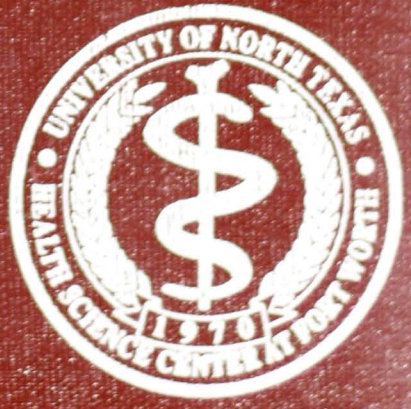
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Glaucoma presents a distinctive dysfunction in anterograde axonal transport that disproportionately affects the delivery of specific types of cargo(s) into the optic nerve. Previous models for the pathogenesis of glaucoma have failed to provide an adequate mechanism to explain the characteristic cargo-selectivity. A new theoretical model, the "endothelin receptor-mediated model of neuropathogenesis," was developed to explain the cargo-selective axonal transport dysfunction seen in glaucomtous optic neuropathy. In addition, a new experimental animal model, the "intravitreal endothelin/axonal transport" model was developed to test hypotheses generated by the new theoretical model.

Intravitreal endothelin-1 significantly affected all of the known rate components and subcomponents of anterograde axonal transport in the rat optic nerve. Changes were seen in anterograde fast axonal transport for both the fastest moving small tubulovesicles, and slightly slower membrane bound organelles (MBOs), as well as in the slow transport of cytoplasmic matrix and cytoskeletal materials. Endothelin-1's predominant effect was a severe depression in the mitochondrial subcomponent of fast anterograde axonal transport, which was most pronounced at 28 hours post-treatment. At that time, the effects of endothelin-1 were mimicked by endothelin-3, characteristic of the non-ischemic endothelin-B type of receptor. In addition, analysis of a cohort of 11 distinctive

protein bands moving with the mitochondrial subcomponent demonstrated a cargo-selective effect of endothelin-1 and the delayed movement into the optic nerve for a chemically distinct subset of proteins, but not the majority of protein, in transport during this timeframe. These results appear to be consistent with what is known about the pathology of glaucomatous optic neuropathy and the neurochemistry of anterograde axonal transport and suggest that intravitreal may be an excellent model to study the mechanisms of neurodegeneration that occurs in glaucoma.

EFFECTS OF INTRAVITREAL ENDOTHELIN-1 ON ANTEROGRADE AXONAL TRANSPORT IN RAT OPTIC NERVE: EVALUATING A POSSIBLE MECHANISM FOR GLAUCOMATOUS OPTIC NEUROPATHY.

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DISSERTATION

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INTRODUCTION

Statement of the problem

Glaucoma is a disease that shows a characteristic dysfunction in anterograde axonal transport disproportionately affecting the delivery of a few specific types of cargo(s) into the optic nerve. Previous theoretical models for the pathogenesis of glaucoma failed to provide an adequate mechanism to explain this cargo-selectivity.

Glaucoma

Glaucoma is a stereotypic optic neuropathy leading to blindness which affects 67 million people worldwide [1]. It is characterized by cupping of the optic nerve head, optic nerve head palor [2], astrogliosis in the region of the non-superficial optic nerve head [3], loss of retinal ganglion cells [4], and a dysfunction in anterograde axonal transport [5].

Glaucoma and axonal transport

In glaucoma, the anterograde axonal transport of specific cargos important for ganglion cell survival is impaired, most notably the transport of mitochondria. This impairment affects axonal transport of mitochondria in retinal ganglion cell axons that are traversing the peri-laminar region of the optic nerve head [5]. Because axons lack the machinery required for local synthesis of proteins [6], the timely delivery of specific cargos to their appropriate destinations, represents a complex logistical burden for the

neuron [7]. For example, mitochondria must be delivered to those axonal regions with intense ATP consumption [8]. At the same time, nascent synaptic vesicles must be delivered to the axon terminals [9]. Targeted delivery of specific cargos to different axonal destinations implies that axonal transport is regulated by a number of discrete mechanisms. Misregulation of these targeted deliveries could result in the "fingerprint" cargo-selective axonal transport dysfunctions seen in glaucoma [5], and other neurodegenerative diseases [9, 10].

Anterograde axonal transport is the intricately regulated process that neurons use to provide proteins necessary for maintenance and survival to their axons, whose long fiber tracts integrate the nervous system. Anterograde axonal transport is composed of several distinct rate components that deliver specific types of cargo to their appropriate axonal domains. Kinesins are the family of mechanochemical motor proteins associated with cargos moving in the fast component [11], whereas motors for the slow components are uncertain [12-15]. Fast anterograde axonal transport delivers a variety of membrane-bound organelle cargos (MBOs) and may be divided into several subcomponents, including 1) very fast, small tubulovesicles that include synaptic vesicle precursor proteins, and 2) slower moving MBOs that include mitochondrial marker proteins [16, 17]. Regulation of fast anterograde axonal transport is only partially understood [9-11, 18-24], however, it is known that markers for mitochondria and synaptic vesicle precursors co-segregate with different isoforms of the kinesin motor heavy chain [16, 25]. The majority of total protein delivered by anterograde axonal transport moves within the slow components. Slow component b (SCb) delivers the cytoplasmic matrix, including

actin microfilaments and most of the "soluble" proteins and enzymes [12, 13, 16, 26-35]. Slow component a (SCa) delivers cytoskeletal elements, such as neurofilaments and microtubules [26, 27, 31-34, 36-38]. Because of this, a generalized impairment of anterograde axonal transport may be expected to present large proximal swellings in regions with unmyelinated axons, disorganized microtubules, and prominent accumulations of neurofilaments [39, 40].

In glaucoma, peri-laminar accumulations are predominantly mitochondrial-vesicular in nature. This suggests that glaucoma selectively impairs anterograde transport for some of the fast component cargos to a greater extent than its impairment of slow component cargos. In addition, visual loss develops slowly in glaucoma, indicating that, unlike mitochondria, the delivery of synaptic vesicle precursors is not seriously compromised during the early phases of the disease. Both of these cargos move with the fast component, are membrane bound, and are transported in association with the kinesin mechanochemical motor. This suggests a mechanism that selectively affects different classes of kinesin-associated cargo.

Previous models fail to explain cargo-selective effects on axonal transport:

Direct compression of axons by elevated intraocular pressure

The most common risk factor associated with glaucoma is elevated intraocular pressure [41-43]. The early assumptions were that direct mechanical compression of retinal ganglion cell axons pinched against the laminar beams of the optic nerve head resulted in inhibition of axonal transport [44, 45]. Primate studies, carried out at

intraocular pressures in excess of 50 mm Hg [46-48], have been interpreted as evidence favoring the direct compression model [49]. Lower intraocular pressures (below 50 mm Hg) appear to act through a less direct mechanism [50-52]. However, studies on the early effects (4 hours) of pressure-induced optic neuropathy in primates indicated that the distribution of axonal transport dysfunction within a given axon bundle was not associated with proximity to the laminar beams, apparently contradicting the direct compression hypothesis [50]. Additional evidence against the direct compression hypothesis came from studies of the effects of direct compression upon axonal transport in exposed axons. These findings indicated relatively high pressures, 50-60 mm Hg, were required for inhibition of transport by compression alone. At relatively high pressures (50-60 mm Hg) all axonal transport was indiscriminately "derailed" by the loss of linear microtubule arrays [53], in contrast to the cargo-selective axonal transport dysfunction seen in human glaucomatous donor tissue [5]. In addition, distinctively glaucomatous neuropathology is seen in patients without elevated intraocular pressure [42, 43]. This "normal tension glaucoma" [54] suggests that, although elevated pressure may be sufficient to cause glaucomatous optic neuropathy in animal models [52, 55], it is apparently not necessary for its neuropathogenesis [42]. Human glaucomas typically involve quite moderate intraocular pressures, and more than half of all glaucoma patients show no pressure elevation at time of first diagnosis [56]. For these reasons, a model for glaucoma that does not depend upon direct compression of axons is needed.

Previous models fail to explain cargo-selective effects on axonal transport:

Chronic Retinal Ischemia or the Vascular Hypothesis

Chronic retinal ischemia and/or ischemia-reperfusion injury, resulting from vascular dysregulation, are commonly postulated as a pressure-independent model for the pathogenesis of glaucoma [43]. Studies suggesting that temperature-induced changes in peripheral blood flow may be more pronounced in some patients with normal tension glaucoma [57], have been interpreted as supporting the ischemic/vascular hypothesis. Short duration experiments (2 to 24 hours) have demonstrated that an inhibition of axonal transport is associated with central artery occlusion in primates [58]. However, clinical studies on the delayed effects (1-3 days delay) of transient ischemia in the central nervous system (CNS), have suggested that ischemia alone, like direct compression, may act on axonal transport through loss of linear microtubule arrays, "derailing" all axonal transport. This loss of linear microtubule arrays, produces a generalized inhibition of anterograde axonal transport. Neuropathies known to operate by this mechanism present with large proximal swellings in regions with unmyelinated axons, disorganized microtubules, and prominent accumulations of neurofilaments [39, 40], unlike the mitochondrial/vesicular accumulations characteristic of glaucoma [5]. For these reasons, a model for glaucoma that does not depend on loss of microtubule linear arrays is needed.

Glaucoma and endothelins

Glaucoma is a stereotypic optic neuropathy that selectively affects the retinal ganglion cells whose axons leave the retina to form the optic nerve [4]. Increased endothelins are associated with glaucoma [54, 59] and intravitreal endothelin-1 has been specifically used to model glaucomatous optic nerve head damage [60, 61]. The

endothelins, endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3), represent a family of 21 amino acid isopeptides [62, 63]. ET-1 is a dually neuroactive [64] and vasoactive [62] peptide, that is present in normal eyes [65], but increases in animal models of glaucoma with elevated intraocular pressure [66, 67]. In humans with glaucoma, endothelin-1 concentrations are known to be significantly elevated in the aqueous humor of primary open angle glaucoma patients [59], and in plasma taken from patients with normal tension glaucoma [54]. Endothelins act through the G-protein coupled receptors ET_A and ET_B [68]. The ET_A receptor has 1000-fold greater affinity for ET-1 than for ET-3 [69], while the ET_B receptor has comparable affinities for ET-1 and ET-3 [68, 70, 71]. ET-1, its cognate ET-3, and their receptors are normally present in ocular sites that could affect the axons of retinal ganglion cells [65, 72-74].

Specific Aims

In this study, a new theoretical model for glaucoma has been proposed to explain the cargo-selective effects on axonal transport, which are characteristic of glaucoma. It has been proposed that increased endothelins could serve as pathogenic intermediaries, either independent of, or mechanistically downstream from, elevated pressure and/or ischemia [66]. As pathogenic intermediaries, endothelins might contribute to glaucomatous optic neuropathy by inducing receptor-mediated, cargo-selective misregulation(s) of anterograde axonal transport [75-80]. The long-term goal of this research is to understand the mechanisms that contribute to the misregulation of anterograde axonal transport, producing the "fingerprint" cargo-selective axonal transport dysfunctions seen in many neuropathies. If mechanisms for the regulation-misregulation

of anterograde axonal transport can be deciphered, significant advances may be possible in the treatment of neurodegenerative diseases, such as glaucoma [79, 80], Huntington's, Alzheimer's [9], diabetic neuropathy [10], and ALS.

In this study, the following hypotheses were tested:

1) Exogenously elevated endothelin-1, in the rat vitreous, can access a neuropathogenic site and produce dysfunctions in anterograde axonal transport within the rat optic nerve.

2) ET-1's effects on anterograde axonal transport are receptor-mediated.

3) ET-1's effects on anterograde axonal transport are cargo-selective.

The following specific aims were used to test these hypotheses:

Part I. Effects of Intravitreal Endothelin-1 on Components of Anterograde Axonal Transport in Optic Nerve.

Specific Aim 1: Determine whether intravitreal ET-1 can significantly affect the various rate components of anterograde axonal transport.

Intravitreal injections of 35S-Methionine plus or minus ET-1 were used for a series of *in vivo* pulse-chase experiments performed in young, adult, male Harlan

Sprague-Dawley rats (200-250 g, N=7 for experimentals, N=7 for controls). A series of injection-sacrifice intervals were selected to represent important components and subcomponents of anterograde axonal transport in rat optic nerve. Optic nerves were harvested, flash frozen, sectioned, homogenized, and pulse-labeled material quantitated by liquid scintillation count. Data were statistically analyzed by ANOVA.

Specific Aim 2: Determine whether ET-1's predominant effect is receptor-mediated, and characterize the mediating receptor's activation profile as either ET_A or ET_B type.

At the time associated with ET-1's most pronounced effect upon anterograde axonal transport the effect of the ET_B-selective agonist ET-3 was evaluated, using the same protocol. All data was normalized to the group of control animals simultaneously treated and processed, and the normalized data for ET-1's and ET-3's effects on anterograde axonal transport were statistically compared by ANOVA.

Part II. Intravitreal Endothelin-1: Effects on anterograde fast axonal transport are cargo-selective.

Specific Aim 3: Determine whether ET-1's effect(s) on anterograde fast axonal transport are cargo-selective, in a manner consistent with what is known about glaucoma and axonal transport.

Aliquots from the homogenized nerve segments from Specific Aim 1 were separated by gradient sodium dodecyl-sulfate polyacrylamide gel electrophoesis (SDS-

PAGE). The Coomassie stained gels were impregnated with fluor, dried, and exposed to high resolution X-ray film at -80°C. Templates were made from the fluorographs and 11 protein bands of calculated SDS-PAGE molecular weights were excised from 8 lanes (representing segments 1-4 for one control and one experimental rat) for each of the twenty-eight gels, dissolved, and quantitated by liquid scintillation count (N=7 rats/nerves for experimentals, N=7 rats/nerves for controls, at each of 4 injection-sacrifice intervals). Movement of the 11 protein bands through the rat optic nerve and its relation to the movement of total protein was analyzed for cargo-selective characteristics.

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Figure 1. Schematic of the experimental design used in the following studies. The first study, covered in chapter 2 uses only the upper portion of the design, through the first liquid scintillation counting procedure. The second study, covered in chapter 3, uses the entire experimental design.

Schematic of Experimental Design

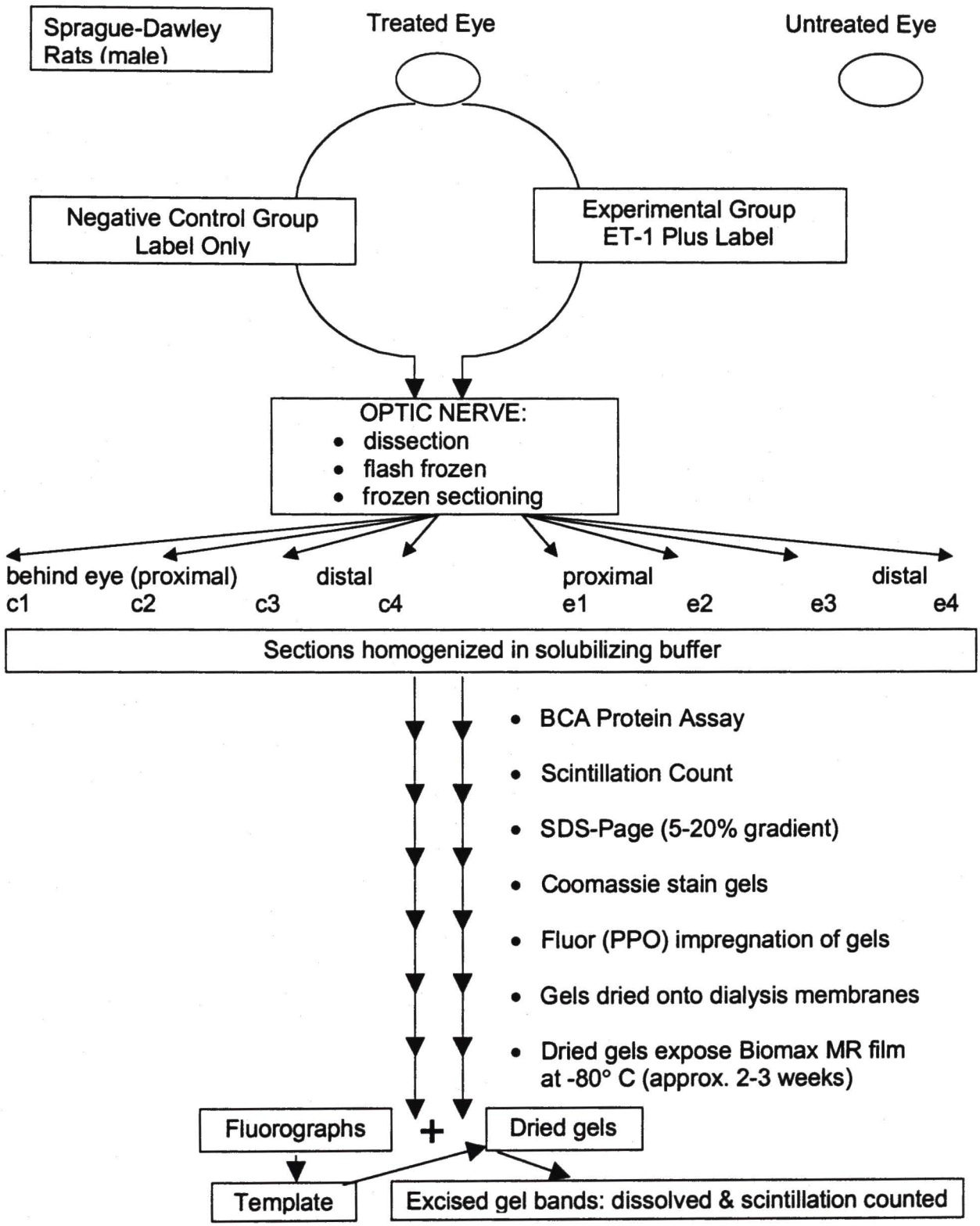
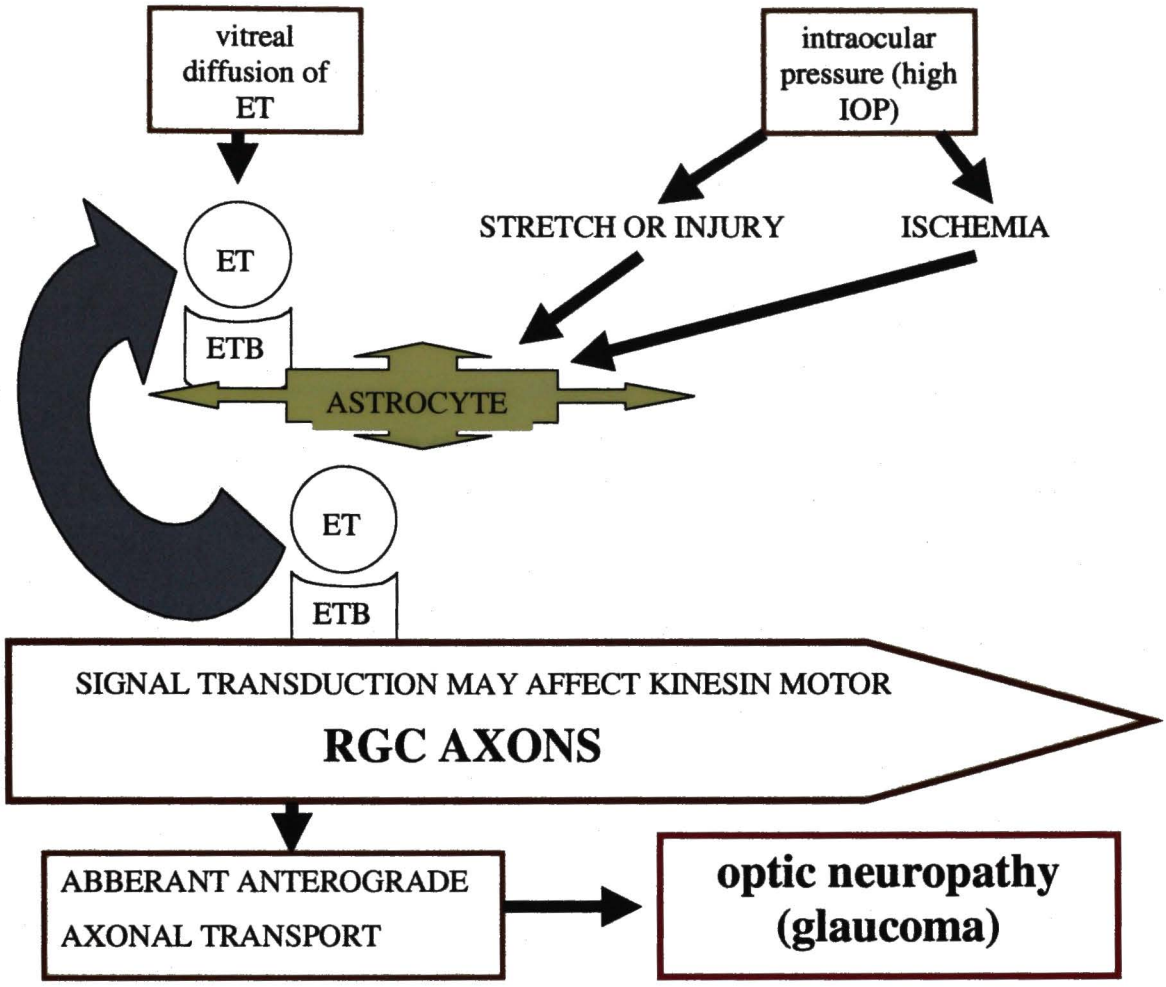


Figure 2. Endothelin receptor-mediated theoretical model for the pathogenesis of glaucomatous optic neuropathy.


vitreal diffusion of ET
intraocular pressure (high IOP)
STRETCH OR INJURY
ISCHEMIA
ET
ETB
ASTROCYTE
ET
ETB
SIGNAL TRANSDUCTION MAY AFFECT KINESIN MOTOR
RGC AXONS
ABBERANT ANTEROGRADE AXONAL TRANSPORT
optic neuropathy (glaucoma)

Chapter II

EFFECTS OF ENDOTHELIN-1 ON COMPONENTS OF ANTEROGRADE AXONAL TRANSPORT IN OPTIC NERVE

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The following manuscript was submitted to Investigative Ophthalmology and Visual Science. Martha E. Stokely performed all of the experimental work while Dr. Scott T. Brady and Dr. Thomas Yorio assisted in editing and functioned in an advisory capacity.

Effects of Endothelin-1 on Components of Anterograde Axonal Transport in Optic Nerve

ABSTRACT

PURPOSE. Increased endothelins are associated with glaucoma and have been proposed to contribute to the development of glaucomatous optic neuropathy. In glaucoma, movement of selected components of anterograde axonal transport important for ganglion cell survival is impaired, specifically the transport of mitochondria. This study evaluates the effect(s) of a single administration of intravitreal endothelin-1 (ET-1) on anterograde axonal transport in rat optic nerve.

METHODS. Proteins for anterograde axonal transport were pulse-labeled by intravitreal injection of ^{35}S-methionine plus or minus ET-1 (2 nmols) in HEPES buffer, pH 7.4. At appropriate time intervals, optic nerves were dissected, sectioned frozen, homogenized in denaturing buffer, and transported protein was quantitated by liquid scintillation counting. Counts corrected for efficiency, quench, background, and decay were statistically evaluated (ANOVA, N=7).

RESULTS. Effects of intravitreal endothelin-1 treatment on anterograde axonal transport were significant, biphasic, and prolonged (4 hours to 21 days). The initial phase was a significant enhancement of transport at times normally associated with small, fast-moving tubulovesicles (4, 24 hours), followed by significant impairments at times normally associated with transport of mitochondria (28 to 36 hours), cytoplasmic matrix (4 days), and cytoskeletal proteins (21 days). The most pronounced effect of ET-1 was

decreased axonal transport at times associated with normal anterograde transport of mitochondrial proteins (28, 32, 36 hours, ps = <.001, .015, <.001, respectively). This was mimicked by ET-3 at 28 hours.

CONCLUSIONS. Effects of intravitreal ET-1 are consistent with a receptor-mediated role for elevated endothelins in pathological misregulation(s) of anterograde axonal transport.

INTRODUCTION

Glaucoma is a stereotypic optic neuropathy involving loss of retinal ganglion cells whose axons leave the retina to form the optic nerve.[1] One characteristic of glaucoma is a dysfunction in the regulated delivery of mitochondria and uncharacterized tubulovesicles from their sites of synthesis and assembly in the cell body to sites of proper function that are situated along the axon and in terminals.[2] The observed disruption in anterograde axonal transport might result from nerve compression, as a consequence of elevated intraocular pressure,[3] or it might result from anoxic conditions[4, 5] during ischemia.[6] The effect of direct compression upon axonal transport appears to involve the loss of linear microtubule arrays in exposed axons,[7] a mechanism also suggested to result from ischemia.[5] However, experimental neuropathies known to act by this mechanism[8] present with large proximal swellings in regions with unmyelinated axons, disorganized microtubules, and prominent accumulations of neurofilaments.[9] In contrast, accumulations seen in glaucomatous human donor tissue appear to be predominantly mitochondrial/vesicular in nature.[2] This would suggest that glaucomatous pathology represents a selective misregulation of axonal transport, rather than an indiscriminate inhibition resulting from loss of microtubular arrays. Some other mechanism(s) could be involved in the full development of glaucomatous pathophysiology, which would contribute to glaucoma by subtly perturbing important physiological functions in the proximal optic nerve. One candidate agent to affect anterograde axonal transport is endothelin-1, a dually neuroactive[10] and vasoactive[11] peptide, present in the normal eye,[12]

and reported to increase in experimental animal models of glaucoma with elevated intraocular pressure.[13, 14]

Endothelin-1, its cognate endothelin-3, and their receptors are normally present in the appropriate ocular regions to affect axonal compartments of retinal ganglion cells.[12, 15-17] Endothelin-1, best-studied member of the endothelin family of isopepetides,[11, 18] acts through G-protein coupled receptors ET_A and ET_B with specificities of ET-1>>ET-3 and ET-1≅ET-3, respectively.[19] These receptors are located at the optic nerve head,[20] as well as within the ganglion cell and nerve fiber layers of the retina.[21] Because of its vasoconstrictive properties, endothelin-1 has been extensively used experimentally to model retinal ischemia.[22-24] However, indications from other regions of the central nervous system (CNS) are that either ischemic events or mechanical injury can induce the secretion of ET-1 from resident astrocytes[25, 26] and alter the expression of endothelin receptors in CNS tissues, [27, 28-30] including the retina.[30] Additionally, non-selective endothelin receptor antagonists can block secondary axonal degeneration in long fiber tracts,[31] a mechanism believed to contribute to retinal ganglion cell loss in some experimental models of glaucoma.[32] These data suggest additional roles for endothelins in CNS tissue such as the optic nerve that may include alterations in anterograde axonal transport.

Anterograde axonal transport is a complex and tightly regulated process by which neurons supply the axons of their long fiber tracts with protein elements required for maintenance and survival. Because axons lack the machinery required for protein synthesis, the timely delivery of specific cargos to their functional domains, represents a

complex logistical burden for the neuron. Axonal transport comprises multiple distinct rate components that deliver specific types of cargo to axonal domains.[33, 34] Fast anterograde axonal transport delivers a variety of membrane-bound organelle cargoes (MBOs) and may be divided into several subcomponents, including 1) very fast, small tubulovesicles moving with synaptic vesicle precursor proteins, and 2) slower moving MBOs with mitochondrial proteins.[35] Regulation of fast anterograde axonal transport is only partially understood.[36-38]

In glaucoma, the anterograde axonal transport of mitochondria is seriously compromised as axons traverse the peri-laminar region of the optic nerve head,[2] presumably leading to bio-energetic perturbations as a consequence of inappropriate supply. The specific type(s) of tubulovesicles affected in glaucoma are unknown,[2] making it difficult to assess their contribution(s) to development of the neuropathy. In the present study we report that elevated endothelin-1 induces a complex pattern of aberrations, affecting all the distinct rate components of anterograde axonal transport, with its most pronounced effect(s) upon the mitochondrial subcomponent.

METHODS

Rats

Young, adult, male Sprague-Dawley rats (N=7 rats for controls, and N=7 rats for experimentals, for each time point or injection-sacrifice interval, ISI) weighing 200 to 250 grams were purchased from Harlan Sprague-Dawley (Indianapolis, IN), and acclimatized to the animal facility for two weeks prior to use in this study. Rats were selected for use in this study because their retinal ganglion cell axons, like human, are unmyelinated prior to leaving the retina, and anterograde axonal transport in the optic nerve has been extensively characterized for this species in previously published work by the authors.[39-43] All studies were conducted in accordance with the NIH guidelines and the ARVO statement on the Care and Use of Animals in Ophthalmic and Vision Research.

Intravitreal injection of radiolabeled precursors plus or minus endothelin (ET)

Newly synthesized proteins undergoing anterograde axonal transport in the optic nerve were pulse-labeled in either the presence or absence of endothelin as modified from previously published methods.[34, 35, 44] (Modifications to previously published methods were minimal and involved the replacement of a distilled water vehicle for resuspension of radiolabeled precursors with either HEPES buffered ET-1 or HEPES vehicle buffer alone). ^{35}S-Methionine (Easytag EXPRESS PROTEIN LABELING MIX, Dupont-NEN Life Sciences, Boston, MA) was lyophilized and resuspended in either vehicle alone (10 mM HEPES, pH7.4, Sigma Chemical Co., St Louis, MO) or in vehicle containing 500

μM ET-1 (Bachem, Belmont, CA). Rats were anesthetized by Metofane inhalation, and 0.8 mCi (4 μl) of radiolabel in vehicle either plus or minus ET-1 (final dose 2 nmols), was injected into the vitreous of the left eye using a 30 gauge needle attached to a Hamilton syringe (microliter #710, 22s gauge, Hamilton Co., Reno, Nevada) by polyethylene tubing (PE-20, Clay Adams Brand, Becton Dickson and Co., Sparks, MD).[34, 44] In one experiment, ET-3 was substituted for ET-1, using the same methods (2 nmol dose, 28 hour ISI, N=7 for controls, N=7 for experimentals). During intravitreal injections, retinas were observed through the pupil with a Zeiss surgical microscope, model Stiffuss S. During introduction of the resuspended label into the vitreous, a transient blanching of the retina was observed for all animals, both controls and experimentals, which did not appear noticeably greater in the ET-1 treated animals, and began to recover immediately after the injection was complete. One minute after injection, all retinas appeared normal in color. (Based upon these initial observations, further observations of the retinas were not performed). Information on the dose-related effect(s) of intravitreal ET-1 in this species (rat) were unavailable, and physiological/pathological concentrations of endothelin in the optic nerve head's microenvironment are generally unknown. Therefore, dose selection was made on the basis of a small pilot study, using these methods and measuring the total pulse-labeled protein axonally transported into the rat optic nerve. (An N of 3 rats in every group was used only for the pilot study, 4 hour ISI, data not shown). The pilot study evaluated 0.3, 0.4, and 2 nmol doses of intravitreal ET-1 and showed a trend of increasingly enhanced axonal transport, compared to control, as the dose of ET-1 increased. However,

significant effects on axonal transport (4 hour ISI) were only seen in the pilot study for the 2 nmol dose. The combination of a non-significant trend at lower doses with a large variance seen at the lowest significantly effective dose (2 nmols), was interpreted to mean that the 2 nmol dose was centrally located within the effective pharmacological dose range, for anterograde axonal transport in rat optic nerve, at the 4 hour ISI. Possible effect(s) on non-assayed ocular tissues were not considered in dose selection, as data on these were unavailable for either acute or chronic intravitreal ET-1 administration in rats.

Harvest and preparation of pulse-labeled optic nerves

Animals were anesthetized with Metofane at specified times after injection and sacrificed by decapitation. Injection-sacrifice intervals were selected based upon the published characterizations of anterograde axonal transport in rat optic nerve for specific marker proteins associated with specific classes of axonally transported materials (Table 1).[35, 36, 39, 40, 45-50] Seven vehicle-treated animals and seven endothelin-treated animals were sacrificed at each of the specified times (4, 24, 28, 32, 36 hours and 4, 21 days). Optic nerves were removed, flash frozen with crushed dry ice, and sectioned frozen. Nerves were sectioned to aid complete homogenization, and to provide additional data for future studies. The frozen sections (2mm in length) were numbered as segments 1 - 4 (from proximal to distal), and glass-on glass homogenized in 100 µl of BUST sample buffer (2% β-mercaptoethanol, 8M urea, 1% SDS, 0.1M Tris, 0.02% phenol red, pH 7.4).[51] A 25% aliquot of each homogenized segment was counted in a liquid scintillation counter, and counts were corrected for decay, quench, and counting efficiency.

Statistical analysis

Corrected decays per minute (dpms) were analyzed by ANOVA (N=7) using the Systat 5 statistical package. All statistical analyses were performed for whole optic nerve (the sum of all 4 segments from an individual optic nerve). For the comparison of the effect of ET-3 with ET-1 at the 28 hr ISI, corrected dpms for the ET-1 group (7 rats) were normalized to the group of control rats simultaneously treated (7 rats), and the same thing was done for the ET-3 group (7 rats) and its simultaneously treated group of control rats (7 rats). Normalized data for ET-1 and ET-3 were compared (ANOVA, N=7) using the Systat 5 statistical package.

RESULTS

ET-1: alterations in all components of anterograde axonal transport

The effects of intravitreal endothelin-1 treatment were significant, biphasic, and prolonged (Table 1, $p < 0.05$, and Figures 1-4, N=7 for controls, N=7 for experimentals, at each time point). The most profound effect of ET-1 was seen at 28 hours. At the 28 hour ISI, this effect was mimicked by the ET_B-receptor-selective agonist ET-3 (no significant difference between ET-1 and ET-3, $p>.999$, ANOVA, N=7, Figure 5), and suggests a receptor-mediated phenomenon, with similar effects for ET-1 and ET-3.

The direction and magnitude of ET-1's effects varied over time and with the cargo being transported (Table 1, Figures 3 and 4), suggesting a selective misregulation, as opposed to an indiscriminate inhibition of anterograde axonal transport. Distributions of radiolabel within optic nerves were monitored (Figures 2 and 3) but all statistical comparisons (Table 1 and Figure 4) were made on data for whole optic nerve (N=7 for each time point).

ET-1 moderately enhances axonal transport of some small, fast tubulovesicles

There was a moderate, but significant enhancement of axonal transport into the optic nerve at times normally associated with small, fast-moving tubulovesicles, but little or no mitochondrial marker proteins (4 and 24 hour ISIs, Table 1, Figures 1-4).[35] The magnitude of ET-1's enhancement was greater for the 4 hour ISI than for the 24 hour ISI (Figure 1). The 4 and 24 hour ISIs were selected for use in this study because they are normally associated with similar amounts of total anterogradely transported material,[35]

but the chemical compositions of transported material are different.[39] Typically, a single form of the kinesin motor[52] is associated with transport at the 4 hour ISI, while multiple isoforms of the motor are associated with the 24 hour ISI.[35, 39] The possibility of a differential regulation in the transport of various classes of tubulovesicles during this subcomponent were the basis for our use of the 4 and 24 hour ISIs. In this study, ET-1's effects on anterograde axonal transport at the 4 and 24 hour ISIs were consistent with a hypothesized differential misregulation in the transport of various classes of small, fast transported tubulovesicles.

ET-1 and ET-3 severely decrease axonal transport in the mitochondrial subcomponent

Intravitreal ET-1's effects were most severe within a 28-36 hour window (Table 1, Figures 1, 3, and 4). In this time interval, a large reduction in transport was seen. At these times, a large pulse (Figure 1) of mitochondrial proteins normally moves through the rat optic nerve.[35] A closely related endothelin, ET-3, was also tested for an effect on transport at 28 hours. ET-3 has 1000-fold less affinity than ET-1 for vasoconstrictive ET_A receptors,[53] but has comparable affinity for ET_B receptors.[54] The ET_B-selective agonist ET-3 had effects on axonal transport at 28 hours that were comparable to those seen with ET-1 (Figure 5).

ET-1 moderately decreases axonal transport in the slow components

At times associated with both slow components of axonal transport, ET-1's effect(s) remained significant (Table 1), but were more moderate (Figures 2 and 4) than those seen during the mitochondrial subcomponent of fast transport (Figure 2). This

suggests a mechanism that is either somewhat component specific or partially reversible, and could result in less accumulation of cytoplasmic matrix and cytoskeletal proteins,[35, 39, 45-49] than might be expected from a generalized loss of transport.[8, 9]

Biphasic effects of intravitreal ET-1

The effects of intravitreal endothelin-1 treatment upon anterograde axonal transport were biphasic (Table 1 and Figures 1, 3, and 4). The initial, rapid effect of ET-1 treatment was a significant enhancement of anterograde axonal transport into the optic nerve at 4 and 24 hours (Figures 1 and 4). The slower, but more prolonged effect of ET-1 was a significant reduction of anterograde axonal transport into the optic nerve at 28, 32, and 36 hours, 4 days, and 21 days (Figures 1 and 4).

Prolonged effects of ET-1

Intravitreal ET-1, administered as a single bolus, exerted significant effects (Table 1) upon anterograde axonal transport as early as 4 hours post-treatment (Figures 1 and 3) and as late as 21 days post-treatment (Figure 4), inducing an extended period of aberrant axonal transport within the retinal ganglion cell axons of the optic nerve (Figure 4). Chronic administration was not required to achieve this effect.

DISCUSSION

A single application of intravitreal ET-1 had a complex and profound effect on anterograde fast axonal transport (Figure 1). Changes were seen in both the very fastest moving material thought to represent movement of small tubulovesicular structures including synaptic vesicle precursors through the slower moving subcomponents of fast anterograde transport that contain mitochondrial markers.[35] The most pronounced effect of intravitreal ET-1 was a sharp reduction in the mitochondrial subcomponent of anterograde transport (Figures 1 and 3). This sharp reduction in the mitochondrial subcomponent contrasted with a modest increase in material transported at the fastest rate (Figures 1 and 3).

Although, ischemia inhibits axonal transport,[55] ET_A-mediated vasoconstriction is apparently not a prerequisite for endothelin's effects on axonal transport. The effects of the ET_B-receptor-selective agonist ET-3 on the mitochondrial subcomponent of axonal transport at 28 hours were comparable to the effects obtained with ET-1 suggesting that endothelin's actions on axonal transport (Figure 5) are an ET_B-mediated effect (Figure 5). This suggests the possibility that ET_B-activation could lie in a direct mechanistic line downstream from ischemia, perhaps "shortening" a pathological ischemic "circuit."

The complexity of ET-1 effects, with early increases in transported material followed by later decreases in transport (Figures 1 and 3), would appear to reflect a mechanism that perturbs the neuron's ability to regulate the timely delivery of specific cargos to their functional domains. The early increases, which were greater at the 4 hour

ISI than at the 24 hour ISI (Figure 1), might reflect the markedly increased transport for a subset of the fastest tubulovesicles, highly enriched at the 4 hour ISI but less well represented at the 24 hour ISI. Markedly increased transport for a subset of vesicles, might partially obscure decreased transport for other types of vesicles that happen to be simultaneously transitioning the optic nerve at the 24 hour ISI. This interpretation would not be inconsistent with the previously published neurochemical analyses on the composition of transported materials in the optic nerves of normal rats at these ISIs.[35, 39, 40] Those studies have indicated that multiple distinct populations of MBOs move in fast axonal transport, with differences in protein content, motor isoforms, and destination.[35, 36, 39, 40] Targeted delivery of MBOs to different destinations, implies a different regulatory control. Such an explanation is compatible with a receptor-mediated event,[36, 56] and reconciles the early effects of ET-1 with observed glaucomatous pathology.[2]

Effects of ET-1 and ET-3 on the subcomponent that contains mitochondrial proteins[35, 39] (Figures 3 and 4), appear consistent with evidence that a reduced percentage of mitochondria successfully transition the peri-laminar region in glaucoma.[2] The peri-laminar region is both: 1) adjacent to the vitreal site of ET-1 and ET-3 applications, and 2) within the primary site of glaucomatous pathology, the optic nerve head.[32] The results from this study suggest the hypothesis that increases in vitreal endothelins could contribute to the development of glaucomatous optic neuropathy,[13] through pathologic misregulation of anterograde axonal transport.

All components of anterograde axonal transport were significantly affected by ET-1 treatment, including the transport of cytoskeletal materials moved in slow

component a (Table 1, Figures 2 and 4). This would appear consistent with the occasionally observed fibrillary changes reported in human glaucomatous donor tissue.[2] The fact that intravitreal endothelin had lesser effects upon the slow components of axonal transport than were seen in the earlier mitochondrial-associated subcomponent may result from continued reductions in axonal energy supply due to reduced mitochondrial transport, reductions in synthesis of cytoskeletal proteins, or a subcomponent-specific action.

The ability of a single intravitreal administration of ET-1 to elicit a biphasic response (Figures 1 and 3) may reflect activation of two separate signal transduction pathways. Activation of multiple endothelin receptors present on retinal ganglion cell soma and/or axons,[15, 21] or of a single class of receptors able to activate dual signaling pathways[57, 58] could produce a biphasic effect. Less direct mechanism(s) might also contribute by activating endothelin receptors on retinal vasculature[15] and resident glia,[59, 60] initiating processes that selectively alter either anterograde transport alone,[36, 61] or the coordinated synthesis and anterograde transport of specific neuronal proteins.[62, 63] One type of resident glia, optic nerve head astrocytes, share intimate contact with retinal ganglion cell axons in the peri-laminar region,[59, 60] express endothelin receptors,[20] and undergo both morphological and secretory changes in glaucoma.[64]

These results demonstrate that a single exposure to elevated levels of endothelin-1 in the vitreous can produce an extended series of effects (Figure 2) that may impinge on neuronal physiology and energy supply for retinal ganglion cell axons in the optic nerve. The simplest interpretive model would suggest that ET-1 might act directly upon

receptors that may be located on retinal ganglion cell axons in the peri-laminar region, locally affecting anterograde axonal transport, perhaps inducing cytoskeletal modification[65] and/or aberrant phosphorylation of proteins[36, 56, 66, 67] that are critical to transport. However, the effects of endothelins in other regions of the CNS are generally not simple.

A less simple, but more probable model, would suggest that both direct and indirect effects of elevated vitreal endothelins contribute to the prolonged, multiple-component dysfunctions in anterograde transport observed in this study. In many regions of the CNS, the synthesis and secretion of endothelins have a dynamic and complex interrelationship with both ischemia[25, 68] and mechanical injury.[69, 70] By analogy to other CNS regions, ET-1 synthesis and release from astrocytes might be stimulated by either mechanical injury, possibly from elevated intraocular pressure, or retinal ischemia. Elevated endothelin could then induce a pathological dysregulation of the mitochondrial subcomponent of anterograde axonal transport, presumably resulting in energy perturbations within retinal ganglion cell axons. ET-1 could stimulate astrocytic ET_B-mediated responses, including increased endothelin synthesis and release,[26] enhanced secretion of cytokines[71] and efflux of glutamate.[72] Vascular responses to elevated endothelin could include ET_A-mediated rapid vasoconstriction (with a possible subsequent ischemic event and reiterative astrocytic responses), and ET_B-induced vasodilation mediated by nitric oxide and possibly TNF-alpha.[11, 71, 73] This series of events would appear much like the vasospasms reported for some glaucoma patients,[74] as

elevated endothelins and nitric oxide alternated with diminished retinal perfusion and glucose-oxygen deprivation.

The demonstration that elevated levels of ET-1 in the vitreous can produce an extended period of aberrant anterograde axonal transport (Table 1, Figures 1 and 4) within the optic nerve has a number of implications for the pathogenesis of glaucoma. Endothelin-sensitive sites might be accessed through vitreal diffusion and/or local secretion of the peptide with similar pathological consequences. Elevated endothelins may exert direct receptor-mediated effects upon retinal ganglion cells, resident glial cells, and retinal vasculature simultaneously or affect a subset of these targets. In either case, increased activation of endothelin pathways would initiate a shower of cascading events that interact to produce the stereotypic neuropathology of glaucoma. If accurate, this model would predict an important place for the regulation of endothelin and its receptors in glaucoma therapy.

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ISI	component	CARGO/subtype	effect of ET-1	p =
4 hr	fast	MBO/small tubulovesicles	increased transport	**.010**
24 hr	fast	MBO/small tubulovesicles	increased transport	**.020**
28 hr	fast	MBO/mitochondria	decreased transport	**<.001**
32 hr	fast	MBO/mitochondria	decreased transport	**.015**
36 hr	fast	MBO/mitochondria	decreased transport	**<.001**
4 days	SCb	cytoplasmic matrix proteins	decreased transport	**.001**
21 days	SCa	cytoskeletal proteins	decreased transport	**.010**

TABLE 1. Summary and significance of ET-1's effect on anterograde axonal transport. (ANOVA, N=7, for every time and every treatment condition)

FIGURE 1. Intravitreal injection of ET-1 alters delivery of axonally transported proteins to optic nerve for an extended period. Plotting the amount of radiolabeled proteins detectable in the proximal two mm of the optic nerve provides a "window in the nerve" view of axonal transport. Such a window shows the timed movements of radiolabeled materials through the most proximal 2 mm segment of the rat optic nerve (adjacent to the eye, designated as segment 1 in the text and in Figures 2 and 3) for vehicle-treated controls and ET-1-treated experimentals. A single injection produces a significant increase in the amount of material delivered by axonal transport in the first 24 hours after injection, followed by a dramatic decline in transported material delivered to the nerve at subsequent time points. The most dramatic decline was between 28 and 36 hours, an interval previously shown to include transport of mitochondrial protein markers. Data shown is the mean of 7 values obtained from seven different animals, for each point plotted (N=7 for controls, N=7 for experimentals). Error bars are plus the standard error of the mean (SEM).

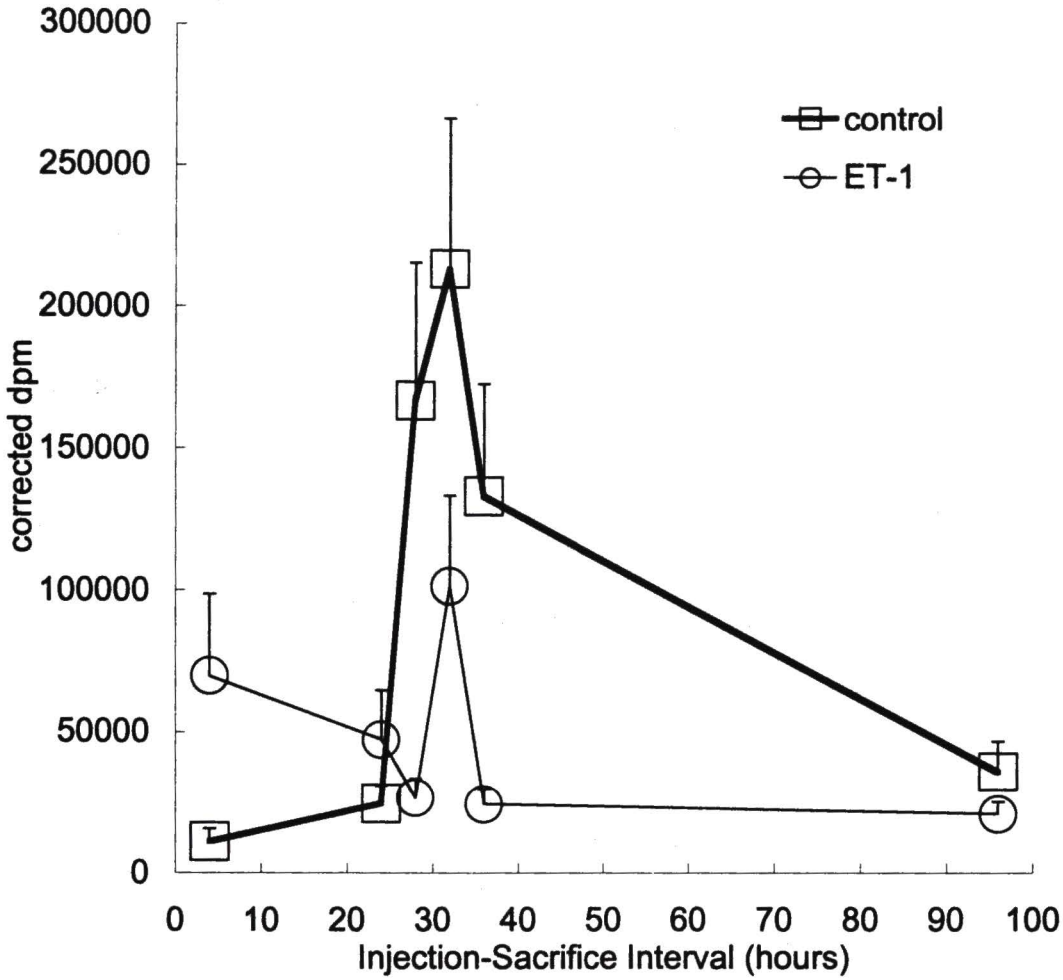
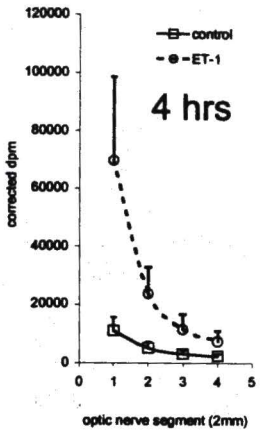
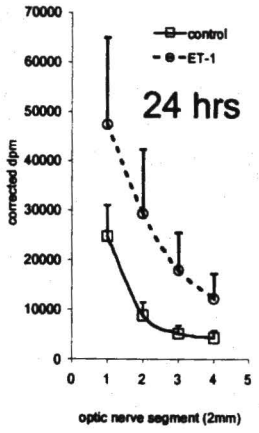

300000
250000
200000
150000
100000
50000
0
corrected dpm
control
ET-1
0
10
20
30
40
50
60
70
80
90
100
Injection-Sacrifice Interval (hours)

FIGURE 2. A single intravitreal ET-1 injection produced significant alterations in the distribution of radiolabel within the optic nerve for all times evaluated. The changes are biphasic and may reflect changes in both the amount of material transported (amount of radiolabel protein at each time point) being transported and in the delivery of transported material to the nerve (changes in distribution at different times after labeling). This figure is a graphical representation of the raw data set (N=7 for controls, N=7 for experimentals, at every time point), as the only corrections made were for radioactive decay and calibration of the liquid scintillation counting technique. Optic nerve segments, 2 mm in length, are numbered consecutively from immediately behind the eye (segment 1). Error bars are plus standard error of the mean (SEM). Evaluation for statistical significance of ET-1's effects upon individual nerve segments was not considered appropriate, due to their physical continuity at the time of treatment. Statistical comparisons for whole optic nerve were addressed in Figure 4.



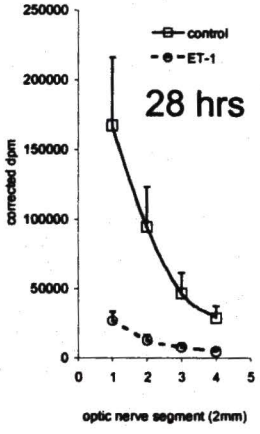


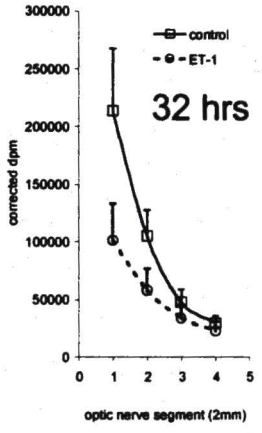
<u>4 and 24 hrs</u>:

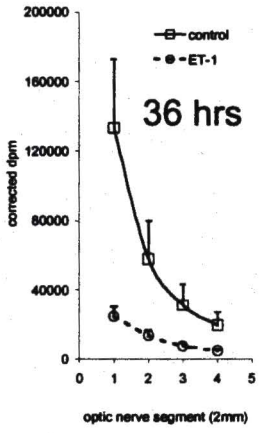
Fast MBOs

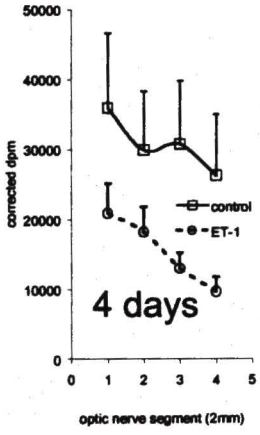
<u>28-36 hrs</u>:

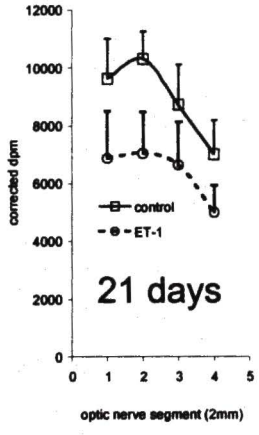
Mitochondria











<u>4 days</u>:

Cytoplasmic matrix

<u>21 days</u>:

Cytoskeleton

FIGURE 3. Biphasic effect(s) of intravitreal ET-1's on anterograde axonal transport were detected in all regions of the optic nerve (segments 1-4, 2 mm in length, numbered from the eye, N=7 controls, N=7 experimentals, for every time point). The differential response for different subcomponents of axonal transport suggests a complex response to elevated vitreal endothelins. Magnitude of the effect (mean endothelin-treated minus mean vehicle-treated control) is expressed in corrected dpms (decays per minute). Direction of the effect is positive if the endothelin-treated value exceeded the control value (at 4 and 24 hours), and negative if the endothelin-treated value was less than the control value (28, 32, and 36 hours, also 4 and 21 days). It should be noted that (unlike Figure 1) the x-axis in this figure is a "category axis" and not to scale. Standard errors of the mean for individual nerve segments were provided in Figure 2. Evaluation for statistical significance of ET-1's effects upon individual nerve segments was not considered appropriate, due to their physical continuity at the time of treatment.

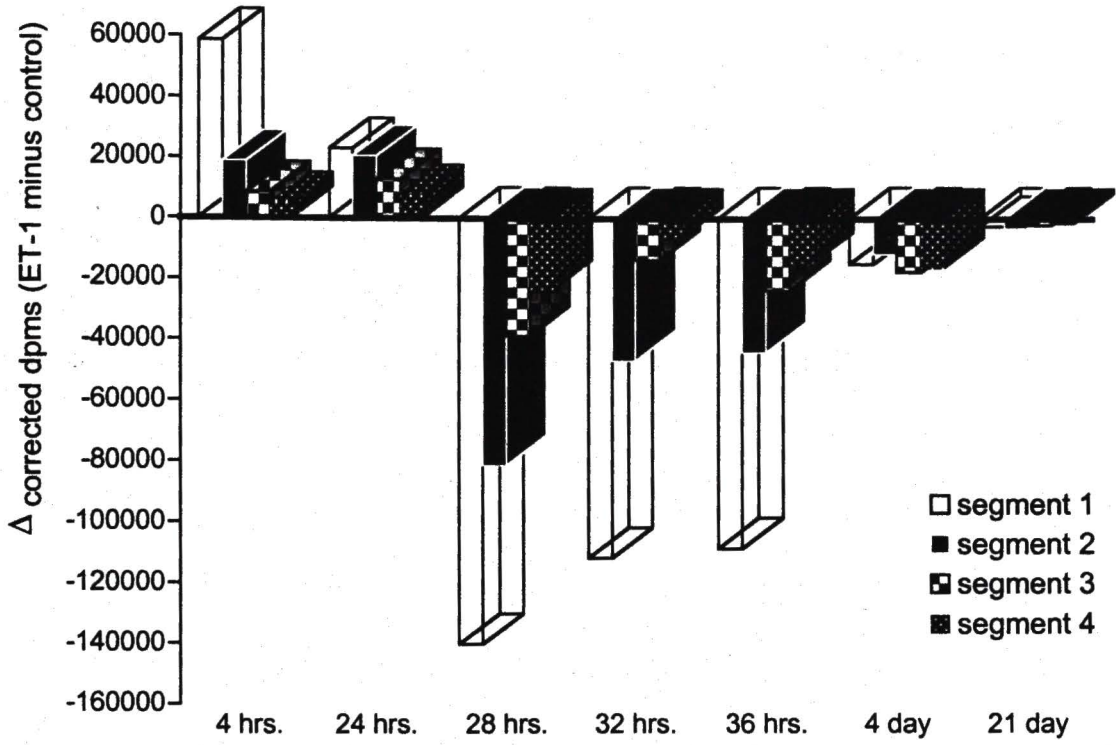

Δ corrected dpms (ET-1 minus control)
60000
40000
20000
0
-20000
-40000
-60000
-80000
-100000
-120000
-140000
-160000
4 hrs.
24 hrs.
28 hrs.
32 hrs.
36 hrs.
4 day
21 day
segment 1
segment 2
segment 3
segment 4

FIGURE 4. Elevated intravitreal ET-1 significantly increased the amount of material in transport at early times after injection, but profoundly decreased delivery of transported material to the nerve at times consistent with transport of mitochondria. Effects of intravitreal ET-1 on anterograde axonal transport in rat optic nerve, for each time interval examined, are expressed as corrected decays per minute (dpms). N=7 for controls; N=7 for experimentals, at every time point. Error bars are plus the standard error of the mean (SEM). (★ denotes statistical significance at $p < .05$, and ★ ★ denotes statistical significance at $p < .01$). Because the x-axis is a "category axis" and not to scale, the large time difference, inherent in presenting slow transport data within the same figure as fast transport data, is marked by the appearance of white dots upon the black bars for the ET-1 treatment group, at the slow component time intervals.

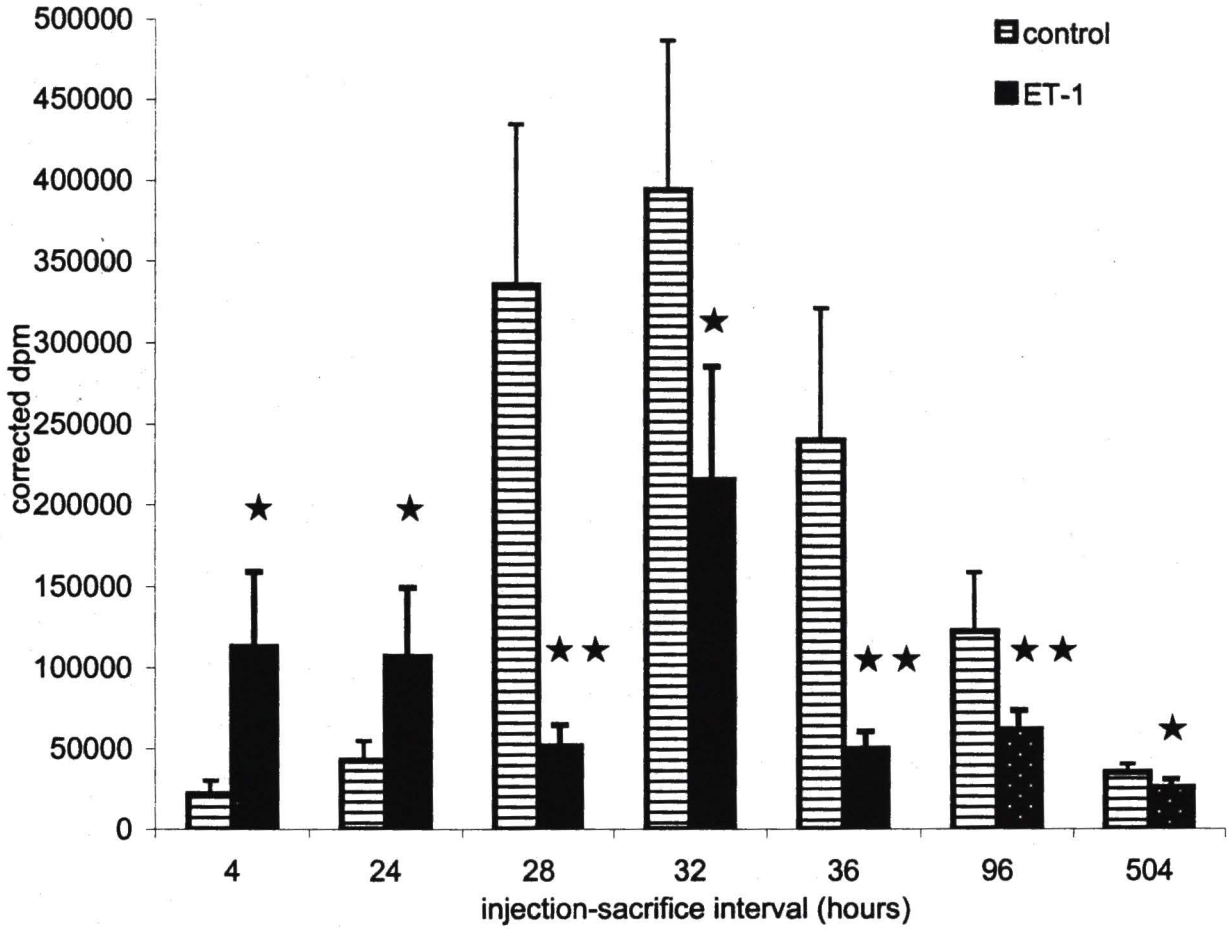
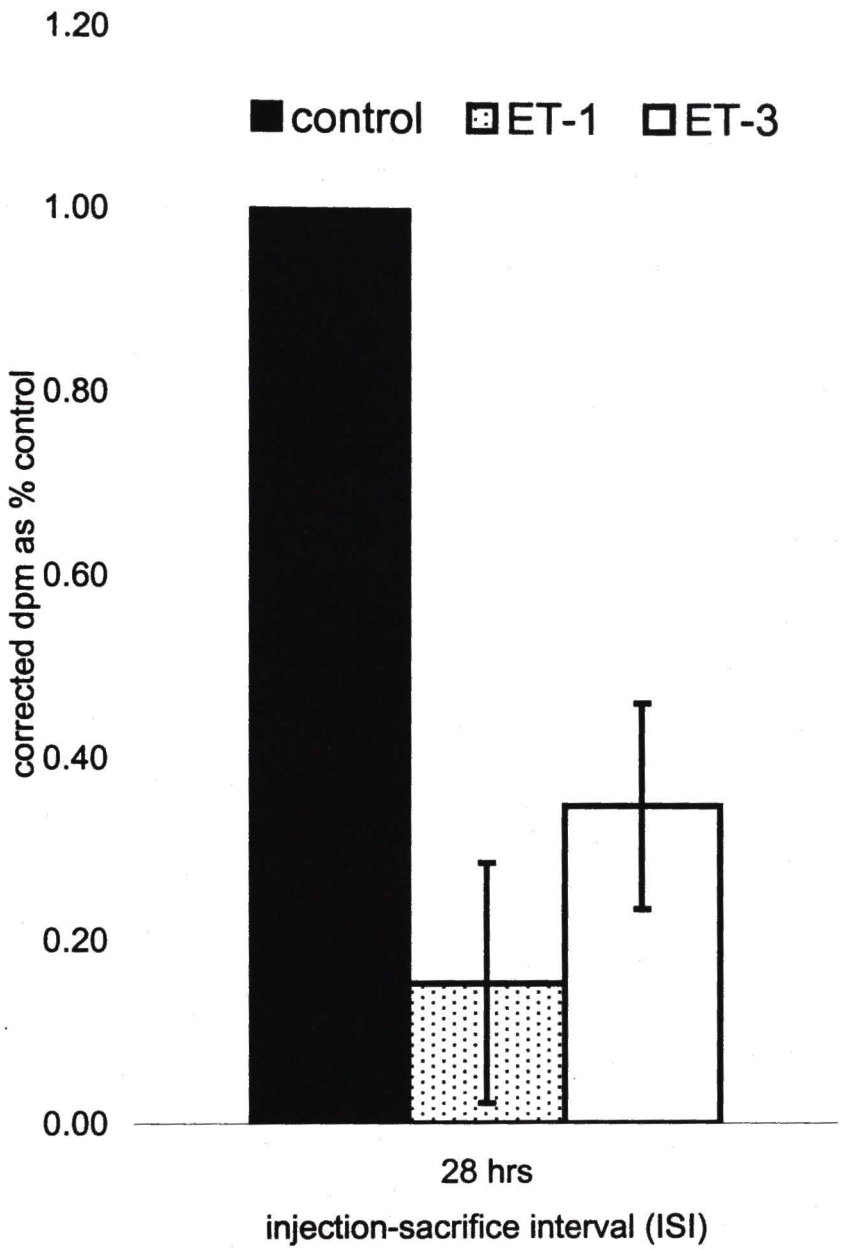

500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
corrected dpm
control
ET-1
4
24
28
32
36
96
504
injection-sacrifice interval (hours)

FIGURE 5. No significant difference was seen between the effects of intravitreal ET-3 and intravitreal ET-1 on anterograde axonal transport at the 28 hour ISI ($p>.999$, ANOVA, normalized data, N=7). The similar effects of ET-1, which activates both ET_A- and ET_B-receptors, and ET-3, which selectively activates ET_B-receptors, indicate that changes in axonal transport are largely ET_B-receptor mediated. Data for ET-1 and ET-3 groups were normalized to their appropriate simultaneously treated and processed vehicle-treated negative control groups' data (N=7 for the vehicle-treated control group that was treated and processed simultaneously with the ET-1 group, N=7 for the ET-1-treated group; N=7 for the vehicle-treated control group that was treated and processed simultaneously with the ET-3 group, N=7 for the ET-3-treated group). Values plotted and statistically compared were for whole optic nerve (sum of all segments 1-4 for each individual optic nerve). Standard error bars are plus or minus SEM. No standard error could be calculated for the control value because all data in this figure was normalized to control.


1.20
control
ET-1
ET-3
1.00
0.80
0.60
0.40
0.20
0.00
corrected dpm as % control
28 hrs
injection-sacrifice interval (ISI)

MOVING FROM GLAUCOMA TO OTHER NEUROPATHIES: ENDOTHELINS MAY PROVIDE A KEY

Glaucoma is the second leading cause of blindness and affects 67 million people worldwide [1], but it is only one of the neuropathies which present with some type of characteristic anterograde axonal transport dysfunction [2-6]. It is possible that these neuropathies share a common feature, the pathologic misregulation of anterograde axonal transport. Endothelin and its receptors might prove to be important targets for future therapies in some of these diseases. However, even if endothelin receptor activation does not prove to be an important contributor to non-glaucomatous neuropathies, the animal model developed in these studies may provide basic scientific insights that advance our understanding of the underlying mechanisms for neurodegenerative diseases. Some of these underlying mechanisms involve misregulation of anterograde axonal transport.

Results from these studies suggest that receptor-mediated events may cause misregulations in anterograde axonal transport that are similar to those indicative of neurodegenerative disease. Receptor-mediated events activate signal transduction pathways that initiate neurochemical changes within the retinal ganglion cell and its axon and may affect axonal transport for specific types of cargos. Signal transduction pathways normally cross-talk with other signal transduction pathways complicating the issue. If we can unravel the signal transduction pathways that regulate and misregulate anterograde axonal transport, new therapeutic targets could be identified for the treatment

of neurodegenerative diseases. However, unraveling these pathways requires a method that is able to distinguish between the various signal transduction pathways that regulate the transport and delivery for specific cargos to their specific axonal destinations [2]. If the effects of intravitreal endothelin upon axonal transport in the rat optic nerve are cargo-selective, then the intravitreal endothelin/axonal transport experimental model could prove to be a valuable tool in future studies of the underlying mechanisms for neurodegenerative disease.

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Chapter III

INTRAVITREAL ENDOTHELIN-1: EFFECTS ON ANTEROGRADE FAST AXONAL TRANSPORT ARE CARGO-SELECTIVE.

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Intravitreal endothelin-1: effects on anterograde fast axonal transport are cargo-selective

ABSTRACT

Glaucoma is a neurodegenerative disease that may involve a misregulation of anterograde axonal transport and result in disproportionate failure to deliver mitochondria to sites of proper function within the optic nerve. Increased endothelins (ETs) have been associated with glaucoma, and are suggested to play a role in its pathogenesis. Standard pulse-labeling and segmental analysis techniques were used to evaluate the effects of intravitreal endothelin-1 (ET-1) on anterograde fast axonal transport in the rat optic nerve at 4, 24, 28, 32, and 36 hour injection-sacrifice intervals (ISIs). Intravitreal ET-1 caused pronounced and novel disruptions of anterograde fast axonal transport in the rat optic nerve, first increasing transport at post-labeling times normally associated with movement of nascent synaptic vesicles (4 hr ISI, $p = .01$), then decreasing transport at times normally associated with a large pulse of mitochondrial markers plus continued transport of nascent synaptic vesicles (28, 32, and 36 hr ISIs, ps = 0.001, 0.02, <0.001, respectively). Concomitant with this decrease in the transport of total pulse-labeled protein (28-36 hr ISIs), ET-1 caused a selective delay for movement of a distinctive cohort of 11 SDS-PAGE protein bands into the optic nerve (consistently shifting times for their peak delivery from 28 to 32 hours). The peak delivery time for total pulse-labeled protein was simultaneously unaffected by ET-1 treatment (32 hours), suggesting

ET-1's effect may be selective for a chemically distinct class of cargo. Cargo-selective misregulation of anterograde fast axonal transport has not been previously demonstrated in a genetically normal animal model.

INTRODUCTION

Misregulation of anterograde axonal transport presents as a common theme in many neuropathies, including glaucoma. In glaucoma, mitochondria-associated anterograde fast axonal transport appears to be impaired for ganglion cell axons transversing the region of the lamina cribrosa within the optic nerve head (Hollander 1995). The affected axonal compartment lies adjacent to the vitreous, immediately proximal to where these axons become the optic nerve.

Increased endothelins (ETs) in aqueous humor (Kallberg 2002a; Kallberg 2002b; Noske 1997) and vitreous (Kallberg 2002a; Kallberg 2002b) are associated with glaucoma, correlate with glaucomatous retinal damage (Kallberg 2002a), and experimentally induce damage to the optic nerve head (Cioffi 1996; Orgul 1996). While some have suggested that these effects are caused by ET_A-receptor-mediated ischemia (Cioffi 1996; Orgul 1996) this interpretation fails to consider the non-vasoconstrictive ET_B-receptors located at sites important to glaucomatous pathology, such as the retinal ganglion cell layer and nerve fiber layer of the retina (MacCumber 1994; Stitt 1995), optic nerve head astrocytes (Yorio 2000), and the proximal optic nerve (Ripodas 2001). Recent evidence indicates that ET_B-mediated responses to elevated vitreal endothelins may contribute to neuropathogeneis in the proximal optic nerve by inducing misregulation(s) in anterograde fast axonal transport (Stokely 2002a; Stokely 2002b; Yorio 2002). It is possible that these misregulations could be cargo-selective in nature.

Distinctive features of glaucomatous impairment(s) to axonal transport include the discrete site at which axonal transport is compromised, and the apparent selectivity of affected cargo (Hollander 1995). The location of this discrete site, adjacent to the vitreous, suggested that endothelins introduced exogenously into the vitreous may be able to access the neuropathogenic site (Cioffi 1996; Orgul 1996), thereby affecting anterograde axonal transport (Stokely 2002a), perhaps in a cargo-selective manner. Cargo-selective misregulation(s) of anterograde fast axonal transport have not previously been demonstrated in a genetically normal animal model. The ability to induce these types of misregulation(s) in a normal animal model may provide a new and subtle tool for dissecting the mechanism(s) involved in cargo-selective misregulations of anterograde axonal transport.

METHODS

Rats

Prior to use in this study, young adult male Sprague-Dawley rats (N=7 rats for controls and N=7 rats for experimentals, for each time point or injection-sacrifice interval, ISI) weighing 200 to 250 grams were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and acclimatized to the animal facility. Like humans, the retinal ganglion cell axons of rats are unmyelinated prior to exiting the retina. Additionally, anterograde axonal transport in the optic nerve has been well characterized, using essentially the same protocol, in previously published work by one of the authors (Elluru 1995a; Elluru 1995b; McQuarrie 1986; McQuarrie 1989; Oblinger 1987). All animals used in this study were acquired and cared for in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985) and the principals presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience.

Intravitreal injection of radiolabeled precursors plus or minus endothelin-1 (ET-1)

Anterograde axonal transport of newly synthesized proteins in the optic nerve was detected by intravitreal pulse-labeling with ^{35}S-methionine amino acid precursors in either the presence or absence of endothelin-1 and analyzed. Methods used were modified (Stokely 2002a) from published methods (Brady 1985; Brady and Lasek 1982; Elluru 1994). Modifications were minimal and involved the replacement of a distilled

water vehicle for resuspension of radiolabeled precursors with either HEPES buffered ET-1 or HEPES vehicle buffer alone. ^{35}S-Methionine amino acid precursors (Easytag EXPRESS PROTEIN LABELING MIX, Dupont-NEN Life Sciences, Boston, MA) were lyophilized and then resuspended in either vehicle alone (10 mM HEPES, pH7.4, Sigma Chemical Co., St Louis, MO) or in vehicle containing 500 μM ET-1 (Bachem, Belmont, CA). Rats were anesthetized by inhalation of methoxyflurane and 0.8 mCi (4 μl) of radiolabel in vehicle either plus or minus ET-1 (final dose 2 nmols) was injected into the vitreous of the left eye by means of a 30 gauge needle attached to a Hamilton syringe (microliter #710, 22s gauge, Hamilton Co., Reno, Nevada) by polyethylene tubing (PE-20, Clay Adams Brand, Becton Dickson and Co., Sparks, MD) (Brady 1985; Brady and Lasek 1982). The 2 nmol dose was selected based upon our earlier studies into the effects of ET-1 on anterograde axonal transport in the rat optic nerve (Stokely 2002a) which indicated that this dose was within the pharmacological dose range for ET-1's effects upon anterograde fast axonal transport, when administered intravitreally.

Harvest and preparation of pulse-labeled optic nerves

At specified times after injection, animals were anesthetized by inhalation of methoxyflurane then sacrificed by decapitation. Selected injection-sacrifice intervals were based upon published characterizations of anterograde axonal transport in rat optic nerve and the transport of marker proteins associated with specific classes of membrane bound organelle cargos (Brady 1986; Elluru 1994; Elluru 1995a; Elluru 1995b; Jahn 1985; Stokely 2002a; Wilson 1985). Seven vehicle-only animals and seven vehicle-plus-endothelin animals were sacrificed at each of the selected injection-sacrifice intervals (4,

24, 28, 32, and 36 hours). Tissue was dissected and optic nerves were removed, flash frozen with crushed dry ice, then sectioned frozen. The frozen sections (2mm in length) were numbered as segments 1 - 4 (proximally from just behind the eye, to distally adjacent to the optic chiasm) then glass-on glass homogenized in 100 µl of BUST sample buffer (2% β-mercaptoethanol, 8M urea, 1% SDS, 0.1M Tris, 0.02% phenol red, pH 7.4) (Brady 1999). An equal aliquot (25%) from each homogenized segment was analyzed for ^{35}S-content in a liquid scintillation counter and counts were corrected for decay, quench, background, and counting efficiency.

Separation and quantitation of labeled proteins by SDS-PAGE molecular weights

Equal aliquots from each homogenized segment were separated by SDS-PAGE on 5-20% gradient gels. The gels were stained with Coomassie blue and destained, then processed for fluorography by dehydration in dimethylsulfoxide (DMSO; 3 x 20 minutes), impregnation with diphenyl oxazole (22% in DMSO, 2 hr) and rehydration (Laskey 1975). The gels were then dried and exposed to Biomax-MR high resolution x-ray film (Eastman Kodak, Rochester, NY) for the appropriate time (17-24 days, -80°C). The amount of radioactivity incorporated into a protein band of known SDS-PAGE molecular weight was quantitated by excising the appropriate band from the gel, using the fluorograph as a template. The bands were solubilized in 30% hydrogen peroxide for 2 days at 60°C and counted in a liquid scintillation counter. Decays per minute (dpms) were corrected for background, decay, quench, and counting efficiency. Time of entry for a given protein band into the optic nerve was considered to be the post-injection time

(ISI) at which the sum of corrected dpms (for all optic nerve segments) presented its maximum value.

Statistical analysis

Corrected decays per minute (dpms) were analyzed by ANOVA, using the Systat 5 statistical package. N=7.

RESULTS

Recent studies have suggested that intravitreal ET-1's effects on anterograde fast axonal transport may be directly receptor-mediated (Stokely 2002a; Stokely 2002b), and that ET-1's effects are more pronounced upon anterogradely transported material moving with the fast component than on material moving with slow components A and B (Stokely 2002a).

ET-1 selects between fast transport cargos by time (ISI)

The direction and magnitude of ET-1's effects varied with the pulse-labeled protein cargo(s) transported through the optic nerve at selected injection-sacrifice intervals (in figures 1-2 compare 4 hour vs. 28-36 hour ISIs). Intravitreal ET-1 significantly increased the transport of pulse-labeled material at times known to contain proteins associated with synaptic vesicle precursors, but not mitochondrial markers (4 hour ISI, $p = .01$) (Elluru 1994; Elluru 1995a). In contrast, during the 28-36 hour window, total newly synthesized protein transitioning the rat optic nerve was significantly diminished (ps = <0.001, 0.02, <0.001, respectively). This window represents the mitochondrial subcomponent of anterograde fast transport and normally contains a large pulse of mitochondrial markers that simultaneously transition the optic nerve in concert with continued movement of markers for nascent synaptic vesicles, and other uncharacterized cargos (Elluru 1994; Elluru 1995a).

ET-1 selectively delays cargo of distinctive protein composition

A cohort of 11 SDS-PAGE protein bands (139, 118, 89, 80, 64, 59, 51, 45, 42, 37, and 25 kDa) was selectively delayed for 4 hours by intravitreal ET-1 treatment (figure 4, bar graphs) while the majority of protein experienced no delays (figure 4, top left). This ET-1-induced 4 hour delay shifted the peak for the 11 affected proteins from 28 hours to 32 hours in endothelin-treated nerves as compared to control (figure 4, ET-1-treatment shown as white bars, vehicle-treatment shown as black bars). Endothelin-treatment did not shift the peak for total radiolabeled protein (figure 4, top left corner) which transitioned the nerve at 32 hours, regardless. The 11 bands shown were selected for analysis because they represented a distinctive banding pattern, first seen at 24 hours when the first appearance of small amounts of mitochondrial proteins was expected (Elluru 1994; Elluru 1995a). The coherent movement of these 11 bands during the 28-36 hour window, and their selective delay by ET-1 (figure 4), suggest that a chemically distinct class of cargo was selectively affected.

DISCUSSION

Intravitreal ET-1 produced pronounced and novel misregulations of anterograde fast axonal transport in the rat optic nerve. Three misregulations occured. 1) There was an early increase in total pulse-labeled protein transported into the rat optic nerve (figures 1A-1B), followed by 2) a profound decrease in transport of total radiolabeled protein (figures 1-2). This significant decrease in total transported material was concommitant with 3) a 4 hour delay that selectively affected a chemically distinctive subset (figure 4: bar graphs, cohort of 11 bands), but not the majority (figure 4, top left), of transported proteins. Selectively delayed transport, affecting only a subset of chemically distinct cargo, suggests a novel misregulation of anterograde fast axonal transport not previously seen in genetically normal animal models.

In combination, the first two ET-1-induced misregulations represent an effect that is both biphasic and cargo-selective. ET-1 significantly increased the amount of pulse-labeled protein transported during the early phase of fast anterograde transport, a time associated with synaptic vesicle precursor proteins but not mitochondrial markers (4 hour ISI, $p = 0.01$) (Elluru 1994; Elluru 1995a). In contrast, ET-1 significantly decreased the transport of pulse-labeled protein (28, 32, and 36 hour ISIs, ps = <0.001, 0.02, <0.001, respectively) at times associated with the mitochondrial subcomponent of fast anterograde transport, which represents a complex array of cargos distinguished by a large pulse of mitochondrial marker proteins, plus the continued transport of nascent synaptic vesicles and uncharacterized cargo (Elluru 1994; Elluru 1995a).

The biphasic aspects of ET-1's effect could result from either activation of multiple receptors, or of a single class of receptors capable of evoking multiple signaling pathways (Hawes 1996; van Biesen 1995; van Biesen 1996). These might initiate affects on either: 1) axonal transport in combination with changes in protein synthesis and/or cargo packaging, or 2) axonal transport alone.

ET-1's novel cargo-selective delay, which distinguished between chemically distinctive cargos in simultaneous transport (24-36 hour ISIs, figure 4) indicates that the cargo-selective aspects of intravitreal ET-1's effects may be more complex than a simple temporal sequence of receptor activations. Recent evidence that some of ET-1's effects on axonal transport (total pulse-labeled protein, 28 hour ISI) may be mediated by the non-ischemic ET_B receptor (Stokely 2002a; Stokely 2002b) suggests that trimeric G-protein-associated signaling may have the potential to activate pathways that are cargo-selective. Cargo-selective regulation of axonal transport might be accomplished through mechanisms involving either the phosphorylation (Hollenbeck 1993; Lee 1995) or the dephosphorylation of: 1) cargo-associated isoform(s) of the kinesin motor's heavy chains (Elluru 1995a), 2) kinesin's cargo-binding light chains (Morfini 2002), and/or 3) associated regulatory proteins (Fang 2000; Morfini 2002; Ratner 1998; Sanchez 2001; Tsai 2000).

Selectively delayed transport of a chemically distinct class of cargo might result from mechanisms involving: 1) transient stoppage of the selected cargo (Brady 2000; Ratner 1998; Roy 2000), 2) a decreased rate of transport for the selected cargo (de Waegh 1990; de Waegh 1992; Kirkpatrick 2001; Stein 1991a; Stein 1991b), or 3) transiently

retrograde transport for a selected cargo (Morris 1993; Trinczek 1999). Underlying mechanisms for these events could be dependent on cytoskeletal changes (de Waegh 1990; Kirkpatrick 1994; Morris 1995), diminished motor function, and/or decreased motor-cargo association (Morfini 2002; Ratner 1998).

The effect(s) of intravitreal ET-1 on anterograde axonal transport in the rat optic nerve represents a unique model for studying axonal transport function in the mammalian CNS. The complex cargo-selectivity demonstrated in this study suggests that this model may be useful in unraveling the underlying mechanisms involved in selective misregulation(s) of axonal transport.

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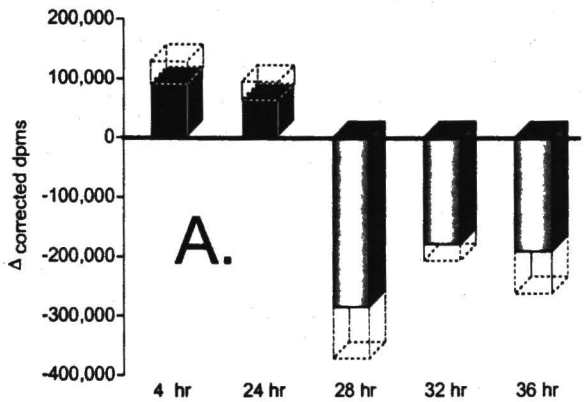
FIGURE 1. Effects of Intravitreal Endothelin-1 on Total Radiolabeled Protein.

A. The direction and magnitude of ET-1's effects (endothelin-treated minus control) were different for cargos transported through the optic nerve at different times (injection-sacrifice intervals or ISIs, in hours). Dark 3-D bars represent early membrane-bound cargos (MBOs), and light bars represent cargos moving with the mitochondrial subcomponent (Elluru 1994; Elluru 1995a). Values plotted are means (N=7; transparent stacked bar represents plus the standard error of the mean (SEM) for positive values and minus SEM for negative values). B. The effects of ET-1 were significant at all times (ISIs) examined. As in A, values plotted are means (N=7) and error bars are plus SEM. ★ denotes statistical significance at $p < .05$, ★★ denotes significance at $p < .01$.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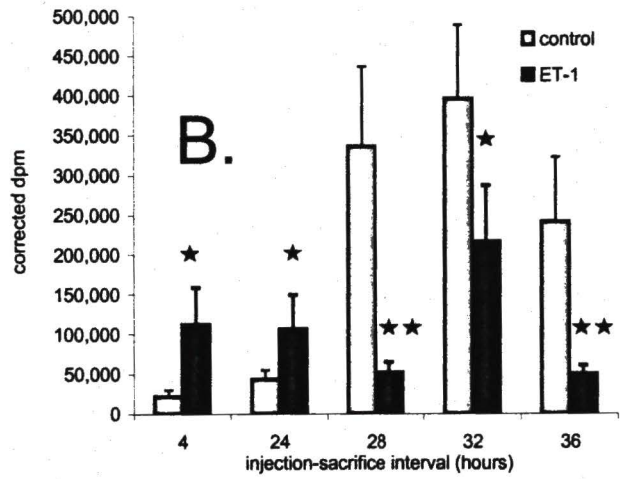
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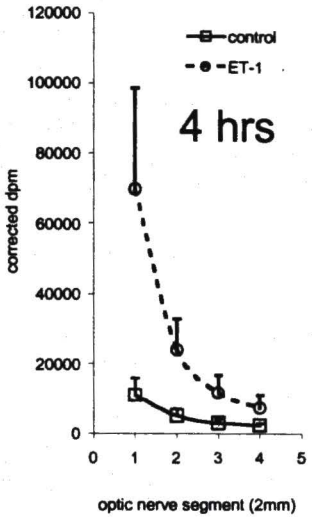
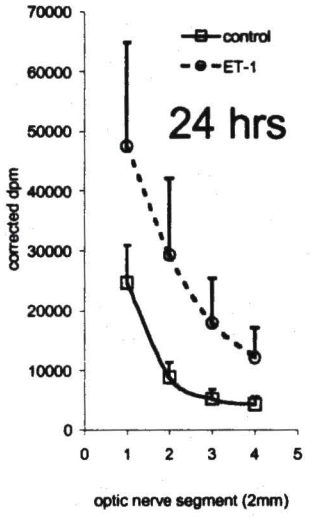
B.
control
ET-1
4
24
28
32
36
injection-sacrifice interval (hours)

FIGURE 2. Distribution of radiolabel throughout the optic nerve (2mm segments, numbered from proximal, behind the eye, to distally adjacent to the optic chaism) at all times examined. Values plotted are means (N=7) and error bars are plus SEM. This is a graphical representation of the raw data as it was collected, since the only corrections made were for radioactive decay and calibration of the scintillation counting technique.





4 hrs:

synaptic vesicles

no mitochondria

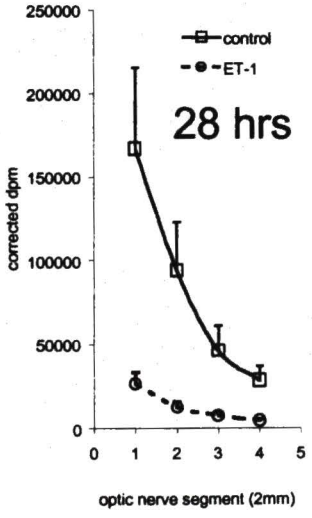
24 hrs:

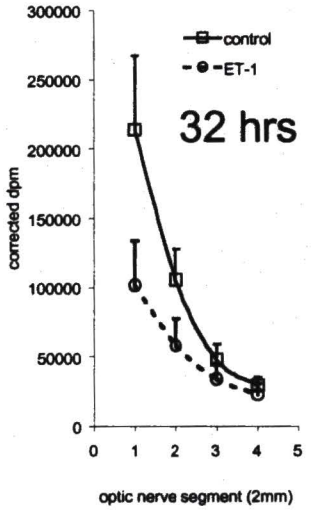
trace mitochondrial proteins

28-36 hrs:

mitochondria

synaptic vesicles





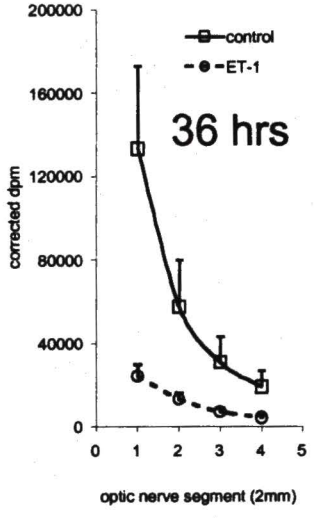
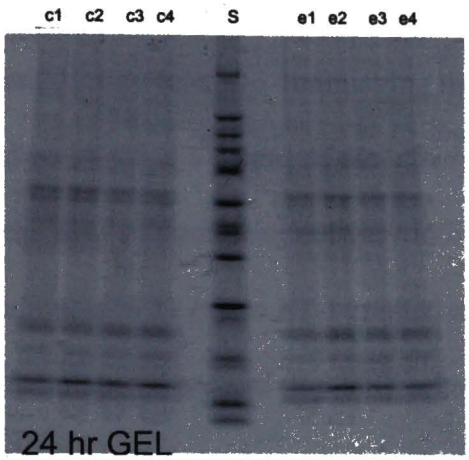


FIGURE 3. Typical coomassie-stained gel (top left, 24 hr ISI) and representative flurographs (24-36 hr ISIs) show the effects of intravitreal endothelin during the 24-36 hour ISI window. Every fluorograph shown is typical of 7 fluorographs, one gel for two animals. The 11 marked bands were excised from all 8 lanes of each corresponding gel (corresponding to the 4 segments from each optic nerve, for one control and one ET-1 treated rat, per gel). A band-sized background region from the molecular standard lane (lane S) of each gel was also excised (89 excised bands per gel). Bands were dissolved, and scintillation counted (3115 excised bands, data shown in figure 4, bar graphs). For the gel and each fluorograph: Left half: segments 1-4 (left to right) for vehicle-treated controls; Right half: segments 1-4 (left to right) from ET-1-treated rats. SDS-PAGE Bands 1-11 to be excised from gels and analyzed (139, 118, 89, 80, 64, 59, 51, 45, 42, 37, and 25 kDa, respectively) are marked in the center lane of the fluorographs, where the molecular weight standards (Sigmamarker, wide range) were run on the corresponding gel (lane S). Fluorographs were sometimes overexposed to facilitate making templates.



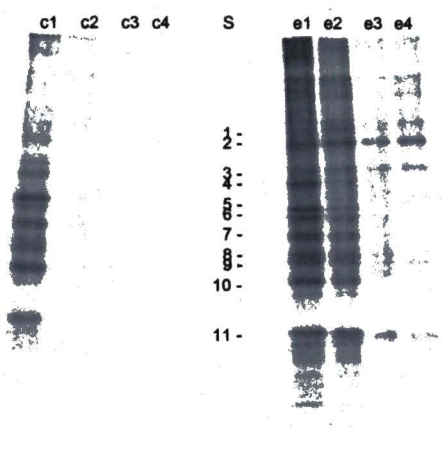
lanes c1-c4:
segments 1-4 control rats

lane S:

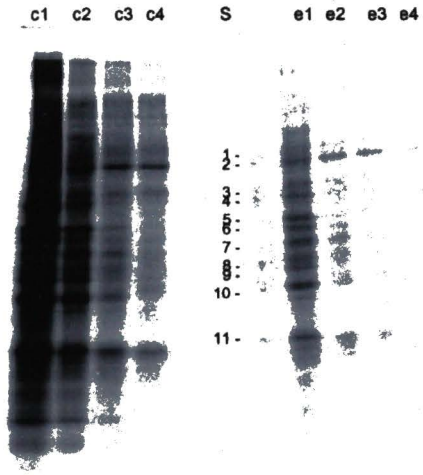
gels:
molecular weight standards

fluorographs:
11 bands for analysis

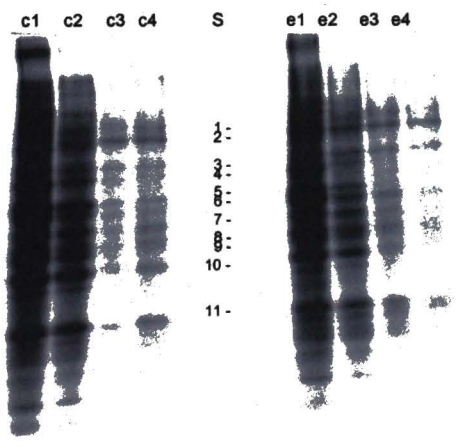
lanes e1-e4:
segments 1-4 ET-1 rats



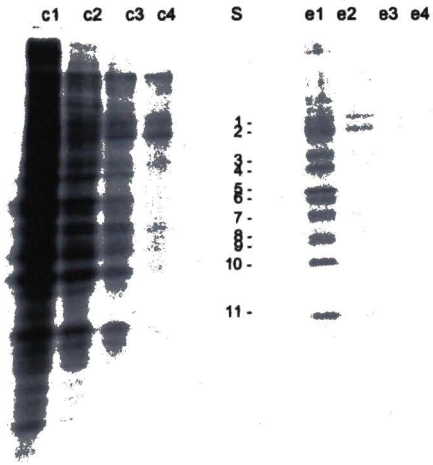
24 hr FLU



28 hr FLU

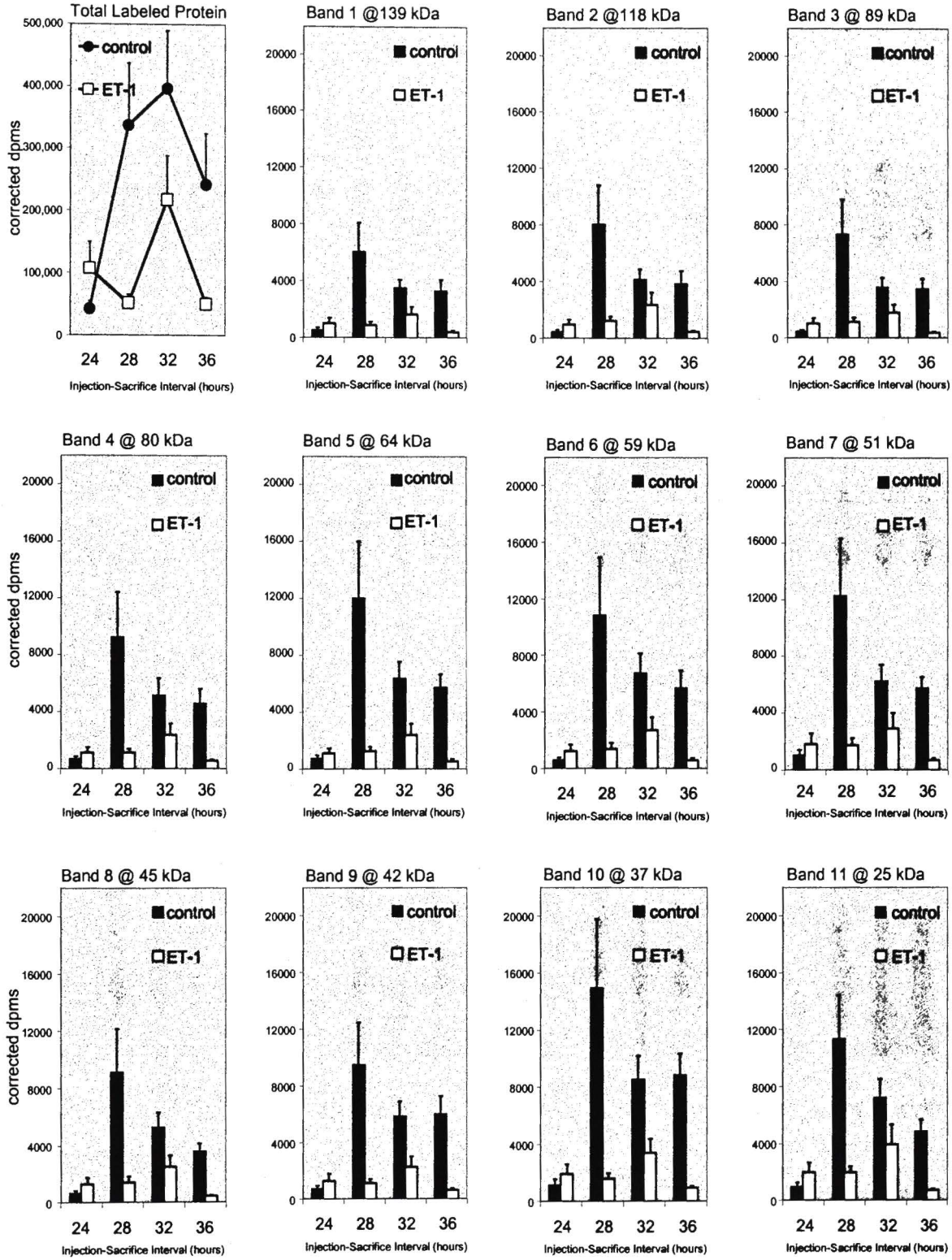


32 hr FLU



36 hr FLU

FIGURE 4. Eleven SDS-PAGE bands move through the optic nerve (24-36 hr ISIs). In addition to severely diminishing the amount of transported material, ET-1 delayed the movement of a cohort of eleven protein bands into the optic nerve for 4 hours, shifting the time of their peak delivery into the nerve from 28 hrs. in vehicle-treated control animals to 32 hrs in ET-1-treated animals. This was not true for ET-1's effect on total radiolabeled protein (top left), where the time of peak delivery into the nerve was at 32 hrs for both vehicle-treated controls and ET-1 treated animals, suggesting that ET-1 delayed a chemically distinct subset of proteins, while not affecting the majority of proteins in transport during this timeframe. This figure compares ET-1's effects on total radiolabeled protein (top left) with the effects of ET-1 on 11 coherently moving SDS-PAGE protein bands (subsequent panels, labeled by SDS-PAGE molecular weights). Values plotted are means (N=7 for controls, N=7 for experimentals) and error bars are plus SEM. For all bands: Black bars represent vehicle-treated controls, and White bars represent ET-1-treated experimentals.


Total Labeled Protein
control
ET-1
corrected dpms
500,000
400,000
300,000
200,000
100,000
0
24 28 32 36
Injection-Sacrifice Interval (hours)
Band 1 @139 kDa
Band 2 @118 kDa
Band 3 @ 89 kDa
Band 4 @ 80 kDa
Band 5 @ 64 kDa
Band 6 @ 59 kDa
Band 7 @ 51 kDa
Band 8 @ 45 kDa
Band 9 @ 42 kDa
Band 10 @ 37 kDa
Band 11 @ 25 kDa
20000
16000
12000
8000
4000
0

Chapter IV

CONCLUSIONS AND FUTURE PERSPECTIVES

Summary and Conclusions:

Previous models for the pathogenesis of glaucoma, that relied on direct compression of axons or chronic retinal ischemia, have failed to provide an adequate mechanism to explain the characteristic cargo-selective effects of this disease on axonal transport, specifically the selective impairment of mitochondria transitioning the peri-laminar region [1].

The direct compression model was based upon early studies, made prior to our current understanding, which examined the effects of elevated intraocular pressure on axonal transport. These early studies suggested that relatively high intraocular pressures (≥50 mm Hg) might be necessary for a significant inhibition of anterograde axonal transport [2-5]. However, the 4-fold difference between the axonal transport rates for the different types of MBO cargos was not well understood, and those studies typically evaluated post-labeling times that were suitable to detect effects upon the anterograde axonal transport of very fast small tubulovesicles, such as nascent synaptic vesicles, but were not suitable to detect effects on transport of mitochondria [6-8]. This misunderstanding gave rise to a series of studies evaluating the effects of relatively high intraocular pressures (≥50 mm Hg) on axonal transport. These studies did not

distinguish between effects upon axonal transport of the specific types of MBO cargo [9-11], and effectively equated traumatic compression of the optic nerve head with glaucomatous optic neuropathy. However, studies on the effect of direct compression upon exposed axons have suggested that pressures in this range (50-60 mm Hg) inhibit anterograde axonal transport by an indiscriminate mechanism, loss of microtubule linear arrays [12]. This mechanism inhibits all axonal transport and would not be expected to produce the apparently cargo-selective accumulations seen in the peri-laminar region of human glaucomatous donor tissue [1, 13, 14]. Lower pressure animal models, that more closely represent the moderate intraocular pressures seen in most human glaucoma [15], appear to affect axonal transport by a different mechanism [16, 17].

The chronic retinal ischemia model was based upon clinical observations that optic disc palor is commonly associated with glaucoma [18]. These observations gave rise to studies evaluating the effects of occluding either the central retinal artery or the short posterior ciliary arteries, then evaluating the apparent accumulations of MBOs in the peri-laminar region by electron microscopy, as an indicator of axonal transport inhibition [19, 20]. These studies did not distinguish material actually in axonal transport during the experimental period from material previously delivered to peri-laminar sites. In addition, the severe tissue damage, reported to result from several hours of arterial occlusion [19, 20], was difficult to visually distinguish from inadequate fixation, a common problem for electron microscopy in CNS tissues. However, clinical studies showing delayed effects of transient ischemia in other regions of the CNS have suggested that ischemia alone may affect axonal transport through the loss of linear microtubule

arrays [21]. This mechanism would indiscriminately affect all types of axonal transport [13] and could not adequately explain the predominately mitochondrial/vesicular accumulations seen in human glaucomatous donor tissue [1, 14]. Recent "ischemia" models have used the dually neuroactive [22] and vasoactive [23] peptide, ET-1, to induce glaucoma-like optic nerve head damage [24-27]. This "ischemia" model disregards possible effects from activation of the non-ischemic ET_B receptors at sites central to the neuropathology of glaucoma, such as the retinal ganglion cell and nerve fiber layers of the retina, optic nerve head astrocytes, and within the proximal optic nerve [28-34].

In this study, a new theoretical model for glaucomatous optic neuropathy was developed and tested, the "endothelin receptor-mediated model of neuropathogenesis." This theoretical model is able to explain the cargo-selectivity of glaucoma's effects on axonal transport [35, 36]. To test the endothelin receptor-mediated theory, a new experimental animal model was developed, measuring the effects of intravitreal endothelin (ET-1 or ET-3) on the anterograde axonal transport of pulse-labeled proteins in rat optic nerve [35-41]. Dose selection was made on the basis of a small pilot study (N=3 for pilot study only, data not shown) that measured the total pulse-labeled protein axonally transported into the rat optic nerve. The pilot study evaluated three doses of intravitreal ET-1 (0.3, 0.4, and 2 nmol) at the 4 hour ISI. The lowest significantly effective dose (2 nmols) was used throughout these studies. The intravitreal endothelin/axonal transport animal model was used to test three hypotheses generated by the endothelin receptor-mediated theoretical model. 1) Exogenously elevated endothelin-

1, in the rat vitreous, can access a neuropathogenic site and produce dysfunctions in anterograde axonal transport within the rat optic nerve. 2) ET-1's effects on anterograde axonal transport are receptor-mediated. 3) ET-1's effects on anterograde axonal transport are cargo-selective. Three specific aims were structured to test these hypotheses.

Specific Aim 1: Determine whether intravitreal ET-1 can significantly affect the various rate components of anterograde axonal transport.

Intravitreal administration of exogenous ET-1 affected all components of anterograde axonal transport in the rat optic nerve. Intravitreal ET-1 produced effects were significant, biphasic, and prolonged (4 hours to 21 days). The initial phase of ET-1's effect produced a significant enhancement of transport at times normally associated with small, fast-moving tubulovesicles (4, 24 hours) This was followed by significant impairments at times which are normally associated with the transport of mitochondria (28 to 36 hours), cytoplasmic matrix (4 days), and cytoskeletal proteins (21 days) [35].

Specific Aim 2: Determine whether ET-1's predominant effect is receptor-mediated, and characterize the mediating receptor's activation profile as either ET_A or ET_B type.

The most pronounced effect of intravitreal ET-1 was a decrease in axonal transport at those times associated with the normal anterograde transport of mitochondrial proteins (28, 32, 36 hours, ps = <.001, .015, <.001, respectively). This effect was mimicked at 28 hours by the ET_B- selective agonist, ET-3, suggesting that this effect was receptor-mediated [35, 41]. These data were consistent with an ET_B-receptor-mediated role for endothelins in pathological misregulation(s) of anterograde axonal transport [35].

The receptor-selective agonist approach to this question was used because of problems with antagonist solubility within the volume limitations (4 μl) imposed by the rat vitreous [42].

Specific Aim 3: Determine whether ET-1's effect(s) on anterograde fast axonal transport are cargo-selective, in a manner consistent with what is known about glaucoma and axonal transport.

Intravitreal ET-1 caused a series of pronounced and novel misregulations in anterograde fast axonal transport, first increasing transport at post-labeling times normally associated with the movement of nascent synaptic vesicles (4 hr ISI, $p = .01$), and then decreasing transport at times normally associated with both a large pulse of mitochondrial markers and the continued transport of nascent synaptic vesicles (28, 32, and 36 hr ISIs, $ps = .001, .02, <.001$, respectively) [35, 36]. Concomitant with this pronounced decrease in the transport of total pulse-labeled protein (28-36 hr ISIs), ET-1 caused a selective delay for a distinctive cohort of 11 SDS-PAGE bands. This delay consistently shifted the times of their peak delivery into the optic nerve from 28 hours in vehicle-treated control animals to 32 hours in ET-1-treated animals. During this same interval, the peak delivery time for total pulse-labeled protein was unaffected by ET-1 treatment (32 hours), suggesting that ET-1's effect may be selective for a chemically distinct class of cargo [36]. Cargo-selective alterations in anterograde fast axonal transport had not previously been demonstrated in a genetically normal animal model.

Visual loss develops slowly in glaucoma [15, 43]. This suggests that the delivery of nascent synaptic vesicles, unlike mitochondria, is not seriously compromised during

early phases of the disease. Both of these cargos move with the fast component, and are membrane bound [8]. However, their transport is associated with different isoforms of the kinesin motor heavy chain [6]. This may indicate a mechanism that selectively affects different classes of kinesin-associated cargo [44], possibly through their association with kinesin heavy chains [6]. This type of mechanism would be consistent with endothelin's increasing anterograde axonal transport in rat optic nerve at the 4 hour ISI, but decreasing anterograde transport at 28, 32, and 36 hour ISIs. It would also be consistent with endothelin's selective delay of a chemically distinct subset of cargo, represented by the cohort of 11 protein bands (28-36 hour ISIs). Because the kinesin heavy chain proteins are phosphorylated in vivo [6, 45-47], this type of mechanism would be compatible with receptor-mediated signal transduction pathways [44, 48-53] and might produce results consistent with glaucomatous neuropathology [1]. An alternative interpretation of these results would be that ET_B receptors located on resident glia such as astrocytes stimulate release of some other substance(s) in addition to endothelin and that one or more of these other substances acts in a paracrine fashion upon retinal ganglion cells to produce aberrant anterograde axonal transport. However, this alternative explanation lacks the ET-stimulated-ET amplification loop located in the focal site of glaucomatous pathology that may be provided by optic nerve head astrocytes in the endothelin receptor-mediated model nor does it provide a good explanatory mechanism for effects of elevated intraocular pressure and retinal ischemia upon anterograde axonal transport. These results support the endothelin receptor-mediated theoretical model for neuropathogenesis in the

optic nerve, and suggest that this model may accurately predict some of the mechanisms involved in the development of glaucomatous optic neuropathy (Figure 1).

Future Perspectives:

The intravitreal endothelin/axonal transport animal model will be used in future studies to help unravel the signal transduction pathways involved in intravitreal ET-1's misregulation of anterograde axonal transport. Many of the signal transduction pathways reportedly activated by ET_B receptors appear to be PKC-dependent pathways [54-56] that result in phosphorylation of substrate proteins at serine and/or threonine residues, but some are microfilament dependent and result in tyrosine phosphorylation of substrate proteins [57]. Initially, these pathways might be explored in either the presence or absence of PKC inhibitors [56, 58, 59], by immunoprecipitation of pulse-labeled proteins from the optic nerve and/or retina (endothelin-treated vs. control) perhaps using phosphoserine and/or phosphotyrosine monoclonal antibodies [60-62]. These pulse-labeled proteins may be separated, using SDS-PAGE, for visualization by fluorography and/or quantitated by liquid scintillation counting. Special interest would be given to candidate phosphoproteins that may be associated with the actin cytoskeleton [57, 63, 64], dynamin [50, 65-67], or small GTPases [50, 66] of the MAP kinase pathway [49, 54, 55, 65, 68]. The objective would be to define biochemical pathways linking activation of the ET_B receptor to mechanisms regulating axonal transport.

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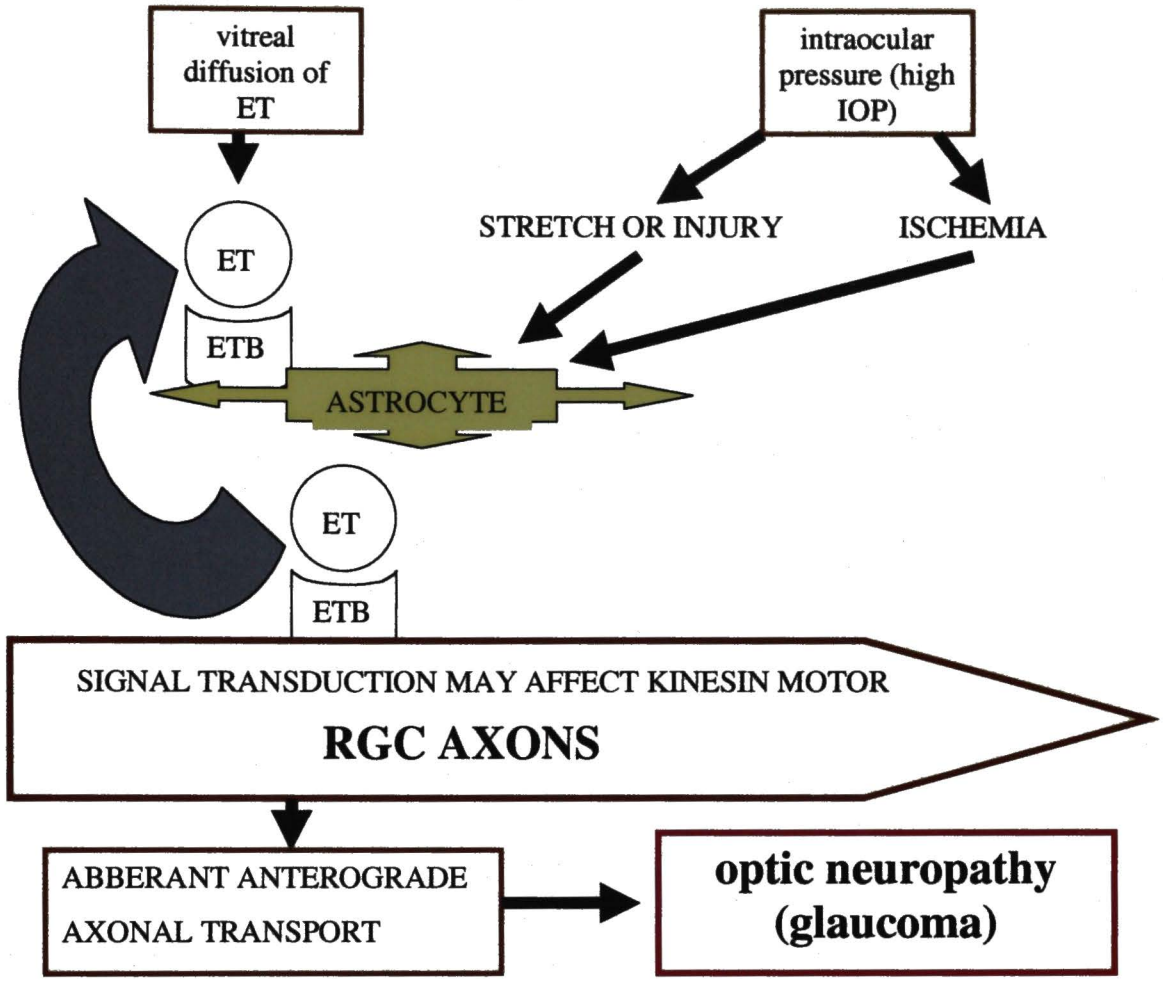
Figure 1. Endothelin receptor-mediated theoretical model for the pathogenesis of glaucomatous optic neuropathy.


vitreal diffusion of ET
intraocular pressure (high IOP)
STRETCH OR INJURY
ISCHEMIA
ET
ETB
ASTROCYTE
ET
ETB
SIGNAL TRANSDUCTION MAY AFFECT KINESIN MOTOR
RGC AXONS
ABBERANT ANTEROGRADE AXONAL TRANSPORT
optic neuropathy (glaucoma)