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Differential roles of Aβ 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 (Aβ) 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 Aβ is a necessary step in structural compromise of retinal ganglion cell (RGC) axons induced by the disease-relevant stressor hypoxia. However, identical blockade of Aβ production was not sufficient to protect axons from associated hypoxia-induced reduction in axonal transport. Thus, Aβ 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.

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 (Coleman, 2005). While clearly associated with physical trauma to the nervous system (Rotshenker, 2011), axonopathy has also been increasingly appreciated as an early event in neurodegeneration in neurological disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and glaucoma (Coleman, 2005; Morfini et al., 2009). 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 (Aβ) from amyloid precursor protein (APP) as a common pathway associated with axonopathy. While Aβ has been studied most extensively in relation to AD (Hardy & Selkoe, 2002), aberrant processing through this cascade has now also been reported in axonopathic diseases such as glaucoma (Yoneda et al., 2005), multiple sclerosis (Ferguson et al., 1997), ALS (Calingasan et al., 2005), epilepsy (Borges et al., 2003), stroke (Ohgami et al., 1992), HIV-dementia (Raja et al., 1997), Creutzfeld–Jakob disease (Liberski & Budka, 1999), and traumatic brain and optic nerve injury (Olsson et al., 2004; Reichard et al., 2004). Aβ itself impairs axonal structure and function in a variety of experimental paradigms (Kasa et al., 2000; Pike et al., 1992; Stokin et al., 2005), and blockade of the enzymes necessary for Aβ production from APP (BACE1 and the γ-secretase complex (Hardy and Selkoe, 2002; Scheuner et al., 1996; Vassar et al., 1999) protects central axons from diverse stressors (Farah et al., 2011; Nikolaev et al., 2009; Yoon et al., 2006; Jurynczyk et al., 2005). Together, these data imply a broad role for Aβ as an effector of axonopathy across CNS degenerative diseases.

Such a role for Aβ would have particular relevance in the setting of CNS hypoxia. Hypoxia has long been known to induce both structural and functional axonopathy (Def Webster & Ames, 1965; Ochs & Ranish, 1970), and the enzymatic pathway necessary for Aβ production is sensitive to tissue oxygen status (Peers et al., 2009). CNS hypoxia in fact predisposes central neurons to degeneration in well-studied diseases like Alzheimer's and optic neuropathy (Grimm & Willmann, 2012; Zlokovic, 2011), and such hypoxia-induced stress mechanisms as hypoxia-inducible factor-mediated pathways (Koh & Powis, 2012), heat-shock-factor-mediated pathways (Baird et al., 2006; Shen et al., 2005), and the unfolded protein response (Kim et al., 2008) 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 (Powers et al., 2009; Mehta et al., 2009). 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 Aβ is a critical downstream effector of hypoxic stress on central axons,

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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 (Dowling, 2012). Using hypoxic stress to induce early-stage axonopathy in these mature CNS neurons within their native tissue environment in explanted retinas, we found that Aβ 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 Aβ 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 Aβ 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.

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 C57Bl6 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% CO_2 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. The 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% CO_2 at 37 °C. For experiments involving hypoxic stress, explants were incubated in 12-well plates placed in the chamber under 0% O_2, 5% CO_2, and 95% N_2 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 (Christianson & Lo, 2011). 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) ± 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 (Luppi et al., 1990; Angelucci et al., 1996).

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 μl 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× 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 ± 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