Published in final edited form as:

Neurochem Int. 2013 December ; 63(8): 782–789. doi:10.1016/j.neuint.2013.08.013.

Axon degeneration is key component of neuronal death in amyloid - β toxicity

Wilson M. Alobuia, Wei Xia, and Bhupinder P. S. Vohra

Biology Department, University of Central Arkansas, Conway, AR 72035

Abstract

Depending upon the stimulus, neuronal cell death can either be triggered from the cell body (soma) or the axon. We investigated the origin of the degeneration signal in amyloid β ($A\beta$) induced neuronal cell death in cultured *in vitro* hippocampal neurons. We discovered that $A\beta_{1-42}$ toxicity-induced axon degeneration precedes cell death in hippocampal neurons. Overexpression of Bcl-xl inhibited both axonal and cell body degeneration in the $A\beta$ -42 treated neurons. Nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) blocks axon degeneration in a variety of paradigms, but it cannot block neuronal cell body death. Therefore, if the neuronal death signals in $A\beta_{1-42}$ toxicity originate from degenerating axons, we should be able to block neuronal death by inhibiting axon degeneration. To explore this possibility we over-expressed Nmnat1 in hippocampal neurons. We found that inhibition of axon degeneration in $A\beta_{1-42}$ treated neurons prevented neuronal cell death. Thus, we conclude that axon degeneration is the key component of $A\beta_{1-42}$ induced neuronal degeneration, and therapies targeting axonal protection can be important in finding a treatment for Alzheimer's disease.

Keywords

Amyloid - β ; Nmnat; Axon degeneration; Bcl-xl; Alzheimer's

1. Introduction

The pathological hallmarks of Alzheimer's disease (AD) include the presence of extracellular senile plaques primarily composed of amyloid- β ($A\beta$) peptide, and intracellular neurofibrillary tangles constituted by hyper-phosphorylated aggregates of the microtubule-associated protein, tau (Cavallucci et al. 2012). The $A\beta$ protein fragments (39 to 43 amino acids) which are deposited as amyloid in the brains of patients with Alzheimer's disease are derived from the proteolytic processing/cleavage of Amyloid Precursor Protein (APP) (Shoji et al. 1992). APP is cleaved by β and γ secretase to produce N-terminus $A\beta$ cleavage products in the extracellular spaces (Zhang et al. 2011). $A\beta_{1-42}$ (the most common isoform of $A\beta$) is implicated in the development and progression of AD (Younkin et al. 1998, Lippa et al. 1998). In the trophic factor deprivation paradigm, APP cleavage by Beta-site APP cleavage enzyme (BACE1) activates caspase-6 and caspase-3 pathways in axons and

© 2013 Elsevier Ltd. All rights reserved.

Corresponding author: Bhupinder P. S. Vohra, Biology Department, University of Central Arkansas, Conway, AR 72035 Phone: 501-450-5846 E. Bvohra@uca.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interests Statement The authors declare that there are no conflicts of interests.

neuronal cell body, respectively (Vohra et al. 2010, Nikolaev et al. 2009). The accumulation of A β causes neuronal damage in Alzheimer's disease (O'Brein & Wong 2011, Zhou et al. 2011) and A β also contributes to the formation of intracellular neurofibrillary tangles, which further accelerates the process of neuronal loss and causes the symptoms of dementia (Takata & Kitamura 2012).

Oxidative stress, altered calcium homeostasis, mitochondrial dysfunction, and activation of caspases are implicated in the origination and progression of AD (Cai et al. 2011, Ferreira et al. 2012, Devi and Ohno 2012, Graham 2011). However, depending upon the stimulus, neuronal cell death can either be triggered from the cell body (soma) or axon. Degeneration of neuronal soma and axons is regulated by different sets of executioner molecules (Nikolaev et al. 2009, Vohra et al 2010), but the sequence of these events (what dies first; soma or axon) is not known in AD.

Axonal destruction shares morphological features with apoptosis, but it is a caspase-independent process as manipulation of the mitochondrial apoptotic machinery or caspase inhibitors fail to block axonal degeneration in the case of axotomy and vincristine treatment (Sagot et al., 1995; Finn et al., 2000; Watts et al., 2003; Whitmore et al., 2003). However, in the paradigms of trophic factor deprivation, apoptotic activation in the form of caspase-6 activity indeed takes place in axons (Nikolaev et al. 2009, Vohra et al. 2010). Further, different caspases are compartmentalized in different components of neurons; for example caspase-3 is activated only in neuronal cell body and caspase-6 activation is restricted to the axons. Moreover, inhibition of caspase-6 can protect axons, while caspase-3 inhibition can only block neuronal cell body degeneration (Nikolaev et al. 2009, Vohra et al. 2010). Caspase-6 activation has also been reported in hippocampus and cerebral cortex of mild, moderate, severe and very severe sporadic Alzheimer's disease (Albrecht et al. 2009). Caspases have been directly implicated in the cleavage of amyloid-beta precursor protein during apoptosis, resulting in elevated A β peptide formation (Stone et al. 2002). Caspase-6 deficient neurons are protected against excitotoxicity, nerve growth factor deprivation and myelin-induced axonal degeneration (Uribe et al. 2012). Caspase-6 is also activated in familial AD brains (Albrecht et al 2009), and caspase-6 mediates axon degeneration in the primary human neurons expressing familial Alzheimer disease-associated autosomal dominant mutants (Sivananthan et al. 2010). Thus, caspases appear to be involved in both the processing of APP, and hence A β peptide formation, as well as in neuronal death.

Many members of the Bcl-2 family, including Bcl-xl, are potent inhibitors of programmed cell death and inhibit the activation of caspases in cells (Garcia-Saez AJ 2012). Bcl-xl blocks the opening of the mitochondrial permeability pore, thereby blocking activation of caspases (Sharpe et al. 2004). Bcl-xl also blocks A β -evoked cell death in differentiated PC12 cells (Song et al. 2004). In human primary neurons, A β ₁₋₄₂ treatment down-regulates anti-apoptotic Bcl-2 and upregulates pro-apoptotic Bax (Paradis et al 1996). Thus, we hypothesize that Bcl-xl overexpression might protect against A β ₁₋₄₂-induced degeneration in hippocampal neurons.

Although Bcl-xl can effectively block cell death in a mouse model of motor neuron disease, overexpression of Bcl-xl could only save the neuronal cell body. Surprisingly, overexpression of "slow Wallerian degeneration" (Wlds) could save both axons and cell body and prolonged the life span of these mice (Ferri et al. 2003). Wlds cannot inhibit apoptosis but it can effectively block axon degeneration in various paradigms (Beirowski et al. 2008, Meyer zu Horste et al. 2011). Therefore if the degeneration signal is initiated in the axons, axonal protection can be the key to the neuronal protection. Therefore, we performed experiments to explore whether the initial degeneration signals emanate from the axon or neuronal cell body in A β ₁₋₄₂ toxicity.

Nmnat1 is responsible for the axon protective properties of Wlds fusion protein (Araki et al 2004). Nmnat1 prevents axonal degeneration in various paradigms (Sasaki et al. 2009, Vohra et al. 2010, Yan et al. 2010). The ability of Nmnat1 to protect the axon from various perturbations suggests that Nmnat1 controls the central mechanism of axonal degeneration (Vohra et al 2010). Nmnat1 protects axon degeneration in various kinds of insults, but it cannot thwart cell body death (Vohra et al. 2010). Therefore, we investigated whether Nmnat1 can prevent axonal degeneration caused by A β ₁₋₄₂ and also whether we can prevent neuronal cell death by blocking axonal degeneration in A β -treated neurons. During the course of this study we found that A β -induced neurodegeneration begins in the axons, and by protecting the axon we were able to prevent neuronal death.

2. Material and methods

2.1 Materials

CD1 mice were obtained from Charles River Laboratories (Wilmington, MA), neuronal tubulin specific antibody (Tuj1) and anti-A β antibody 6E10 was purchased from Covance (Madison, WI), Hoechst 33342 and other reagents were procured from Sigma (Saint Louis, MO).

2.2 Culture of hippocampal neurons

Hippocampi were dissected from CD1 (Charles River) mice at postnatal day 0 (P0). Animals were anesthetized on ice and sacrificed by decapitation. Hippocampi were removed and incubated in 40 μ L/ml of papain. Hippocampi were triturated by passing repeatedly through a 1 ml pipette tip and filtered through 40 μ m nylon mesh. Neurons were washed three times in 500 μ l of culture medium consisting of neurobasal medium (Invitrogen) supplemented with B-27, penicillin, streptomycin and L-glutamine. Neurons were plated (5×10^4) in 24-well chambered poly-D lysine and laminin coated culture plates in 500 μ l of culture medium containing anti-mitotic 1 μ M 5-fluoro-2-deoxyuridine and 1 μ M uridine to inhibit proliferation of astrocytes and other dividing cells. Neuronal cultures were incubated at 37°C with 5% CO₂ for the duration of experiments.

2.3 Treatment of neurons with fibrillar and oligomeric A β

Fibrillar A β ₁₋₄₂ and control reverse peptide A β ₄₂₋₁ (American Peptides) were prepared by incubating freshly solubilized peptide at 25 μ M in sterile ammonia (0.5%) at 37 °C for 5 days. (Pike et al 1993). After incubation period A β preparations were centrifuged at room temperature (10 min at 15,000g), and the pellet containing fibrillar A β was resuspended in Hepes buffer (pH 7.5). The formation of fibrillar A β was evaluated by a Thioflavin T (ThT) fluorometric assay (Dahlgren et al 2002). 1 ml of ThT (3mM in 50 mM sodium phosphate buffer, pH 6.0) was added to fibrillar A β preparations and fluorescence was measured at excitation and emission wavelengths of 450 nm and 482 nm. We observed that β -sheet content of fibrillar A β , prepared after incubation of the peptide solution for 5 days at 37° C, were significantly higher in comparison with the fresh A β , used immediately after peptide solubilization (Supplemental Figure 1A).

To generate oligomeric we dissolved A β peptide in cold hexafluoro-2-propanol (HFIP) to a final concentration of 1 mM (Chromy et al 2003). The peptide was aliquoted and dried under vacuum and stored at -20° C. The peptide was resuspended in DMSO then diluted to 100 μ M in Ham's/F12 media and incubated overnight at 4 °C. The preparation was centrifuged (15,000g for 10 min at 4 °C) to remove insoluble aggregates. Supernatant containing soluble oligomers was transferred to clean tubes and stored at 4° C.

Gel electrophoresis and Western blot analyses were performed to determine the presence and purity of oligomers and fibrils used in our system. To confirm that the A β ₁₋₄₂ peptide maintained the appropriate fibrillar or oligomeric conformation throughout the period of treatment, we incubated the peptide in B27 media alone at 37° C for 72 hours. A β ₁₋₄₂ peptide (fibrillar or oligomeric) samples were diluted (1:2) with SDS-PAGE sample buffer (62.5 mM Tris (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol and 0.001% (w/v) bromophenol blue) and were separated by electrophoresis on a 4–16% Tris–Tricine SDS gel. Samples were not boiled to minimize disaggregation prior to electrophoresis. Protein immunoblots were probed using 6E10 mouse monoclonal anti A β antibody (1:1000). In accordance with previous studies (Pike et al 1993, Resende et al 2008, Sondag et al 2009, Itkin et al 2011) the fibrillar peptide did not resolve into the separating gel and remained in the stacking portion of the gel (Supplemental Figure 1B). Oligomeric peptide forms high molecular weight SDS-stable oligomers with incubation (Gong et al 2003). The bulk of the peptide in the oligomeric preparation migrated in the molecular weight range of dimer/trimers (Supplemental Figure 1C).

At 7 days in vitro (DIV), neuronal cultures were treated with 5 μ M of fibrillar or oligomeric A β ₁₋₄₂ for different time periods. Phase-contrast microscopy was performed to monitor neurodegeneration. Neurons were imaged using phase contrast microscopy (Olympus IX71; cell solutions software) with a 20 $\times$ objective lens. Healthy neurons were recognized by their morphology in phase contrast images, axonal segments were considered degenerated if they showed evidence of fragmentation. Images of 6 random field (50-100 cells) per well were typically used. The observer was blind to the condition (n = 5 wells per condition from six experiments).

2.4 Construction of Lentiviral expression Plasmids and Lentiviral infection of hippocampal neurons

cytNmna1-mCherry fusion gene, nuNmna1-mCherry fusion gene and Bcl-xl-Cyan fusion genes were cloned in the lentiviral expression plasmid and lentiviruses expressing transgenes were generated as previously described (Vohra et al 2010). 1 DIV neurons were infected with lentiviruses (10⁵–10⁶ infectious units) expressing Bcl-xl or cytNmna1 or nuNmna1. After infection, cultures were maintained for an additional 6 days. Viral infection and transgene expression were confirmed by fluorescence microscopy of Cyan or mCherry reporter (Supplemental Figure 2 and 3).

2.5 Immunohistochemistry, cell death and tubulin fragmentation analysis

The phase contrast observations were confirmed by Hoechst staining to detect apoptotic neurons and tubulin immunostaining to confirm axon fragmentations. After fixation, cells were washed with PBS and blocked with 4% normal donkey serum in TBST (Tris-buffered saline plus 0.1% Triton X-100) (1 h, 25°C). Cells were incubated in rabbit polyclonal Tuj1 antibody (4°C overnight) and antibody binding was visualized with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (25°C, 1 h). For neuronal cell death, Hoechst 33342 was included in the secondary antibody dilutions. Apoptotic cells were recognized by condensed chromatin, a hallmark of apoptosis. Fluorescence microscopy was performed (Olympus IX71; Cell solutions software). Images of 6 random fields (50-100 cells) per well were typically used to count neurons with condensed chromatin. Axonal segments were considered degenerated if they showed evidence of tubulin fragmentation, n = 5 wells per condition from triplicate experiments (the observer was blinded to the condition).

2.6 Statistical analysis

To detect the importance of time and differences in the effect of oligomeric and fibrillar A β treatments the data were arcsine-transformed to meet the assumptions of parametric testing.

We performed Repeated Measures ANOVA to see the effect of time and two-way ANOVA and Turkey methods for Post-hoc analysis were performed to compare the time dependent effects of fibrillar and oligomeric A β on cell body death and axonal degeneration. For other experiments P values were determined by paired Student's t-test. A p-value < 0.05 was considered significant.

3. Results

3.1 Axonal degeneration precedes somal death in A β -evoked neurotoxicity

Alzheimer's disease, like other neurodegenerative diseases is characterized by neuronal cell death. Whether neuronal cell death is triggered from the soma or axon in A β -induced degeneration is not known. Our quantitative time course analyses revealed that in both fibrillar A β and oligomeric A β induced neurodegeneration axonal degeneration precedes somal death. These data also revealed that at any of the observed time point oligomeric A β was more toxic than fibrillar A β (Figure 1) Twelve hours after fibrillar A β_{1-42} treatment, 22% of the axons were degenerated; however, only 8% of neurons exhibited cell death. This trend continued until 48 h, when we found that 66% of the neurons exhibited the morphological features of neuronal death whereas 86% of axons were degenerated (Figure 1). However, after 60 h and 72 h of A β_{1-42} treatment we observed complete degeneration of both the axons and neuronal somata (Figure 1). In the case of oligomeric A β , by 12 hours of A β treatment 52% of the axons were degenerated and 26% of neurons exhibited cell death. Similarly after 24h of oligomeric A β treatment we observed that 82% axon were degenerated and cell death was observed in 54% neurons. However by 48 h of oligomeric A β treatment we observed complete degeneration of both the axons and neuronal somata. Thus axonal degeneration precedes cell-body death in A β toxicity, suggesting that the degeneration signal might initially begin in the axons.

3.2 Bcl-xL blocks neurodegeneration in mouse hippocampal neurons

Although axon degeneration is largely considered as an apoptosis-independent process, in conditions such as trophic factor depletion, axonal transport blockade and in familial AD, apoptotic pathways in the form of caspase-6 activation do play a pivotal role in axon demise (Nikolaev et al. 2009, Albrecht et al 2009, Vohra et al. 2010). Bcl-xL can block degeneration of either cell soma, or in some cases, both axon and cell soma (Vohra 2010, Sharpe 2004, Simonian 1997, Schott 1995, Song 2004). To investigate whether over-expression of Bcl-xL can protect against degeneration of either axons or somata in A β -induced neurodegeneration, we infected 1 DIV hippocampal neurons with lentiviruses expressing Bcl-xL. Bcl-xL expression was verified by detection of a cyan reporter fusion domain (Supplemental Figure 1). Bcl-xL produced significant protection of neuronal somata against A β_{1-42} toxicity (Figure 2). Quantitative analysis revealed that the fibrillar A β_{1-42} treatment induced chromatin condensation in 66% of the neurons. However, in the Bcl-xL over-expressing group, apoptotic nuclei were observed only in 16% of neurons. Bcl-xL over-expression also inhibited axon degeneration, as fibrillar A β_{1-42} treatment degenerated 86% of axons, but in Bcl-xL overexpressing neurons degeneration was limited to only 10% of axons (Figures 3). Similarly, Bcl-xL overexpression also saved both axon and cell body in the oligomeric A β_{1-42} treated neurons (Figure 6). Therefore, overexpression of Bcl-xL protects both somata and axons in A β -treated neurons.

3.3 Nmnat1 blocks A β -induced axonal degeneration in mouse hippocampal neurons

Although our results indicate that axonal degeneration is a major component of A β toxicity, and Bcl-xL over-expression protects both axonal and somal death (Figure 3 and 4), these data did not provide us with a specific answer about the site of initiation of neurodegeneration in A β -treated neurons. Nmnat1 is known to protect against axon degeneration in various kinds

of insults, but it cannot protect the soma (Vohra et al. 2010). To investigate whether neuronal death signal originates from the axon or soma in A β -treated neurons, we infected hippocampal neurons (1 DIV) with lentiviruses expressing nuNmnat1 or cytNmnat1 to block axonal degeneration. nuNmnat1 is predominantly expressed in the nucleus; cytNmnat1, which is an engineered mutant of Nmnat1, is localized only to the cytoplasm and axon, thereby providing more potent axonal protection than that afforded by Wld(s) or Nmnat1 (Sasaki et al 2009). Expression of these two forms of Nmnat1 proteins was verified by detection of cherry reporter fusion domain in the nucleus for nuNmnat1 and in the cytoplasm and axons for cytNmnat1 (Supplemental Figure 2). We observed that over-expression of either cytNmnat1 or nuNmnat1 blocked the neuronal degeneration caused by A β ₁₋₄₂ (Figure 4). We found that expression of nuNmnat1 and cytNmnat1 restricted axon degeneration to only 17% and 8%, respectively. Similarly, nuNmnat1 and cytNmnat1 overexpression limited the neuronal soma death to 26% and 23%, respectively (Figure 5). We also observed that cytNmnat1 was effective in protecting both axons and cell somata in oligomeric A β -treated neurons (Figure 6). Thus, axonal protection offered by Nmnat1 also inhibited apoptotic processes in the neuronal soma in A β -treated neurons.

4. Discussion

A β has been widely studied and implicated in the cause and progression of the neurodegeneration observed in Alzheimer's disease (Younkin et al. 1998, Lippa et al. 1998). Although it may be debated whether the signal for neurodegeneration emanates from the soma or axon, axon degeneration is considered to be the hallmark of many neurological disorders. Alzheimer's disease also involves both axonal and somal components, but the stepwise degenerative mechanisms are obscure. Depending upon the stimulus, death signals may originate from the neuronal soma or axon and the efforts to prevent neurodegeneration must be specific for each component. We therefore sought to understand how much of each component (axon and soma) is involved in the process of neuronal death induced by A β ₁₋₄₂.

As expected, treatment with A β caused degeneration in mice hippocampal neurons. We also observed that oligomeric A β is more toxic than fibrillar A β and these observations are in accordance with the previous findings (Dahlgren et al 2002, Chafekar et al 2007, Resende et al 2008, Tizon et al 2010, Kitiyanant et al. 2012,). However, we observed that axonal degeneration predominates in both fibrillar and oligomeric A β treated neurons, thereby suggesting that the degeneration signal may emanate from the axons. We also discovered that neurons expressing either Nmnat1 or Bcl-xl are protected from both axonal and somal degeneration. Although Bcl-xl is known to protect only the soma in many different paradigms, our results indicate that over-expression of Bcl-xl can protect both soma and axons after A β treatment. Activation of caspase-6 is documented in familial AD brains (Albrecht et al 2009) and caspase-6 also causes axon degeneration in the primary human neurons expressing familial Alzheimer disease (AD)-associated autosomal dominant mutants (Sivananthan et al. 2010). The caspase-6 deficient neurons are protected against excitotoxicity, nerve growth factor deprivation and myelin-induced axonal degeneration (Uribe et al. 2012, Vohra et al. 2012). Bcl-xl mediated axonal protection in the present study might be attributed to the potent antiapoptotic activity of Bcl-xl because Bcl-xl blocks the opening of the mitochondrial permeability pore, and inhibits the activation of caspases (Sharpe et al. 2004). This is consistent with our previous studies in the paradigms of neurotrophin withdrawal and axon transport blockade, where we have shown that Bcl-xl overexpression inhibits activation of axonal caspase-6. Bcl-xl overexpression, however, also blocked the somal degeneration in the A β -treated neurons. This can be attributed to two possibilities. First, as in the case of neurotrophin withdrawal and axon transport blockade, A β treatment activates simultaneous apoptotic pathways in the soma (activation of caspase-3) and axons (activation of caspase-6) and Bcl-xl can block caspase activation in

both axons and somata. Second, A β triggers a step-wise degeneration in which an initial activation of caspase-6 takes place in the axon, and that triggers apoptosis in the soma; Bcl-xl would prevent somal degeneration by blocking the first step. To test the latter possibility, we over-expressed Nmnat1, which is known to prevent axon degeneration in many paradigms. In the case of neurotrophin withdrawal, where simultaneous activation of caspases take place in the neurons (caspase-3 in cell body and caspase-6 in axon), Nmnat saves only axons (Vohra et al 2010). Thus, if A β stimulates death of the soma independent of the axon, Nmnat1 overexpression should protect the axons only, and the somata should degenerate. We found that over-expression of Nmnat1 protects both axons and somata from A β -induced neurodegeneration. This suggests that the degeneration signal originates first in the axon and then propagates to the soma. These results are similar to the studies in progressive motor neuronopathy (pmn) showing that blocking axon degeneration, is sufficient to block death of the soma (Ferri 2003). Therefore, we argue that Bcl-xl blocks neurodegeneration in A β toxicity by protecting the axons first.

We conclude that the degeneration of axons initiates the onset and progression of neuronal death in AD. This suggests that therapeutic strategies intended to prevent disease progression in AD will benefit from placing more emphasis on the distinct mechanisms of axonal degeneration. Axonal degeneration is also reported as a key feature in Parkinson's disease, where axons and neuronal soma degenerate by distinct mechanisms (Burke et al 2012). Interestingly, it has been speculated that prevention of axonal degeneration may prevent somal demise in the clinical progression of Parkinson's disease (Cheng et al. 2010). Thus, we conclude that similar to Parkinson's disease, axon degeneration is the key step in progression of neuronal death observed in AD. Finally, future therapeutic interventions may benefit from focusing on the axonal degeneration in AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge Dr. Jeff Milbrandt for sharing various viral constructs used in the present study. We acknowledge the Dr. Steven Barger for thoughtful comments about this manuscript. We also acknowledge Dr. Reid Adams for his help in statistical analysis of our data. This publication was made possible by the Arkansas INBRE program, supported by grant funding from the National Institutes of Health (NIH) National Institute of General Medical Sciences (NIGMS) (P20 GM103429) (formerly P20RR016460). This work was also supported by the University of Central Arkansas, University Research council grant to BPSV as well as undergraduate research grants from the University of Central Arkansas College of Natural Science and Mathematics and Biology department to WA and WX.

REFERENCES

- Albrecht S, Bogdanovic N, Ghetti B, Winblad B, LeBlanc AC. Caspase-6 activation in familial Alzheimer disease brains carrying amyloid precursor protein or presenilin I or presenilin II mutations. *J Neuropathol Exp Neurol*. 2009; 68:1282–93. [PubMed: 19915487]
- Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science*. 2004; 305:1010–3. [PubMed: 15310905]
- Beirowski B, Babetto E, Coleman MP, Martin KR. The Wlds gene delays axonal but not somatic degeneration in a rat glaucoma model. *Eur J Neurosci*. 2008; 28:1166–79. [PubMed: 18783366]
- Burke RE, O'Malley K. Axon degeneration in Parkinson's disease. *Exp Neurol*. 2012 PMID: 22285449.
- Cai Z, Zhao B, Ratka A. Oxidative stress and β -amyloid protein in Alzheimer's disease. *Neuromolecular Med*. 2011; 13:223–50. [PubMed: 21901428]

- Cavallucci V, D'Amelio M, Cecconi F. A β Toxicity in Alzheimer's Disease. *Mol Neurobiology*. 2012; 45:366–378.
- Cheng HC, Ulane CM, Burke RE. Clinical progression in Parkinson disease and the neurobiology of axons. *Ann Neurol*. 2010; 67:715–25. [PubMed: 20517933]
- Chafekar SM, Hoozemans JJ, Zwart R, Baas F, Scheper W. Abeta 1-42 induces mild endoplasmic reticulum stress in an aggregation state-dependent manner. *Antioxid Redox Signal*. 2007; 9:2245–54. [PubMed: 17979527]
- Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, Jones BW, Fernandez SJ, Lacor PN, Horowitz P, Finch CE, Krafft GA, Klein WL. Self-assembly of Abeta(1–42) into globular neurotoxins. *Biochemistry*. 2003; 42:12749–12760. [PubMed: 14596589]
- Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem*. 2002; 277:32046–32053. [PubMed: 12058030]
- Devi L, Ohno M. Mitochondrial dysfunction and accumulation of the β -secretase-cleaved C-terminal fragment of APP in Alzheimer's disease transgenic mice. *Neurobiol Dis*. 2012; 45:417–24. [PubMed: 21933711]
- Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC. Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. *J Neurosci*. 2000; 4:1333–1341. [PubMed: 10662823]
- Ferreira IL, Bajouco LM, Mota SI, Auberson YP, Oliveira CR, Rego AC. Amyloid beta peptide 1-42 disturbs intracellular calcium homeostasis through activation of GluN2B-containing N-methyl-D-aspartate receptors in cortical cultures. *Cell Calcium*. 2012; 51:95–106. [PubMed: 22177709]
- Ferri A, Sanes JR, Coleman MP, Cunningham JM, Kato AC. Inhibiting axon degeneration and synapse loss attenuates apoptosis and disease progression in a mouse model of motoneuron disease. *Curr Biol*. 2003; 13:669–73. [PubMed: 12699624]
- Garcia-Saez AJ. The secrets of the Bcl-2 family. *Cell Death Differ*. 2012; 11:1733–40. [PubMed: 22935609]
- Graham RK, Ehrnhoefer DE, Hayden MR. Caspase-6 and neurodegeneration. *Trends Neurosci*. 2011; 34:646–56. [PubMed: 22018804]
- Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA, Klein WL. Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc Natl Acad Sci USA*. 2003; 100:10417–10422. [PubMed: 12925731]
- Itkin A, Dupres V, Dufrêne YF, Bechinger B, Ruyschaert JM, Raussens V. Calcium ions promote formation of amyloid β -peptide (1-40) oligomers causally implicated in neuronal toxicity of Alzheimer's disease. *PLoS One*. 2011; 6:18250–59.
- Kitiyanant N, Kitiyanant Y, Svendsen CN, Thangnipon W. BDNF-, IGF-1- and GDNF-secreting human neural progenitor cells rescue amyloid β -induced toxicity in cultured rat septal neurons. *Neurochem Res*. 2012; 37:143–52. [PubMed: 21909955]
- Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci*. 1993; 13:1676–87. [PubMed: 8463843]
- Resende R, Ferreira E, Pereira C, Resende de Oliveira C. Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. *Neuroscience*. 2008; 155:725–37. [PubMed: 18621106]
- Lippa CF, Nee LE, Mori H, St George-Hyslop P. Abeta-42 deposition precedes other changes in PS-1 Alzheimer's disease. *Lancet*. 1998; 352:1117–8. [PubMed: 9798591]
- Meyer, zu Horste G.; Miesbach, TA.; Muller, JI.; Fledrich, R.; Stassart, RM.; Kieseier, BC.; Coleman, MP.; Sereda, MW. The Wlds transgene reduces axon loss in a Charcot-Marie-Tooth disease 1A rat model and nicotinamide delays post-traumatic axonal degeneration. *Neurobiol Dis*. 2011; 42:1–8. [PubMed: 21168501]
- Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature*. 2009; 457:981–9. [PubMed: 19225519]

- O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci.* 2011; 34:185–204. [PubMed: 21456963]
- Ronicke R, Klemm A, Meinhardt J, Schröder UH, Fändrich M, Reymann KG. Abeta mediated diminution of MTT reduction--an artefact of single cell culture? *PLoS One.* 2008; 3:e3236–41. [PubMed: 18800168]
- Roth KA. Caspases, apoptosis, and Alzheimer disease: causation, correlation, and confusion. *J Neuropathol Exp Neurol.* 2001; 60:829–38. [PubMed: 11556539]
- Sagot Y, Dubois-Dauphin M, Tan SA, de Bilbao F, Aebischer P, Martinou JC, Kato AC. Bcl-2 overexpression prevents motoneuron cell body loss but not axonal degeneration in a mouse model of a neurodegenerative disease. *J Neurosci.* 1995; 11:7727–7733. [PubMed: 7472523]
- Sasaki Y, Vohra BP, Baloh RH, Milbrandt J. Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. *J Neurosci.* 2009; 29:6526–34. [PubMed: 19458223]
- Sasaki Y, Vohra BP, Lund FE, Milbrandt J. Nicotinamide mononucleotide adenyltransferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. *J Neurosci.* 2009a; 29:5525–5535. [PubMed: 19403820]
- Schott AF, Apel IJ, Nuñez G, Clarke MF. Bcl-XL protects cancer cells from p53-mediated apoptosis. *Oncogene.* 1995; 11:1389–94. [PubMed: 7478561]
- Sharpe JC, Arnoult D, Youle RJ. Control of mitochondrial permeability by Bcl-2 family members. *Biochim Biophys Acta.* 2004; 1644:107–13. [PubMed: 14996495]
- Shoji M, Golde TE, Ghiso J, Cheung TT, Estus S, Shaffer LM, Cai XD, McKay DM, Tintner R, Frangione B, et al. Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science.* 1992; 258:126–9. [PubMed: 1439760]
- Simonian PL, Grillot DA, Nuñez G. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. *Blood.* 1997; 90:1208–16. [PubMed: 9242554]
- Song YS, Park HJ, Kim SY, Lee SH, Yoo HS, Lee HS, Lee MK, Oh KW, Kang SK, Lee SE, Hong JT. Protective role of Bcl-2 on beta-amyloid-induced cell death of differentiated PC12 cells: reduction of NF-kappaB and p38 MAP kinase activation. *Neurosci Res.* 2004; 49:69–80. [PubMed: 15099705]
- Sivananthan SN, Lee AW, Goodyer CG, LeBlanc AC. Familial amyloid precursor protein mutants cause caspase-6-dependent but amyloid β -peptide-independent neuronal degeneration in primary human neuron cultures. *Cell Death Dis.* 2010; 1:100.
- Sondag CM, Dhawan G, Combs CK. Beta amyloid oligomers and fibrils stimulate differential activation of primary microglia. *Journal of Neuroinflammation.* 2009; 6:1–13. [PubMed: 19123954]
- Stone JR, Okonkwo DO, Singleton RH, Mutlu LK, Helm GA, Povlishock JT. Caspase-3-mediated cleavage of amyloid precursor protein and formation of amyloid Beta peptide in traumatic axonal injury. *J Neurotrauma.* 2002; 19:601–14. [PubMed: 12042095]
- Takata K, Kitamura Y. Molecular approaches to the treatment, prophylaxis, and diagnosis of Alzheimer's disease: tangle formation, amyloid- β , and microglia in Alzheimer's disease. *J Pharmacol Sci.* 2012; 118:331–7. [PubMed: 22382659]
- Tizon B, Ribe EM, Mi W, Troy CM, Levy E. Cystatin C protects neuronal cells from amyloid-beta-induced toxicity. *J Alzheimers Dis.* 2010; 19:885–94. [PubMed: 20157244]
- Uribe V, Wong BK, Graham RK, Cusack CL, Skotte NH, Pouladi MA, Xie Y, Feinberg K, Ou Y, Ouyang Y, Deng Y, Franciosi S, Bissada N, Spreew A, Zhang W, Ehrnhoefer DE, Vaid K, Miller FD, Deshmukh M, Howland D, Hayden MR. Rescue from excitotoxicity and axonal degeneration accompanied by age-dependent behavioral and neuroanatomical alterations in caspase-6-deficient mice. *Hum Mol Genet.* 2012; 21:1954–67. [PubMed: 22262731]
- Vohra BP, Sasaki Y, Miller BR, Chang J, DiAntonio A, Milbrandt J. Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenyltransferase 1-sensitive pathway. *J Neurosci.* 2010; 30:13729–38. [PubMed: 20943913]
- Watts RJ, Hoopfer ED, Luo L. Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron.* 2003; 6:871–885. [PubMed: 12818174]

- Whitmore AV, Lindsten T, Raff MC, Thompson CB. The proapoptotic proteins Bax and Bak are not involved in Wallerian degeneration. *Cell Death and Differentiation*. 2003; 10:260–261. [PubMed: 12700655]
- Yan T, Feng Y, Zheng J, Ge X, Zhang Y, Wu D, Zhao J, Zhai Q. Nmnat2 delays axon degeneration in superior cervical ganglia dependent on its NAD synthesis activity. *Neurochem Int*. 2010; 56:101–6. [PubMed: 19778564]
- Younkin SG. The role of A-beta 42 in Alzheimer's disease. *J Physiol Paris*. 1998; 92:289–92. [PubMed: 9789825]
- Zhai RG, Zhang F, Hiesinger PR, Cao Y, Haueter CM, Bellen HJ. NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration. *Nature*. 2008; 452:887–91. [PubMed: 18344983]
- Zhang YW, Thompson R, Zhang H, Xu H. APP processing in Alzheimer's disease. *Mol Brain*. 2011; 4:3. [PubMed: 21214928]
- Zhou ZD, Chan CH, Ma QH, Xu XH, Xiao ZC, Tan EK. The roles of amyloid precursor protein (APP) in neurogenesis: Implications to pathogenesis and therapy of Alzheimer disease. *Cell Adh Migr*. Jul-Aug;2011 5(4):280–92. 2011. [PubMed: 21785276]

Highlights

Axon degeneration is key component of neuronal death in amyloid- β toxicity

We investigated the origin of the degeneration signal in amyloid β ($A\beta$) induced neuronal cell death in cultured *in vitro* hippocampal neurons.

1. $A\beta_{1-42}$ toxicity-induced axon degeneration precedes cell death in hippocampal neurons.
2. Overexpression of Bcl-xl inhibited both axonal and cell body degeneration in the $A\beta$ -42 treated neurons.
3. We found that inhibition of axon degeneration in $A\beta_{1-42}$ treated neurons prevented neuronal cell death.

Conclusion

Axon degeneration is the key component of $A\beta_{1-42}$ induced neuronal degeneration.

Figure 1. Axonal degeneration precedes somal death in Aβ₁₋₄₂ toxicity

Hippocampal neurons (7 DIV) were treated with 5 μM fibrillar Aβ₁₋₄₂ or oligomeric Aβ₁₋₄₂ or control reverse peptide Aβ₄₂₋₁, and phase-contrast microscopy was performed to analyze somal death and axonal degeneration. Quantitative analysis revealed that the Aβ₁₋₄₂ oligomers were more neurotoxic than fibrillar Aβ₁₋₄₂ and axon degenerated at a significantly earlier time point than the soma death. The data were arcsine-transformed to meet the assumptions of parametric testing and Repeated Measures ANOVA was performed to see the effect of time ($F=5850.9475$, $p<0.0001$) and two-way ANOVA and Turkey methods for Post-hoc analysis were performed to compare the time dependent effects of fibrillar and oligomeric Aβ on cell body death and axonal degeneration. * indicates statistically different from cell body degeneration in each treatment group, # indicate significant different from fibrillar Aβ₁₋₄₂ treatment groups. ($p < 0.0001$).

Ab 42

Figure 2. Bcl-xl overexpression prevents Aβ-induced neuronal degeneration

Neurons (7 DIV) expressing Bcl-xl or empty vector were treated with 5 μ M A β_{1-42} for 48 h. Representative phase-contrast images revealed that Bcl-xl overexpression prevented axonal degeneration caused by A β_{1-42} (B).

Figure 3. Bcl-xL over-expression prevents both axonal degeneration and cell death induced by fibrillar Aβ

Neurons (7 DIV) were treated with 5 μM fibrillar Aβ₁₋₄₂ (A, B and C), and fluorescence microscopy was performed to visualize Hoechst 33342 staining to score dead neurons [condensed chromatin (arrows) and surviving neurons (arrow heads)]. Axonal degeneration was measured via neuron-specific tubulin fragmentation. Bcl-xL overexpression blocked both axonal degeneration and somal death (arrowheads) in Aβ-induced neurodegeneration (D, E and F). Quantitative analysis showed significant axonal protection (G) and significant cell death protection (H) in Bcl-xL overexpressing neurons. * indicates statistically different from control (reverse peptide, Aβ₄₂₋₁), # indicates statistically different from Aβ₄₂₋₁-treated neurons.

Figure 4. Nmnat1 over-expression blocks fibrillar $A\beta_{1-42}$ -induced axon degeneration
nuNmnat or cytNmnat expressing hippocampal neurons were treated with 5 μ M $A\beta_{1-42}$. Phase-contrast microscopy was performed to visualize the protective effects of Nmnat1 on $A\beta$ -treated neurons. Representative images of phase contrast microscopy revealed that fibrillar $A\beta_{1-42}$ caused extensive neuronal degeneration (A). nuNmnat overexpression (B) or cytNmnat overexpression (C) each prevented neuronal degeneration caused by $A\beta_{1-42}$.

Figure 5. Nmnat1 over-expression prevents both axon degeneration and cell death induced by fibrillar Aβ₁₋₄₂ toxicity

Hippocampal neurons were infected at 1 DIV with lentiviruses expressing nuNmnat (A, B and C) and cytNmnat (D, E and F). Six days after infection, neurons were treated with 5 μM fibrillar Aβ₁₋₄₂. After an additional 48 h, neurons were fixed, and immunofluorescence analysis of Tuj1 and Hoechst 33342 was performed. Representative images of Tuj1 (A), Hoechst 33342 (B) and C (merged image of A and C) of neurons over-expressing nuNmnat1 show that both the axon and cell body were protected in these neurons. Representative images of Tuj1 (D), Hoechst 33342 (E) and F (merged image of D and E) of neurons over-expressing cytNmnat1 show that both the axon and cell body were protected in these neurons as well. Quantitative analysis of axon integrity (G) and somal survival (H) reveals that Nmnat blocked both axonal degeneration and somal death in fibrillar Aβ-treated neurons. * indicates statistically different from control (reverse peptide, Aβ₄₂₋₁), # indicates statistically different from Aβ₄₂₋₁ treated neurons.

Figure 6. Bcl-x1 and cytNmnat1 over-expression prevents both axon degeneration and cell death induced by oligomeric A β ₁₋₄₂ toxicity

Hippocampal neurons were infected at 1 DIV with control lentiviruses (A, B and C) and lentiviruses expressing Bcl-x1 (D, F and F) or cytNmnat (G, H and I). Six days after infection, neurons were treated with 5 μ M oligomeric A β ₁₋₄₂. After an additional 24 h, neurons were fixed, and immunofluorescence analysis of Tuj1 and Hoechst 33342 was performed. Representative images of Tuj1 (D), Hoechst 33342 (E) and F (merged image of D and E) of neurons over-expressing Bcl-x1 show that both the axon and cell body were protected in these neurons. Representative images of Tuj1 (G), Hoechst 33342 (H) and I (merged image of G and H) of neurons over-expressing cytNmnat1 show that both the axon and cell body were protected in these neurons as well. Quantitative analysis of axon integrity (J) and somal survival (K) reveals that Bcl-x1 and Nmnat blocked both axonal degeneration and somal death in oligomeric A β -treated neurons. * indicates statistically different from control (reverse peptide, A β ₄₂₋₁), # indicates statistically different from A β ₄₂₋₁ treated neurons.