74, 2331-2342 (2000)

90. Kerrigan L.A. & D.J. Zack, Use of TUNEL to measure retinal ganglion cell apoptosis, in *Neuromethods, Vol. 29: Apoptosis techniques and protocols*, Poirier, J., Ed., Humana Press Inc., Totowa, NJ, pp. 199-217 (1997).

91. Janicke R.U., P. Ng, M.L. Sprengart & A.G. Porter: Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J Biol Chem* 273, 15540-15545 (1998)

92. Rohn T.T., E. Head, J.H. Su, A.J. Anderson, B.A. Bahr, C.W. Cotman & D.H. Cribbs: Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease. *Am J Pathol* 158, 189-198 (2001)

93. Su J.H., M. Zhao, A.J. Anderson, A. Srinivasan & C.W. Cotman: Activated caspase-3 expression in Alzheimer's and aged control brain: correlation with Alzheimer pathology. *Brain Res* 898, 350-357 (2001)

94. Marx J.: Neuroscience. New leads on the 'how' of Alzheimer's. *Science* 293, 2192-2194 (2001)

95. Tahzib N.G., N.L. Ransom, H.A. Reitsamer & S.J. McKinnon: Alpha-fodrin is cleaved by caspase-3 in a chronic ocular hypertensive (COH) rat model of glaucoma. *Brain Res Bull* (2003, in print)

96. Guy J., X. Qi, N. Muzyczka & W.W. Hauswirth: Reporter expression persists 1 year after adeno-associated virus- mediated gene transfer to the optic nerve. *Arch Ophthalmol* 117, 929-937 (1999)

97. McKinnon S.J., D.M. Lehman, N.G. Tahzib, N.L. Ransom, H.A. Reitsamer, P. Liston, E. LaCasse, Q. Li, R.G. Korneluk & W.W. Hauswirth: Baculoviral IAP Repeat-Containing-4 Protects Optic Nerve Axons in a Rat Glaucoma Model. *Mol Ther* 5, 780-787 (2002)

98. Sadun A.A. & C.J. Bassi: Optic nerve damage in Alzheimer's disease. *Ophthalmology* 97, 9-17 (1990)

99. Vickers J.C., R.A. Schumer, S.M. Podos, R.F. Wang, B.M. Riederer & J.H. Morrison: Differential vulnerability of neurochemically identified subpopulations of retinal neurons in a monkey model of glaucoma. *Brain Res* 680, 23-35 (1995)

100. Vickers J.C., P.R. Hof, R.A. Schumer, R.F. Wang, S.M. Podos & J.H. Morrison: Magnocellular and parvocellular visual pathways are both affected in a macaque monkey model of glaucoma. *Aust N Z J Ophthalmol* 25, 239-243 (1997)

101. Dreyer E.B., Z.H. Pan, S. Storm & S.A. Lipton: Greater sensitivity of larger retinal ganglion cells to NMDA-mediated cell death. *Neuroreport* 5, 629-631 (1994)

102. Ishii K., F. Muelhauser, U. Liebl, M. Picard, S. Kuhl, B. Penke, T. Bayer, M. Wiessler, M. Hennerici, K. Beyreuther, T. Hartmann & K. Fassbender: Subacute NO generation induced by Alzheimer's beta-amyloid in the living brain: reversal by inhibition of the inducible NO synthase. *Faseb J* 14, 1485-1489 (2000)

103. Li S., M. Mallory, M. Alford, S. Tanaka & E. Masliah: Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal APP expression. *J Neuropathol Exp Neurol* 56, 901-911 (1997)

104. Tominaga K., T. Uetsuki, A. Ogura & K. Yoshikawa: Glutamate responsiveness enhanced in neurones expressing amyloid precursor protein. *Neuroreport* 8, 2067-2072 (1997)

105. Masliah E., M. Mallory, M. Alford, S. Tanaka & L.A. Hansen: Caspase dependent DNA fragmentation might be associated with excitotoxicity in Alzheimer disease. *J Neuropathol Exp Neurol* 57, 1041-1052 (1998)

106. Bayer A.U., F. Ferrari & C. Erb: High Occurrence Rate of Glaucoma among Patients with Alzheimer's Disease. *Eur Neurol* 47, 165-168 (2002)

107. Trick G.L., L.R. Trick, P. Morris & M. Wolf: Visual field loss in senile dementia of the Alzheimer's type. *Neurology* 45, 68-74 (1995)

108. Bayer A.U. & F. Ferrari: Severe progression of glaucomatous optic neuropathy in patients with Alzheimer's disease. *Eye* 16, 209-212 (2002)

109. Roses A.D.: Apolipoprotein E alleles as risk factors in Alzheimer's disease. *Annu Rev Med* 47, 387-400 (1996)

110. Copin B., A.P. Brezin, F. Valtot, J.C. Dascotte, A. Bechetoille & H.J. Garchon: Apolipoprotein E-Promoter Single-Nucleotide Polymorphisms Affect the Phenotype of Primary Open-Angle Glaucoma and Demonstrate Interaction with the Myocilin Gene. *Am J Hum Genet* 70, 1575-1581 (2002)

111. Tezel G., L.Y. Li, R.V. Patil & M.B. Wax: Tnf-alpha and tnf-alpha receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci* 42, 1787-1794 (2001)

112. Yan X., G. Tezel, M.B. Wax & D.P. Edward: Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head. *Arch Ophthalmol* 118, 666-673 (2000)

113. Rezaie T., A. Child, R. Hitchings, G. Brice, L. Miller, M. Coca-Prados, E. Heon, T. Krupin, R. Ritch, D. Kreutzer, R.P. Crick & M. Sarfarazi: Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science* 295, 1077-1079 (2002)

114. Mehlen P., S. Rabizadeh, S.J. Snipas, N. Assa-Munt, G.S. Salvesen & D.E. Bredesen: The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. *Nature* 395, 801-804 (1998)

115. Troy C.M. & M.L. Shelanski: Caspase-2 redux. *Cell Death Differ* 10, 101-107 (2003)

116. Troy C.M., S.A. Rabacchi, J.B. Hohl, J.M. Angelastro, L.A. Greene & M.L. Shelanski: Death in the balance: alternative participation of the caspase-2 and -9 pathways in neuronal death induced by nerve growth factor deprivation. *J Neurosci* 21, 5007-5016 (2001)

117. Mancini M., C.E. Machamer, S. Roy, D.W. Nicholson, N.A. Thornberry, L.A. Casciola-Rosen & A. Rosen: Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. *J Cell Biol* 149, 603-612 (2000)

118. Troy C.M., S.A. Rabacchi, Z. Xu, A.C. Maroney, T.J. Connors, M.L. Shelanski & L.A. Greene: beta-Amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activation. *J Neurochem* 77, 157-164 (2001)

119. Mannick J.B., A. Hausladen, L. Liu, D.T. Hess, M. Zeng, Q.X. Miao, L.S. Kane, A.J. Gow & J.S. Stamler: Fas-induced caspase denitrosylation. *Science* 284, 651-654 (1999)

120. Singh M., S.I. Savitz, R. Hoque, G. Gupta, S. Roth, P.S. Rosenbaum & D.M. Rosenbaum: Cell-specific caspase expression by different neuronal phenotypes in transient retinal ischemia. *J Neurochem* 77, 466-475 (2001)

121. Shoji M.: Cerebrospinal fluid Abeta40 and Abeta42: natural course and clinical usefulness. *Front Biosci* 7, d997-1006 (2002)

122. Jen L.S., A.J. Hart, A. Jen, J.B. Relvas, S.M. Gentleman, L.J. Garey & A.J. Patel: Alzheimer's peptide kills cells of retina in vivo. *Nature* 392, 140-141 (1998)

123. Walsh D.T., R.M. Montero, L.G. Bresciani, A.Y. Jen, P.D. Leclercq, D. Saunders, AN E.L-Amir, L. Gbadamoshi, S.M. Gentleman & L.S. Jen: Amyloid-beta peptide is toxic to neurons in vivo via indirect mechanisms. *Neurobiol Dis* 10, 20-27 (2002)

124. Janus C., M.A. Chishti & D. Westaway: Transgenic mouse models of Alzheimer's disease. *Biochim Biophys Acta* 1502, 63-75 (2000)

125. Klein W.L., G.A. Krafft & C.E. Finch: Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci* 24, 219-224 (2001)

126. Wisniewski H.M., J. Frackowiak & B. Mazur-Kolecka: In vitro production of beta-amyloid in smooth muscle cells isolated from amyloid angiopathy-affected vessels. *Neurosci Lett* 183, 120-123 (1995)

127. Mackic J.B., M.H. Weiss, W. Miao, E. Kirkman, J. Ghiso, M. Calero, J. Bading, B. Frangione & B.V. Zlokovic: Cerebrovascular accumulation and increased blood-brain barrier permeability to circulating Alzheimer's amyloid beta peptide in aged squirrel monkey with cerebral amyloid angiopathy. *J Neurochem* 70, 210-215 (1998)

128. Vinters H.V.: Cerebral amyloid angiopathy. A critical review. *Stroke* 18, 311-324 (1987)

129. Prelli F., E. Castano, G.G. Glenner & B. Frangione: Differences between vascular and plaque core amyloid in Alzheimer's disease. *J Neurochem* 51, 648-651 (1988)

130. Van Dorpe J., L. Smeijers, I. Dewachter, D. Nuyens, K. Spittaels, C. Van Den Haute, M. Mercken, D. Moechars, I. Laenen, C. Kuiperi, K. Bruynseels, I. Tesseur, R. Loos, H. Vanderstichele, F. Checler, R. Sciot & F. Van Leuven: Prominent cerebral amyloid angiopathy in transgenic mice overexpressing the london mutant of human APP in neurons. *Am J Pathol* 157, 1283-1298 (2000)

131. Blanc E.M., M. Toborek, R.J. Mark, B. Hennig & M.P. Mattson: Amyloid beta-peptide induces cell monolayer albumin permeability, impairs glucose transport, and induces apoptosis in vascular endothelial cells. *J Neurochem* 68, 1870-1881 (1997)

132. Mattson M.P.: Central role of oxyradicals in the mechanism of amyloid β-peptide cytotoxicity. *Alz Dis Rev* 2, 1-14 (1997)

133. Tamaoka A., R.N. Kalaria, I. Lieberburg & D.J. Selkoe: Identification of a stable fragment of the Alzheimer amyloid precursor containing the beta-protein in brain microvessels. *Proc Natl Acad Sci U S A* 89, 1345-1349 (1992)

134. Russo C., S. Salis, V. Dolcini, V. Venezia, X.H. Song, J.K. Teller & G. Schettini: Identification of amino-terminally and phosphotyrosine-modified carboxy- terminal fragments of the amyloid precursor protein in Alzheimer's disease and Down's syndrome brain. *Neurobiol Dis* 8, 173-180 (2001)

135. Zhang Y., C. Goodyer & A. LeBlanc: Selective and protracted apoptosis in human primary neurons microinjected with active caspase-3, -6, -7, and -8. *J Neurosci* 20, 8384-8389 (2000)

Key Words: Alzheimer's disease, Amyloid, Amyloid-Beta, Amyloid Precursor Protein, Apoptosis, Axonal Transport, Caspase, Glaucoma, Neurodegeneration, Nerve, Optic Nerve, Retinal Ganglion Cell, Rat, Glaucoma, Model, Review

Send correspondence to: Dr. Stuart J. McKinnon, Department of Ophthalmology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, MC 6230, San Antonio, Texas 78229-3900, USA, Tel.: 210-567-8463, Fax: 210-567-8413, E-mail: mckinnon@uthscsa.edu