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Differential roles of A β processing in hypoxia-induced axonal damage

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Abstract

Axonopathy is a common and early phase in neurodegenerative and traumatic CNS diseases. Recent work suggests that amyloid β (A β) produced from amyloid precursor protein (APP) may be a critical downstream mediator of CNS axonopathy in CNS diseases, particularly those associated with hypoxia. We critically tested this hypothesis in an adult retinal explant system that preserves the three-dimensional organization of the retina while permitting direct imaging of two cardinal features of early-stage axonopathy: axonal structural integrity and axonal transport capacity. Using this system, we found via pharmacological inhibition and genetic deletion of APP that production of A β is a necessary step in structural compromise of retinal ganglion cell (RGC) axons induced by the disease-relevant stressor hypoxia. However, identical blockade of A β production was not sufficient to protect axons from associated hypoxia-induced reduction in axonal transport. Thus, A β mediates distinct facets of hypoxia-induced axonopathy and may represent a functionally selective pharmacological target for therapies directed against early-stage axonopathy in CNS diseases.

Keywords

Axonopathy; axonal degeneration; axonal transport; amyloid precursor protein; amyloid beta; retinal explant

1. Introduction

Axonopathy, encompassing compromise of both axonal structure and function, is an early consequence of stress across a broad range of central neurons and neuropathological conditions (1). While clearly associated with physical trauma to the nervous system (2), axonopathy has also been increasingly appreciated as an early event in neurodegeneration in neurological disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis

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(ALS), and glaucoma (1, 3). Common features of compromised axonal structure include the development of axonal varicosities, accumulation of organelles, and loss of synaptic contacts, whereas deficits in transport capacity is a core consequence of impaired axonal function. However, the pathways mediating such structural and functional compromise, and whether these pathways are intersecting or distinct, remain under investigation.

A growing body of evidence has implicated the production of amyloid beta ($A\beta$) from amyloid precursor protein (APP) as a common pathway associated with axonopathy. While $A\beta$ has been studied most extensively in relation to AD (4), aberrant processing through this cascade has now also been reported in axonopathic diseases such as glaucoma (5), multiple sclerosis (6), ALS (7), epilepsy (8), stroke (9), HIV-dementia (10), Creutzfeld-Jakob disease (11), and traumatic brain and optic nerve injury (12, 13). $A\beta$ itself impairs axonal structure and function in a variety of experimental paradigms (14-16), and blockade of the enzymes necessary for $A\beta$ production from APP (BACE1 and the γ -secretase complex (4, 17, 18)) protects central axons from diverse stressors (19-22). Together, these data imply a broad role for $A\beta$ as an effector of axonopathy across CNS degenerative diseases.

Such a role for $A\beta$ would have particular relevance in the setting of CNS hypoxia. Hypoxia has long been known to induce both structural and functional axonopathy (23, 24), and the enzymatic pathway necessary for $A\beta$ production is sensitive to tissue oxygen status (25). CNS hypoxia in fact predisposes central neurons to degeneration in well-studied diseases like Alzheimer's and optic neuropathy (26, 27), and such hypoxia-induced stress mechanisms as hypoxia-inducible factor-mediated pathways (28), heat-shock-factor-mediated pathways (29, 30), and the unfolded protein response (31) are now understood to be central to the protein homeostasis defense mechanisms triggered in a wide range of neurodegenerative and neuroinflammatory conditions in the CNS (32, 33). Thus, elucidating hypoxia-activated axonopathic mechanisms likely to intersect with those in CNS disease remains an important goal.

Therefore, we sought here to test critically the hypothesis that $A\beta$ is a critical downstream effector of hypoxic stress on central axons, focusing on the axons of retinal ganglion cells (RGCs), the long projection neurons of the eye that resemble other projection neurons of the brain and spinal cord in terms of function, connectivity, and susceptibility to neurodegenerative conditions (34). Using hypoxic stress to induce early-stage axonopathy in these mature CNS neurons within their native tissue environment in explanted retinas, we found that $A\beta$ generated from APP is indeed both sufficient and necessary for the structural degeneration of RGC axons in response to hypoxic stress, and that blockade of either BACE1 or γ -secretase activity can provide quantitative structural protection to stressed axons. Surprisingly, whereas blockade of $A\beta$ production maintained the structural integrity of RGC axons, it was not sufficient to restore impaired active transport capacity. Moreover, we found that active transport could still be maintained even through frank distortions in axonal structure induced by $A\beta$ short of overt breakdown of the axon. Thus, our work supports the potential benefit of anti-amyloidogenic therapies in treating neurodegenerative conditions of the eye but suggests that such maintenance of structural integrity is required but not sufficient for full protection of RGC axons against hypoxic stress-associated dysfunction.

2. Materials and Methods

Retinal explant cultures

All experiments were done in explant cultures from adult (>3 months) rats or mice. Briefly, eyes were enucleated from CD Sprague-Dawley rats (Charles River, Wilmington, MA) or C57BI6 or APP-deficient mice (Jackson Labs, Bar Harbor, ME) immediately following sacrifice in accordance with NIH guidelines and under Duke IACUC approval and oversight. A circumferential cut was made 1 mm posterior to the limbus, then the retina was gently coaxed away from the posterior sclera to permit separation of the entire retina and optic nerve head from the scleral tissue via a single cut. Retinas were cut into wedges as sixths (rats) or fourths (mice) and placed RGC-side up onto an interface culture platform composed of filter paper (Sigma-Aldrich Co. LLC, St. Louis, MO) suspended in culture medium (Neurobasal medium supplemented with 10% heat-inactivated pig serum, 5% heat-inactivated rat serum, 10 mM HEPES, 100 µg/ml Primocin, 1 mM MEM sodium pyruvate, and 1 mM GlutaMAX in Neurobasal A (Invitrogen, Carlsbad, CA). In these interface cultures, only the bottom surface of the explant was in direct contact with the culture medium, allowing efficient gas exchange via the upper surface of the tissue. Explant cultures were maintained in humidified incubators under pre-bubbled 5% CO₂ at 37°C. Half of the medium was changed on the first day after dissection and on every second day thereafter. In experiments involving pharmacological treatments, inhibitor compounds were dissolved at the noted concentrations in dimethyl sulfoxide (DMSO) and included in the culture medium throughout the duration of the experiments. All BACE1 and γ-secretase inhibitors used in this study were generously provided by Wyeth/Pfizer (New York, NY) and were used at concentrations that inhibited greater than 90% of Aβ production. Final DMSO concentrations did not exceed 0.1%. In all experiments, retinal explants were divided evenly between treatment conditions such that each treatment condition received an equal number of explants from each eye dissected.

Hypoxic stress

A custom-built hypoxic chamber was used consisting of an inverted pressure cooker (Presto, Eau Claire, WI) with holes for gas inlet and outlet drilled into the base. The entire chamber was placed in a humidified incubator under pre-bubbled 5% CO₂ at 37°C. For experiments involving hypoxic stress, explants were incubated in 12-well plates placed in the chamber under 0% O₂, 5% CO₂, and 95% N₂ for the indicated durations. Unless otherwise noted, hypoxic stress was applied for a period of 4-5 h (rat) or 6 h (mouse).

Transfection of retinal explants

To transfect RGCs in retinal explants, we used a microtargeting biolistic device that avoids the trauma associated with conventional entrainment biolistic methods and permits rapid, efficient, and spatially restricted transfection of RGCs in the adult mammalian retina without damaging their local microenvironment (35). Standard 1.6-µm diameter gold particles (Strem Chemicals, Inc., Kehl, Germany) were used, coated with DNA constructs via calcium/ethanol precipitation. DNA expression constructs for YFP, APP-Wt, APP-Sw, Tau0R, and Tau4R were made in the gWiz expression vector (Genlantis). The APP-Wt and APP-Sw overexpression constructs, including promoters and enhancers, were identical

except for the K595N/M596L mutation. For experiments involving transfection, explants were transfected 30–60 min after dissection.

Assessment of RGC axonal structure

Compromise of RGC axonal structure was assessed visually using a scoring system keying on morphological features of axonal pathology, notably the number of axonal varicosities and the overt loss of axonal continuity. An axonal varicosity was defined as any axonal region that was more than twice as thick as the surrounding axonal segment, giving a “bead on a string” appearance. For clarity, data are presented as the mean percentage of RGC axons in a given explant that did not exhibit axonal pathology (i.e., number of varicosity-free axons/total axonal number*100) $\pm$ standard error of the mean (SEM). In APP overexpression experiments, transfected explants were cultured for 5 days under normoxic conditions, then fixed and processed for assessment of axonal pathology. In experiments examining the effect of hypoxia on RGC axons, transfected explants were exposed on the day after dissection to either normoxia or a brief period of hypoxia, then fixed for processing 24 h later. Assessment of axonal structure was done by a single investigator blinded to experimental treatment condition.

Assessment of RGC axonal transport

RGC axonal transport capacity was assessed using Alexa 488-, 594-, or 647-conjugated cholera toxin B (CTB; Invitrogen), a well-established retrograde neuronal tracer devoid of toxicity due to removal of the α subunit (36, 37).

For experiments examining the retrograde transport capacity of the entire, native RGC population in an explant, retinal explants were exposed on the day after dissection to hypoxic stress and then incubated overnight with a 0.05 μ l drop of CTB (0.5 mg/ml) positioned on the surface of the retinal explant at the optic nerve head. As only the most distal portions of the explanted axons contacted the CTB, the number of peripheral RGC somata accumulating CTB signal via retrograde axonal transport 24 h later served as a metric for axonal transport capacity. To reduce the potential for bias in CTB transport due to axons that, while intact, did not reach the CTB sink because of the geometry of our dissection method, the number and intensity of CTB-positive RGCs were quantified from a 25 $\times$ image taken from the central periphery of each explant using a custom, automated image-analysis program developed in the Matlab software environment (Mathworks, Inc., Natick, MA). Images were taken by a single investigator blinded to experimental treatment condition. Data are presented as the mean number of CTB-positive (transporting) RGCs per explant $\pm$ SEM for each condition or as histograms in which the number of transporting RGCs plotted against RGC intensity for all explants in a given condition.

For experiments examining axonal transport capacity in a specific population of transfected RGCs, explants were dissected and transfected as described above. At 6 h after transfection, explants were pre-labeled with Alexa-488-tagged CTB placed at the optic nerve head. This pre-expression label was used to define the initial population of RGCs in the explant with the capacity to take up and transport the tracer prior to significant expression of transfected constructs. Four 4 days after transfection, explants were post-labeled with Alexa-647-tagged

CTB to assess transport capacity of this same population of RGCs after expression of the transfected genetic construct. Explants were fixed 24 h after application of the post-expression CTB. Accumulation of pre- and post-expression CTB in the somata of transfected RGCs was quantified automatically from images taken with a 63x oil objective using a custom program developed in the Matlab software environment. Data are presented as the mean ratio of the post- to pre-CTB tracer intensities $\pm$ SEM or as the mean CTB intensity ratio of transfected RGCs compared to their untransfected neighbors $\pm$ SEM at the conclusion of the experiments. To reduce bias stemming from the duration of transport or camera sensitivity, data were corrected for overall changes in channel brightness.

Immunofluorescence analyses

Retinal explants were rinsed once in Dulbecco's PBS (Sigma-Aldrich Co. LLC) and fixed with 4% paraformaldehyde in PBS containing 4% sucrose, then incubated overnight in primary antibodies against YFP (rabbit anti-GFP; 1:2000; Abcam, Cambridge, MA or mouse anti-GFP; 1:1000; Millipore, Billerica, MA), phosphorylated neurofilaments (Smi-31; 1:1,000; Covance, Princeton, NJ), calretinin (1:1000; Chemicon International, Billerica, MA), or β -galactosidase (1:1000, Promega, Madison, WI), as indicated. Retinas were then labeled with species-specific secondary IgG antibodies conjugated to Alexa 488, 568, or 594 (Invitrogen), mounted in Hydromount medium (National Diagnostics, Atlanta, GA), and imaged using a Zeiss fluorescence microscope (Carl Zeiss Microimaging, LLC, Thornwood, NY) equipped with a charge-coupled device (CCD) camera (AxioCam MRm, Zeiss). Post-processing and quantification of images were done using ImageJ (National Institutes of Health) and Matlab algorithms, respectively.

Western blot analysis

APP-Sw-transfected cortical brain slices or retinal explants were washed 3 times with PBS on ice and homogenized by trituration through a 25 g needle in homogenization buffer (1% NP-40 in 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA) supplemented with Complete Protease Inhibitor Cocktail (Roche). Samples were cleared twice by centrifugation at 3000 g. For immunoprecipitation (IP), 6E10 antibodies were added to the samples and incubated overnight at 4 °C followed by incubation with Protein G Sepharose (Sigma). The Protein G Sepharose immunocomplex was washed 5 times in homogenization buffer, and eluted in 0.15 mM glycine HCl, pH 2.5–3.0. For Western blot analysis, eluates were neutralized to physiological pH, and proteins separated on 10–20% Tricine gels (Invitrogen). Following transfer to PVDF membranes (0.2 μ m pore size, Bio-Rad), Western blots were done using standard methods with 6E10 and a HRP-conjugated secondary IgG antibody with ECL visualization (Amersham) and documentation on a Bio-Rad VersaDoc MP5000. Densitometric quantification was done using ImageJ software. A β levels normalized to the APP signal in each Western blot to account for variation in transfection efficiency.

ELISA analysis

Levels of A β 1-40 and A β 1-42 in retinal explants were quantified by electroluminescent ELISA (Evotec). Whole retinal explants from P14-16 rats were dissected as described above, incubated for 0, 0.5, 2, or 6 hours as either normoxic interface cultures (5% CO₂) or hypoxic cultures in pre-bubbled media (5% CO₂, 95% N₂), and flash-frozen. For analysis,

retinal tissues were homogenized in 150 μ L of homogenization buffer (10 mM NaF, 1 mM PMSF, 1 $\times$ Phosphatase Inhibitors II and III, and 1 $\times$ Protease Inhibitor cocktail in Mesoscale Tris-Lysis buffer). The dilutions, incubations, and washings steps of the assay were done according to the manufacturer's recommendations, and plates were imaged under electroluminescence (Meso Scale Discovery). All measurements were done in triplicate; statistical significance was determined using a Student's *t*-test with the Bonferroni correction.

3. Results

Hypoxia impairs axonal structure and net retrograde transport in explanted RGCs

The explanted retina offers an accessible experimental context where mature, three-dimensional native tissue architecture is preserved. Acute whole retinal explant cultures have been used for a wide range of physiological and mechanistic studies (38-40), and in general intact tissue models preserve intercellular and local neural circuit interactions to a much greater degree than dissociated cultures and cell lines (41, 42). Using a device for trauma-free transfection of RGCs in adult retinal explants (35), we selectively labeled RGCs with YFP (Figure 1A and Supplementary Figure 1). RGCs thus labeled could be maintained *ex vivo* for one week without compromise of axonal structure (Supplementary Figure 2A-C) or functional active transport capacity (Supplementary Figure 2D,E).

To examine axonal responses of YFP-transfected RGCs to hypoxia, a well-established stressor implicated in a range of neurodegenerative and traumatic CNS diseases (27), we exposed explanted retinas from adult rats to a hypoxic environment (nominal 0% O₂) on the day after transfection and assessed them 24 h later for structural deficits. To focus on the earliest stages of RGC axonal degeneration, we chose a degree of hypoxia that did not cause overt neuronal somatic injury and loss (Figure 1B,C). While RGC axons maintained under normoxic conditions remained intact, those exposed to this level of hypoxia developed structural axonopathy in the form of bead-like varicosities that punctuated the length of the axon and could be observed at the population level via immunostaining against phosphorylated neurofilaments (Figure 1D-F).

We next asked if hypoxia also compromises axonal transport in explanted retinas by measuring the capacity of RGCs to transport fluorescently-tagged cholera toxin B (CTB) from their distal axonal segments into their somata (Figure 2A, Supplementary Figure 3). RGC axons in these hypoxia-stressed retinal explants did indeed show impaired net retrograde CTB transport (Figure 2B). Although axon terminals showed no deficits in CTB uptake (data not shown), CTB accumulated in bright axonal granules instead of being transported efficiently into RGC somata. CTB-labeled somata in hypoxia-stressed explants were thus substantially fewer and dimmer than those in normoxic counterparts (Figure 2C). As above, these changes reflected early-stages of RGC axonopathy, perhaps corresponding to clinical treatment windows during which ocular pathologies may still be reversible. In fact, more prolonged hypoxic stress led to catastrophic failure of axonal transport (Supplementary Figure 3F,G).

A β drives structural compromise of RGC axons in response to hypoxic stress

A role for A β in hypoxia-induced compromise of axonal structure was suggested by reports that hypoxia-induced alterations of APP processing result in increased levels of A β accumulation (43), and we have confirmed that endogenous A β -40 and A β -42 levels are elevated in retinal explants exposed to hypoxic versus normoxic conditions (Supplementary Figure 4). Therefore, we asked whether production of an APP product is necessary for early-stage hypoxia-induced compromise of retinal axonal structure by using pharmacological inhibitors preventing BACE1 (BI131) and γ -secretase (GSI642) cleavage of APP (44, 45). Treatment with BI131 and GSI642 reduced production of A β by over 90% (Figure 3) and protected significantly against structural compromise in hypoxia-stressed retinal explants (Figure 4A,B), consistent with a requirement for endogenous A β in hypoxia-induced impairment of axonal structure.

However, several substrates in addition to APP have been reported for BACE1 and γ -secretase, though outside of APP these substrates generally are not in common for these two enzyme activities (46, 47). Nevertheless, to confirm that APP was the critical substrate for BACE1 and γ -secretase in the induction of axonal structural compromise, we tested the efficacy of BI131 and GSI642 in hypoxia-stressed explants from APP-deficient (“APP-KO”) mice lacking all products of APP proteolytic processing (48). First, we noted that RGCs from adult APP-KO mice developed less severe axonal structural pathology in response to equivalent levels of hypoxic stress than their age- and gender-matched wild-type (WT) counterparts (Figure 4C,D). Furthermore, while axonal varicosities in WT retinal explants were significantly rescued by BI131 and GSI642, treatment with these inhibitors did not protect RGC axons from APP-KO mice (Figure 4C,D), even after exposure to levels of hypoxic stress that were increased to produce a similar degree of structural axonopathy to that observed for WT retinal explants (Supplementary Figure 5). Thus, while we cannot exclude minor contributions from potential non-APP substrates shared by BACE1 and γ -secretase, that genetic deletion of APP occluded the effects of both inhibitors supports a proteolytic product of APP underlying hypoxia-induced structural compromise of RGC axons.

Conversely, we next asked whether overexpression of APP (and consequent A β elevation) was sufficient to drive early-stage structural compromise of RGC axons in retinal explants. RGCs were transfected with DNA constructs encoding either a control plasmid or APP bearing the Swedish familial AD mutation (K595N/M596L; APP-Sw), which generates elevated levels of A β (49). Consistent with *in vivo* models for APP/A β -induced neurodegeneration (16, 50-54), APP-Sw overexpression impaired axonal structure by inducing axonal varicosities (Figure 5A,B). Moreover, the degree of structural deficit was proportional to the amount of A β generated, as an APP-overexpression construct generating lower levels of A β produced a less severe axonal phenotype than did APP-Sw (Figure 5C,D). As there was no measureable loss of RGC axons due to APP transfection (Figure 5E), this structural compromise again likely represents an early phase of axonopathy rather than a secondary consequence of RGC cell death. Finally, APP-Sw-transfected explants were treated with BI131 and GSI642. Treatment of retinal explants with either inhibitor significantly reduced axonal varicosities induced by APP-Sw transfection (Figure 5A,B),

supporting that APP overexpression and A β generation are sufficient to compromise RGC axonal structure in adult retinal explants.

Together, these results demonstrate that APP processing is both necessary and sufficient for hypoxia-induced impairment of axonal structure, and in combination with previous reports of A β impairment of axons (14-16), strongly implicate A β as a key mediator of hypoxia-induced axonal structural impairment. However, we cannot exclude other products conjointly formed upon A β formation (e.g., AICD) also playing a contributory role.

A β does not drive hypoxia-induced compromise of RGC axonal transport

We next asked whether APP processing also mediates hypoxia-induced impairment of net retrograde axonal transport by treating hypoxia-stressed retinal explants with BI131 or GSI642 and assessing their capacity to transport CTB. Surprisingly, treatment with BI131 but not GSI642 rescued hypoxia-induced impairment of axonal transport (Figure 6A,B). These findings were confirmed with three additional BACE1 and two additional γ -secretase inhibitors (Supplementary Figure 6). As blocking the activity of either enzyme necessary for A β generation from APP would be expected to have equivalent effects, these findings raised the question of whether endogenous BACE1 could be acting upon a substrate other than APP in the context of hypoxia-compromised axonal transport.

Therefore, we next tested whether BACE1 inhibition was still protective against hypoxia-induced compromise of axonal transport in APP-KO mice. Because previous work has been divided over whether loss of APP impairs axonal transport capacity (55, 56), we first confirmed that explanted RGCs from wild-type and APP-KO mice did not differ in CTB transport capacity under normoxic conditions (Figure 7A,B). The number and intensity of CTB-transporting RGCs did not differ between control and APP-KO mice, suggesting that loss of APP does not affect basal levels of axonal transport in these explants (Figure 7A,B).

We then exposed BI131-treated retinal explants from adult, age- and gender-matched WT and APP-KO mice to normoxic or hypoxic conditions. We found no differences in hypoxia-driven impairment of RGC axonal transport between the two genotypes (Figure 7C,D). Furthermore, BI131 significantly ameliorated hypoxia-induced impairment of axonal transport even in APP-KO explants, indicating that BACE1 inhibition rescues axonal transport via acting on a substrate other than APP.

Consistent with this finding, axons overexpressing APP-Sw under normoxic conditions, though profusely burdened with varicosities, still accumulated CTB at levels similar to their control-transfected counterparts (Figure 8). In contrast, transfection of RGCs with an isoform of human tau (Tau4R) that impairs axonal transport *in vivo* (57) induced clear reduction of net retrograde CTB transport.

4. Discussion

Together, our results indicate that APP-dependent processes mediate specific and separable components of early-stage axonal damage caused by hypoxic stress: whereas A β generated from APP is both necessary and sufficient for structural compromise of RGC axons induced

by hypoxic stress, A β generation is not obligatory for hypoxia-induced impairment of net retrograde axonal transport. Our findings thus support the existence of parallel axonopathic pathways (58) that selectively recruit either APP-dependent or -independent mechanisms to mediate different facets of early-stage axonal compromise.

The differential APP dependence of hypoxia-induced deficits in axonal structure versus transport adds to a complex literature on the role of APP and its proteolytic processes in axonopathy. Recent work has debated whether axonopathy in APP-transgenic mice depends entirely on APP (59) or instead on A β as well (60). That the degree of hypoxia-induced structural compromise in our studies was proportional to the amount of A β generated is consistent with the latter idea. However, we cannot exclude the possibility that other components of the APP cascade play supplementary roles in hypoxia-induced compromise of axonal structure.

Our findings add to a growing appreciation that BACE1 and γ -secretase inhibition can protect not just neuronal somata (61) but also their axons (19-22) under conditions of stress in the CNS. The distinction between somal and axonal protection has relevance for a broad spectrum of neurodegenerative diseases, given that mechanisms of axonal and somal degeneration may often be independent (1, 62) and that protection of neuronal somata alone has repeatedly proven insufficient to protect axons in models of neurodegeneration (63-65). Although recent data have demonstrated post-injury survival of the proximal axon to be a consequence of RGC somal survival in some circumstances (66), that our hypoxic stressors do not cause somal loss is consistent with our findings being specific for the axonal compartment. Nevertheless, a more careful examination of indices of RGC somal health and activation of relevant signaling pathways (e.g., JNK) would be necessary to determine whether hypoxia-induced axonopathic processes initiate in the axonal compartment itself or instead more broadly across other regions (e.g., the soma) of the RGC. Regardless of its initiation site, because axonopathy frequently precedes overt degeneration of neuronal somata in human disease states (as in the present study), our findings suggest that aberrant APP metabolism may be a first step in the neurodegenerative process in a range of CNS diseases (1, 67).

Our findings of a central role for A β in hypoxia-induced structural axonopathy are supported by previous studies showing sensitivity of APP processing steps to hypoxia. Hypoxia causes HIF-1-mediated increases in BACE1 transcription (68-70), which is associated with elevated protein levels as well as increased BACE1 enzymatic activity (70, 71). Hypoxia also increases basal APP at both the mRNA and protein levels (72-76), increases APP cleavage by γ -secretase (43, 77), decreases APP cleavage by α -secretase (76, 78-80), and reduces the activity of A β -degrading enzymes like neprilysin and endothelin-converting enzyme (76). Together, these changes translate directly into increased levels of A β during hypoxic stress (43, 76, 80). This concordance raises the attractive possibility that BACE1 and γ -secretase inhibitors originally developed in the context of Alzheimer's disease could be repurposed for much-needed use in hypoxia- or ischemia-associated axonopathic diseases of the CNS.

Such potential may be particularly relevant for glaucoma, a neurodegenerative disease of the CNS that is exacerbated by CNS stress (81). Glaucoma is typified by slowly progressive

axonal loss that begins as distal axonopathy and ultimately impairs vision (82). Although the proximate causes of RGC loss in glaucoma are complex, increasing evidence implicates aberrant APP processing as a potential pathogenic mechanism: accumulation of both APP and A β in retinal ganglion cells and their axons has been demonstrated in experimental models (83-87), and anti-amyloid therapy has been reported to ameliorate RGC loss in an animal model of the disease (85).

Our findings build on this work by providing direct evidence that blocking aberrant APP processing mitigates conditions of RGC axonal stress as modeled by hypoxia, as well as by other disease-relevant conditions such as hypoxia-ischemia (Supplementary Figure 7). Such axonal protection by BACE1 and γ -secretase inhibition is especially pertinent for glaucoma therapies, which seek to preserve stressed, but surviving, RGCs and their axons that form the optic nerve. Further, our findings highlight the importance of additional studies examining physiological endpoints of axonal health (e.g., action potential conduction velocity) along with *in vivo* glaucoma models to test inhibitor dosing strategies relevant to the human disease. Although anti-amyloid compounds have been disappointing in trials of AD due to systemic toxicity or poor blood-brain barrier permeability, ease of access—including numerous technologies for localized drug delivery—and immunologic privilege make the eye and its retinal neurons an intrinsically more tractable system than the rest of the CNS for pharmacologic intervention. However, because no currently available glaucoma therapies are known to protect RGCs and their axons directly from degeneration, our findings take an important step toward identifying pharmacological targets with clinical relevance for the glaucoma population.

Finally, although we found that early-stage compromise of axonal structure and transport capacity differentially depend on A β production, we also found that BACE1 inhibition was protective against both deficits. BACE1 cleaves a much broader family of substrates than originally supposed (46), and the recent reports that several BACE1 substrates have physiologically relevant roles within the axonal compartment suggest several potential candidates that may mediate this protection, such as neuregulin 1 and the low density lipoprotein receptor-related protein (88, 89). Thus, identifying the BACE1 substrate responsible for inhibiting axonal transport during hypoxic stress will also be of particular clinical interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Hypoxia induces loss of structural integrity and transport capacity in RGC axons
- A β mediates hypoxia-induced structural compromise of RGC axons
- A β blockade does not restore active axonal transport capacity during hypoxia
- Hypoxia-induced compromise of axonal structure but not transport depends on A β
- A β inhibition could provide clinical benefit for aspects of axonal degeneration

Figure 1. Hypoxia compromises axonal structure in explanted RGCs

(A) Transfected RGCs and axons in an adult retinal explant (48 h in culture). Scale bar, 200 μ m. (B, C) Hypoxia does not cause overt loss of retinal neurons (RGCs and amacrine cells), as quantified by numbers of calretinin-positive neuronal somata (B) or YFP-positive RGC axons (C) 24 h after treatment (48 h in culture) (calretinin: n=6 explants/condition, 2772 RGCs, p=0.80; YFP: n=12 explants/condition, 961 axons, p=0.90). (D, E) YFP-transfected (D) and native (E) RGC axons in retinal explants exposed to normoxic vs. hypoxic conditions 24 h after dissection and fixed 24 h later for analysis of axonal structure (arrows). Scale bar, 25 μ m (D) and 50 μ m (E). (F) Quantification of hypoxia-induced reduction in the percentage of RGC axons without axonal varicosities in (D) (n=12 explants/condition, 1014 axons, ***p<0.001).

Figure 2. Hypoxia compromises net retrograde active axonal transport in explanted RGCs (A) CTB labeling procedure used to assess axonal transport capacity in explanted RGCs. (B) CTB transport and accumulation in RGC somata. Somal (*upper*) and axonal (*lower*) regions of retinal explants are shown at 48 h in culture, 24 h after treatment with normoxic (*left*) or hypoxic (*right*) conditions. Scale bar, 50 μ m. (C) Quantification of numbers (*left*) and intensity distributions (*right*) of RGC somata labeled by transported CTB in normoxic vs. hypoxic conditions (n=12 explants/condition, 1771 RGCs, ***p<0.001).

Figure 3. Pharmacological inhibitors prevent production of Aβ from APP

(A) 6E10 immunoblot (*left*) and densitometry quantification (*right*) of brain slice lysates showing concentration-dependent inhibition by BI131 of β-secretase cleavage of APP to form the C99 C-terminal fragment and consequent inhibition also of Aβ production at 48 h after dissection/transfection. Treatment with BI131 does not significantly affect levels of expression of full-length APP. (B) 6E10 immunoblot (*left*) and densitometry quantification (*right*) of brain slice lysates showing concentration-dependent inhibition by GSI642 of γ-secretase cleavage of C99 and consequent inhibition of Aβ production at 48 h after dissection/transfection. Treatment with GSI642 does not significantly affect levels of expression of either full-length APP or its C99 cleavage product. (C) 6E10 immunoblots of brain slice lysates showing concentration-dependent inhibition of both β-secretase cleavage

of APP as well as A β production by BI116 (*left*), BI954 (*middle*), and BI899 (*right*) at 48 h after dissection/transfection. **(D)** 6E10 immunoblots of brain slice lysates showing concentration-dependent inhibition of both γ -secretase cleavage of APP as well as A β production by GSI953 (*left*) and GSI767 (*right*) at 48 h after dissection/transfection. **(E)** Western blot of retinal explants confirming inhibition of β -secretase cleavage of APP in retinal explants. Lysates from retinal explants transfected with either a control plasmid or a C-terminal, FLAG-tagged APP-Sw construct and treated with a 30 μ M dose of BI131 were immunoprecipitated and immunoblotted using an antibody against FLAG. Production of the C99 band is significantly inhibited by 30 μ M BI131 (*rightmost lane*) at 48 h after dissection/transfection.

Figure 4. Pharmacological blockade of A β production protects against hypoxia-induced structural compromise of RGC axons

(A) YFP-labeled RGC axons are protected 24 h after hypoxia-induced structural compromise (48 h in culture) by concurrent treatment with increasing concentrations of BI131 (*left*) or GSI642 (*right*). Arrows, varicosities. Scale bar, 20 μ m. (B) Quantification of (A) showing the percentage of axons without varicosities, with reduction by hypoxia and rescue by BI131 ($n > 8$ explants/condition, 448 axons, *** $p < 0.001$) and GSI642 ($n = 12$ explants/condition, 1916 axons, *** $p < 0.001$). (C) YFP-labeled RGC axons from WT (*left*) or APP-KO (*right*) retinal explants exposed to normoxic or hypoxic conditions and treated with DMSO, BI131, or GSI642. Arrows, varicosities. Explants are shown 24 h after hypoxia (48 h in culture). Scale bar, 20 μ m. (D) Quantification of (C) showing the percentage of axons without varicosities, with basal resistance to hypoxia evident in APP-KO explants (second pair of bars; *** $p < 0.001$ for WT vs. APP-KO). APP-KO RGC axons are unresponsive to maximal concentrations of inhibitors (WT: $n = 6$ explants/condition, 1016 axons, ** $p < 0.01$ (BI131) and *** $p < 0.001$ (GSI642) vs. hypoxia; APP-KO: $n = 8$ explants/condition, 2416 axons, $p = 0.73$ (BI131) and 0.82 (GSI642) vs. hypoxia).

Figure 5. A β overexpression drives compromise of RGC axonal structure without causing axonal loss

(A) Axonal structure is compromised in retinal explants co-transfected with YFP and APP-Sw, which can be rescued by either inhibition of β -secretase by BI131 or of γ -secretase activity by GSI642. Representative images of transfected RGC axons fixed on day 5 after dissection and transfection showing that transfection with APP-Sw induces axonal structural compromise in the form of varicosities (*arrows*) but not overt axonal loss. Scale bar, 25 μ m. (B) Quantification of (A) showing rescue of APP-induced axonal structural compromise by BI131 or GSI642 ($n=12$ explants/condition, 2936 axons, $**p<0.01$). (C) Transfection with APP-Sw (*bottom*), which produces increased levels of A β , correspondingly increases the severity of axonal compromise observed on day 5 after dissection and transfection compared to that induced by APP-wt (*middle*). Scale bar, 25 μ m. (D) Quantification of (C) showing the mean percentage of axons without varicosities in control transfected explants vs. APP-Wt or APP-Sw ($n=10$ explants per condition, 1555 axons, $**p<0.01$). (E) Transfection with APP isoforms does not induce overt loss of RGC axons, quantified here on day 5 after RGC explantation and transfection ($n>7$ explants/condition, 1194 axons, $p=0.82$).

Figure 6. β - but not γ -secretase inhibitors protect against hypoxia-induced impairment of RGC axonal transport
(A) CTB accumulation in normoxia- or hypoxia-exposed RGC somata 24 h after hypoxia (48 h in culture) after concurrent treatment with BI131 (*left*) or GSI642 (*right*). Scale bar, 50 μ m. **(B)** Quantification of CTB-labeled RGC somata in (A) showing complete rescue of axonal transport by BI131 (n=12 explants/condition, 5651 RGCs, **p<0.01; ***p<0.001) but not by GSI642 (n=12 explants/condition, 3788 RGCs, p=0.68 (3 μ M) and 0.40 (30 μ M)).

Figure 7. β -secretase inhibition still protects against hypoxia-induced impairment of RGC axonal transport in explants from APP-KO mice

(A, B) RGC axonal transport capacity does not differ between RGCs in WT and APP-KO retinal explants. (A) Representative images of CTB accumulation in RGC somata in WT vs. APP-KO retinal explants show no obvious differences under normoxic conditions on day 2 after explantation. (B) Quantification of (A) showing the numbers (*left*) and intensity distributions (*right*) of RGC somata labeled by CTB (12 explants/condition, 4194 RGCs, $p=0.60$). Scale bar, 50 μ m. (C) CTB accumulation in RGC somata showing similar rescue from hypoxia-induced impairment by BI131 in WT and APP-KO retinal explants 24 h after hypoxia (48 h in culture). (D) Quantification of CTB-labeled RGC somata in (C) (WT: $n=12$ explants/condition, 5204 RGCs; APP-KO: $n=12$ explants/condition, 6064 RGCs, *** $p<0.001$). Scale bar, 50 μ m.

Figure 8. CTB accumulation is unaffected by APP overexpression

(A) CTB accumulation is unaffected by APP overexpression-induced disruption of axonal structure. Fluorescence images of a representative RGC transfected with YFP and APP-Sw (top) accumulating CTB (middle) demonstrating intact axonal transport capacity despite the presence of axonal varicosities (bottom, arrows) 5 days after dissection. Scale bar, 50 μ m. (B) RGCs (YFP label false-colored as blue) transfected with a control plasmid (top row), APP-Sw (middle row), or Tau4R (bottom row) and assayed for CTB transport capacity at both pre- (green, 6 h after dissection) and post- (red, 5 d after dissection) expression time points. While CTB accumulation post-expression is not affected by APP-Sw transfection (arrows in red channel), it is completely blocked by Tau4R expression (arrow in red channel, appearing as blue instead of white in the merge). Scale bars, 20 μ m. (C) Quantification of retrogradely transported CTB in (B) showing a significant effect of Tau4R but not APP-Sw transfection vs. controls, analyzed as either mean ratios of CTB intensity during pre- and post-expression periods within the same transfected cells (left) or mean ratios between transfected and non-transfected neighbors during the post-expression period (right) (APP: n=74 RGCs, no significant differences vs. control; Tau4R: n=163 RGCs, **p<0.01, ***p<0.0001 vs. Tau0R). Transf, transfected.