Immediate Axonal Retrograde Signaling in Amyloid-Dependent Neurodegeneration

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ABSTRACT

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The following dissertation herein discusses the role of axonal protein synthesis in $A\beta_{1-42}$-dependent neurodegeneration, which has important implications in AD pathogenesis. In Part 1, I provide a brief introduction to relevant topics including neurodegeneration and axonal protein synthesis. In Part 2, I discuss findings that we published in 2014 describing a mechanism by which axonal exposure to $A\beta_{1-42}$ induces cell death via axonal synthesis and retrograde transport of a transcription factor, ATF4. In Part 3, I discuss a follow-up project that I conducted independently, which is not yet published but is in preparation for submission describing the immediate effect of $A\beta_{1-42}$ on axonal protein synthesis, which mediates the downstream axonal ATF4 signaling events described in Part 2. In Part 4, I discuss the key findings from these two projects including their significance and potential future directions. In the Appendix, I provide details regarding experimental methods and statistical analyses performed in Part 3.

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List of Abbreviations and Acronyms

4EBP1	Eukaryotic Translation Initiation Factor 4E-Binding Protein 1
$A\beta_{1-42}$	β-amyloid (1-42)
AD	Alzheimer's Disease
AHA	L-azidohomoalanine
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
ATF4	Activating Transcription Factor 4
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
CHOP	C/EBP Homologous Protein
CNS	Central Nervous System
DRG	Dorsal Root Ganglion
eIF2α	Eukaryotic Translation Initiation Factor 2A
eIF4	Eukaryotic Translation Initiation Factor 4
EN1 / EN2	Engrailed 1 / Engrailed 2
EOAD	Early-Onset Alzheimer's Disease
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated kinase
FAD	Familial Alzheimer's Disease
FISH	Fluorescent *In Situ* Hybridization
FMRP	Fragile X Mental Retardation Protein
HD	Huntington's Disease

ICC	Immunocytochemistry
LOAD	Late-Onset Alzheimer’s Disease
MCI	Mild Cognitive Impairment
mRNA	Messenger Ribonucleic Acid
mTOR	Mechanistic Target of Rapamycin
NGF	Nerve Growth Factor
NLS	Nuclear Localization Sequence
NT-3	Neurotrophin-3
PD	Parkinson’s Disease
PDC-E1	Pyruvate Dehydrogenase Complex E1
PDK1	Pyruvate Dehydrogenase Kinase 1
PI3K	Phosphoinositide 3-Kinase
PNS	Peripheral Nervous System
PS1 / PS2	Presenilin-1 / Presenilin-2
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RANBP1	Ran (Ras-Related Nuclear Protein)-Binding Protein 1
RanGTP	GTP (Guanosine-5'-Triphosphate)-Bound Ran
RBP	RNA-Binding Protein
RGC	Retinal Ganglion Cell
RNP	Ribonucleoprotein
rRNA	Ribosomal Ribonucleic Acid
S6	Ribosomal Protein S6
S6K	Ribosomal Protein S6 Kinase

SEMA3A	Semaphorin 3A
siRNA	Small/Short Interfering Ribonucleic Acid
STAT3	Signal Transducer and Activator of Transcription 3
TDP-43	TAR DNA-Binding Protein 43
TGN	Trans-Golgi-Network
tRNA	Transfer Ribonucleic Acid
UTR	Untranslated Region
ZBP1	Zipcode Binding Protein 1

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Dedication

In memory of my grandfather, Holland P. Walker

Part 1

Chapter 1. Introduction

1.1. Neurodegeneration

1.1.1. Alzheimer's Disease

Neurodegeneration is a pathological condition that primarily affects neurons and usually results in the functional loss of neurons. There are hundreds of different neurodegenerative disorders, the most common of which are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). This large group of disorders involves the loss of synapses, connections, and neurons; however, the clinical and pathological presentations of each disease are rather heterogeneous. The affected neuronal subtypes and thereby, the associated symptoms vary greatly between neurodegenerative diseases. The chances of developing a neurodegenerative disorder, particularly AD and PD, increase with aging, which is considered to be the most consistent risk factor for development of a neurodegenerative disorder [1-3]. As the growth rate of the population aged 65 and above in first-world countries has exceeded that of the population as a whole, it can be anticipated that the number of people developing neurodegenerative diseases will significantly increase in the coming decades. Although there are drugs that are used to alleviate symptoms to an extent in patients, there are still yet to be any successful therapeutic interventions that can prevent the development or slow the progression of neurodegeneration.

The most common neurodegenerative disease is AD, which will affect approximately 5.3 million Americans aged 65 or older in 2017 [4]. With the aging of the “baby boom” generation, this number is projected to nearly triple by 2050 unless preventative measures are developed. This will be not only be a difficult burden to bear for family members and caregivers of those affected, but it will also be financially burdensome to taxpayers as the total monetary cost of dementia in 2010 was between $157 billion and $215 billion, $11 billion of which was paid by Medicare [5].

AD falls within the category of dementias and is the most common form of dementia [6]. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-V, the core feature of dementia is acquired cognitive decline in one or more domains, including complex attention, executive function, learning and memory, language, perceptual motor or social cognition. AD is specifically characterized by three primary groups of symptoms [7]. The first group encompasses memory loss, difficulties with language, and executive dysfunction. Memory loss is the presenting symptom in most people who develop AD. The second group includes psychiatric symptoms and behavioral disturbances, such as depression, agitation or delusions, which have collectively been termed non-cognitive symptoms [8]. The third group comprises difficulties that affect one’s ability to perform daily activities, such as driving, dressing or eating.

There are two categories of AD: early-onset AD (EOAD) and late-onset AD (LOAD). LOAD accounts for the majority of AD cases whereas a much smaller proportion of cases have EOAD [9]. Though the symptoms are similar

between LOAD and EOAD, patients with EOAD develop the disease earlier in life and are considered to have a more aggressive disease progression and a shorter survival time. Additionally, neuropathological changes found in EOAD brains appear to be more severe with prominent synaptic and neuronal loss [10]. Interestingly, patients with EOAD are much more likely to have family members with dementia as compared to LOAD patients, suggesting an underlying genetic predisposition to EOAD [11]. Indeed, population studies into the genetics of patients with EOAD show that approximately 13% of EOAD patients have a genetic predisposition to the disease [12]. This inherited form of AD has been termed familial AD (FAD). The majority of FAD patients have mutations in the genes encoding the β-amyloid precursor protein (APP) or presenilins (PS1 and PS2) [12-15].

There are distinct structural changes and pathological features that are consistently found in brains of AD patients. Severe brain atrophy is present in AD brains, particularly in the hippocampus, due to neuron loss [16]. Extracellular senile plaques and intraneuronal neurofibrillary tangles are also present in AD brains [17, 18]. Plaques are primarily composed of insoluble deposits of beta-amyloid (Aβ), and tangles are aggregates of predominantly hyperphosphorylated tau proteins. Interestingly, plaques, but not tangles, associate with the earliest symptoms of AD, suggesting that Aβ plays a pathogenic role in the development of AD [19]. This hypothesis is further supported by the fact that most FAD patients have mutations in genes encoding proteins that play crucial roles in Aβ production including APP, PS1 and PS2 [12-15].

1.1.2. APP and the Amyloid Cascade Hypothesis

APP is a type-I transmembrane protein that belongs to a family including APP-like protein 1 (APLP1) and 2 (APLP2), which are all processed similarly [20, 21]. APP is synthesized in the endoplasmic reticulum (ER) and is then transported to the Golgi apparatus and to the trans-Golgi-network (TGN) [22]. The highest concentration of neuronal APP is found within the TGN. Once in the TGN, APP is transported in secretory vesicles to the cell surface and can further be re-internalized via an endosomal/lysosomal degradation pathway [23-25]. APP is subject to cleavage by secretases when at the cell surface and within endosomes.

Two primary secretases cleave APP: α-secretase and β-secretase. When on the cell surface, APP can be cleaved by the membrane-bound α-protease, which generates the soluble molecule, sAPPα [23]. This APP ectodomain plays an important role in neuronal plasticity and survival and also regulates neural stem cell proliferation and CNS development [26-29]. Alternatively, cleavage of APP by β-secretase is the first step in Aβ generation with BACE1 being the primary β-secretase [30-32]. BACE achieves optimal activity at a low pH, and it is largely localized to endosomes and the TGN, which are acidic intracellular compartments [33, 34]. Cleavage of APP by BACE generates a molecule containing the C-terminal fragment of APP, termed βCTF. This βCTF molecule is further cleaved by γ-secretase to generate Aβ [35]. Cleavage of βCTF by γ-secretase can either generate $A\beta_{1-40}$, the majority species, or $A\beta_{1-42}$, the amyloidogenic species. $A\beta_{1-42}$ is a hydrophobic molecule, and therefore is prone

to forming oligomers and fibrils, which eventually accumulate to form plaques that are characteristically found in AD brains [36]. γ-secretase is a high molecular weight complex that consists of four protein components, including PS1 or PS2, which are mutated in the majority of patients with FAD [12]. Overproduction of $A\beta_{1-42}$ is toxic to neurons and induces a neurodegenerative cascade, which leads to axonal dysfunction, intraneuronal fibrillary tangles and neuron loss [37-39]. $A\beta_{1-42}$ is present at much higher levels in senile plaques in AD brains as compared to $A\beta_{1-40}$, providing evidence that the deposition of Aβ into plaques is initially instigated by $A\beta_{1-42}$ [40]. This theory is underscored by the fact that $A\beta_{1-42}$ oligomers, which form due to the hydrophobic nature of the $A\beta_{1-42}$ peptide, are the most neurotoxic form of Aβ [41]. Together, these lines of evidence have led to the amyloid cascade hypothesis being the overarching hypothesis for the pathogenesis of AD, which proposes that Aβ is the causative agent of AD pathology and that axonal dysfunction, neurofibrillary tangles, neuron loss and dementia are all direct results of Aβ deposition [42, 43].

There are many ways in which Aβ deposition can occur within the brain. I have already discussed how many patients with FAD have inherited mutations in genes known to be involved in APP processing, which likely result in increased Aβ formation and deposition. However, this only accounts for a small percentage of patients with AD. Therefore, if the amyloid cascade hypothesis is indeed correct, there must be other factors that result in Aβ overproduction.

Studies from the fields of head trauma and neuronal injury have provided some insight into this possibility. Kainic acid-induced brain damage leads to rapid

increases in APP expression *in vivo*, and this is also the case in reactive astrocytes surrounding lesioned rat hippocampal neurons [44, 45]. Additionally, Aβ deposition is present in human cortices within days of head injury [46]. Consistent with these findings, repetitive traumatic brain injury (TBI) in mice results in axonal damage and numerous APP-immunoreactive axons [47]. Interestingly, head trauma is a known risk factor for developing AD [48]. Studies from patients with dementia pugilistica (DP), a form of chronic traumatic encephalopathy (CTE) often found in boxers and other athletes that experience head trauma, demonstrate that DP brains exhibit neurofibrillary tangles that are also positive for Aβ [49, 50]. Moreover, both moderate and severe head injury in early adulthood is associated with an increased risk of developing AD and other dementias [51]. Together, these studies reveal a role for APP and Aβ overproduction in reacting to neuronal injury and head trauma, an established risk factor for AD and dementia, further supporting the amyloid cascade hypothesis.

Though it is clear that APP plays a crucial, likely causative role in the pathogenesis of AD, the primary biological function of APP is still largely unknown. APP is predominantly localized to synapses, which suggests that the protein exerts its primary role at the synapse [52]. It is trafficked to axons via fast anterograde axonal transport by directly binding to the anterograde motor protein, kinesin I [53, 54]. Hippocampal neurons lacking APP show synaptic dysfunction with fewer synapses formed and thereby decreased synaptic transmission [55]. Similar to APP, the primary biological function of $A\beta_{1-42}$ is not

fully understood, though it is known to positively regulate long-term potentiation and memory at low concentrations in hippocampal neurons [56]. In contrast, $A\beta_{1-42}$ leads to reduced potentiation at higher concentrations. Furthermore, oligomeric $A\beta_{1-42}$ facilitates long-term depression in hippocampal neurons [57]. These studies demonstrate the importance of dendrites, axons, and synapses in the biological functions of APP and $A\beta_{1-42}$, which could explain the synaptic dysfunction that is observed in AD.

1.1.3. Axonal Dysfunction in Alzheimer's Disease

AD is a particularly devastating disease as patients can live with the disease for over 20 years as symptoms progressively worsen. At the beginning of disease progression, patients display symptoms that are clinically categorized as mild cognitive impairment (MCI), which is a transition state between normal aging and early AD [58]. Patients with MCI have significant neuronal loss in the entorhinal cortex, which contains neurons that project to the hippocampus [59, 60]. Neuronal loss is also prevalent within the hippocampus in MCI and early AD, and decreased volume in these areas can be visualized using magnetic resonance imaging (MRI) and are good predictors of conversion from MCI to AD [61-64]. Another prominent feature in MCI is synaptic loss within the CA1 region of the hippocampus [65, 66]. Interestingly, the loss of synapses in brains of MCI and AD patients correlates with cognitive decline and disease severity [67-70]. Using high-resolution imaging techniques, studies in MCI have demonstrated axonal degeneration in the parahippocampal gyrus [71-73]. This effect in the parahippocampal gyrus in MCI is particularly intriguing as this brain region

contains the perforant pathway, which projects to neurons in the entorhinal cortex, one of the earliest brain regions that is affected in AD [74]. White matter degeneration is also present in MCI patients within the cortices, corpus collosum and cholinergic system of MCI patients [75-78]. This loss in white matter integrity correlates with deficits in cognitive and executive function in MCI patients [71-73, 76, 78].

In line with these findings, numerous studies show that dystrophic axons are observed before characteristic tau tangle and amyloid plaque pathologies in AD brains [79]. Further underscoring the important role of axonal and synaptic dysfunction in neurodegenerative disease pathogenesis, recent studies show that affected neurons in AD follow a dying-back pattern of degeneration with axonal dysfunction and synaptic loss preceding the death of the neuron [80]. This dying-back phenomenon has also been observed in PD, HD and ALS [81-83]. AD mouse models harboring established FAD mutations in APP (APP_{V717F} and $APP_{K670N,M671L}$) similarly display axonal pathology, such as axonal swelling, prior to neuronal loss [84, 85]. Synaptic loss also precedes the deposition of amyloid plaques in FAD mouse models [86, 87]. In addition, triple transgenic mice harboring FAD-causing mutations ($PS1_{M146V}$, APP_{SWE}, tau_{P301L}) display significant synaptic loss prior to Aβ plaque and tau tangle deposition [88, 89]. Studies using FAD mouse models harboring five FAD mutations ($APP_{K670N,M671L,I716V,V717I}$; $PS1_{M146L,L286V}$) show axonal swellings before signs of memory deficits are detected [90, 91]. These findings provide insight into the earliest pathogenic events that are occurring in AD brains prior to symptoms appearing, and they

demonstrate that these pathological alterations that occur in axons are likely due to altered APP processing and $A\beta_{1-42}$ production.

Together, these lines of evidence suggest that the presence of increased $A\beta_{1-42}$ within a brain region could lead to axonal dysfunction of neurons that project to that region, thereby leading to the death of those projecting neurons. This concept has been corroborated in multiple studies using AD mouse models. In PDAPP mice, which express mutant APP_{V717F}, neuronal size reduction is present in a portion of the locus coeruleus (LC) containing neurons that project to the cortex and hippocampus, where Aβ pathology is present. Importantly, the LC itself did not contain Aβ pathology, suggesting that the pathological neuronal size reduction is due to axonal rather than somatic exposure to Aβ [92]. In another study utilizing the *APP_{swe}/PS1ΔE9* AD mouse model, significant degeneration and eventual robust loss of monoaminergic neurons occurs in the absence of local Aβ or tau pathology [93]. These affected neurons do, however, project axons to regions that contain Aβ pathology. Together, these studies reveal a possible stress signaling mechanism that originates in axons and propagates retrogradely to the cell body, which thereby induces death of the neuron.

This theory of retrograde toxic signaling from axons to cell bodies is also supported by numerous studies that have demonstrated that AD pathology spreads throughout disease progression along neuronal pathways via connectivity, not proximity. Amyloid plaques first develop within the neocortex and spread from there to allocortical regions, diencephalon, basal forebrain and striatum, midbrain and medulla oblongata, and finally in the pons and the

cerebellum [94]. Neurofibrillary tangles also follow a spatiotemporal spreading pattern beginning in the transentorhinal cortex, then to the entorhinal cortex, the hippocampus, the temporal cortex, and finally to the neocortex [18]. Intriguingly, this spreading pattern of pathology has also been observed in other neurodegenerative diseases, including the spreading of α-synuclein pathology in PD and the spreading of TDP-43 pathology in ALS [95, 96].

In addition to these studies identifying the spreading of AD pathology in a hierarchical manner throughout disease progression, recent functional MRI studies measuring cerebral blood volume (CBV) in patients with pre-clinical AD have illuminated that metabolic defects are present in the entorhinal cortex and parahippocampal gyrus even before AD diagnosis [97]. To determine whether these metabolic defects also spread in a similar manner to the spreading of pathological hallmarks, Duff and colleagues generated a mouse model containing both a pathological APP human transgene and a pathological tau human transgene. Co-expression of these pathological transgenes led to metabolic defects in the lateral entorhinal cortex as well as the perirhinal cortex and posterior parietal cortex, which overlaps with patterns of cortical spread that are observed in pre-clinical AD [18].

An important conclusion that can be made from these studies is that neuronal dysfunction, particularly axonal and synaptic dysfunction, occurs very early in disease progression, often before patients are diagnosed with AD and before plaques and tangles begin to appear within the brain. This highlights the possibility that the pathological hallmarks of AD are not disease-causing but are

rather secondary effects of an earlier, primary pathogenic event that likely begins in part in the axon. There are several lines of evidence that support this theory. First of all, it has been established that Aβ oligomers, as opposed to fibrils, are the most toxic form of Aβ, indicating that plaque precursors rather than plaques themselves are likely responsible for AD pathogenesis [41]. The most convincing support for this hypothesis, however, comes from the frequent failures that have occurred in AD drug development trials.

From 2002 to 2012, there have been 413 AD clinical trials performed: 124 Phase 1 trials, 206 Phase 2 trials, and 83 Phase 3 trials [98]. These trials have had a very high attrition rate and an overall success rate of 0.4%. A little over half of these trials used disease-modifying agents, and 65.6% of these disease-modifying trials targeted Aβ. The most common theory that has been put forth regarding the failure of these trials has been the timing of drug treatment in disease progression. It has been proposed that these anti-amyloid agents may be optimized by focusing on earlier interventions before neurodegeneration begins [99-101]. This is in line with the large amount of evidence demonstrating that axonal and synaptic dysfunction occurs in MCI and preclinical AD. Based on this hypothesis, pharmaceutical companies are aiming to target Aβ using Aβ immunotherapies and BACE inhibitors earlier on in disease progression in patients with early or mild AD [102].

In summary, these data clearly present the importance of understanding the earliest pathogenic events that lead to neurodegeneration in AD. Numerous lines of evidence suggest that $A\beta_{1-42}$ oligomers act as the precipitating agent in

AD neurodegeneration and that the axon is among the primary sites of dysfunction. Upon axonal dysfunction, retrograde stress signaling appears to propagate to the cell body, which causes eventual neuron loss. Therefore, it is important to investigate the early effects of $A\beta_{1\text{-}42}$ on axonal signaling.

1.2. Axonal Protein Synthesis

1.2.1. Axonal Protein Synthesis in Neurodevelopment

Neurons are highly polarized cells consisting of cell bodies, dendrites and axons. In order to properly assemble functional neural circuits, neurons must project axons to the correct targets. Axons achieve this feat via a motile structure at the tip of an axon called a growth cone. Growth cones respond to cues within the extracellular environment that promote either attraction or repulsion [103-107]. Growth cones must respond rapidly to these cues to ensure proper steering of the axon towards the correct target.

In order for growth cones to steer themselves towards or away from an extracellular cue, cytoskeletal and cytosolic proteins must act rapidly within the axon. Growth cones are often a significant distance, up to one meter, from their corresponding cell bodies, which presents a logistical conundrum as to how growth cones are able to respond to guidance cues in a reasonable amount of time to ensure spatiotemporal accuracy. It was long believed that proteins must be supplied to axons via anterograde axonal transport from the cell body [108]. This was supported by early studies that failed to detect ribosomes in axons [109]. However, most cytoskeletal and cytosolic proteins are transported to axons via slow axonal transport, which travels at approximately 0.1-3 mm/day [110]. As the half-lives of proteins range from a few seconds to several days, this slow transport of these proteins makes it difficult to believe that anterograde transport of cell body proteins into axons is the mechanism by which growth cones can rapidly respond to guidance cues. This mystery was addressed in more recent

decades when studies revealed that axons do indeed have the capacity to generate their own proteins.

The first line of evidence that axons may be able to synthesize proteins came from numerous electron microscopy (EM) studies from vertebrate spinal axons, which displayed the presence of protein-synthesizing machinery [111-113]. Subsequent studies revealed tRNA, rRNA, aminoacyl-tRNA synthetases, polypeptide elongation factors, and a variety of mRNA transcripts to be present in invertebrate axons [114-117]. Additionally, released amino acids were reused within invertebrate axons, a process which would require local protein synthesis [118]. Axonal protein synthesis was directly demonstrated in cultured mollusk neurons when an mRNA not present in mollusk neurons was injected into axons and the protein product was detected within hours of injection [119]. Vertebrate axons were later found to contain ribosomes both within the initial axon segment and along the axon shaft [120-122]. Moreover, other translational components were identified in vertebrate axons, including tRNA, initiation factors and mRNA [123-125]. To formally investigate the ability of vertebrate axons to synthesize proteins, Campenot and colleagues used a compartmentalized culturing system, termed “Campenot chambers”, to culture rat sympathetic neurons [126]. These chambers enable axons to extend into compartments isolated from cell bodies, which allows for reagents to be exclusively added to axons. By incubating axons with [^{35}S]methionine and purifying proteins, they determined that axons could produce both actin and β-tubulin proteins, even if cell bodies were removed prior to starting the assay.

These findings led several groups to investigate the function of axonal protein synthesis. Stimulation of chick embryonic hippocampal neurons with the neurotrophin NT-3 induced the rapid localization of *β-actin* mRNA into axonal growth cones, suggesting that local synthesis of β-actin is necessary for growth cone motility in response to NT-3 [127]. The 3' untranslated region (3'UTR) of *β-actin* was identified to contain a sequence, termed a "zipcode", that was required for mRNA localization to the growth cone via the binding of the zipcode to zipcode binding protein 1 (ZBP1) [128-131]. By interfering with this complex formation between the zipcode and ZBP1, growth cones had decreased NT-3-induced motility, providing direct evidence that axonal β-actin synthesis is indeed required for growth cone dynamics in response to an environmental cue [132]. These findings were supported by studies in *Xenopus* retinal ganglion cells (RGCs), which displayed rapid protein synthesis in axons in response to semaphorin 3A (SEMA3A) and netrin-1 [133]. Axonal application of protein synthesis inhibitors was sufficient to block the attractive turning of growth cones toward netrin-1 and the repulsive turning of growth cones away from SEMA3A. In addition to NT-3, netrin-1 and SEMA3A, numerous other guidance cues have been found in recent years to require local protein synthesis to achieve growth cone turning, growth cone collapse, axon elongation, axon branching, and neuronal survival. These include SLIT2, engrailed 1 and 2 (EN1 and EN2), pituitary adenylate cyclase-activating polypeptide (PACAP), nerve growth factor (NGF), and brain-derived neurotophic factor (BDNF) [134-139]. These various extracellular factors immediately induce axonal protein synthesis by activating

the mechanistic target of rapamycin (mTOR) pathway [133-135]. Together, these studies provided intriguing implications for the role of axonal protein synthesis in neurodevelopment.

Once growth cones meet their targets they transform into presynaptic terminals to allow for synapse formation. Several mRNAs have been found within axons that encode proteins involved in synaptogenesis, suggesting that local protein synthesis plays an important role in this process. Transcripts encoding branch-promoting protein and synaptic vesicle proteins become enriched in *Xenopus* RGCs as the cells mature [140]. Correspondingly, BDNF-induced and NT-3-induced potentiation of synaptic vesicle release in *Xenopus* motor neurons requires presynaptic protein synthesis [141, 142]. Axonal protein synthesis is also necessary for synapse formation in mollusk neurons [143-147]. Moreover, fragile X mental retardation protein (FMRP), an RNA binding protein that regulates translation and mediates synaptic plasticity in dendrites, has been found in axons and growth cones [148-150]. Interestingly, defects are observed in the formation of presynaptic terminals in mouse hippocampal neurons lacking FMRP [151].

In summary, these studies refute the long disputed belief that axons are incapable of autonomous protein synthesis and illustrate the importance of localized protein synthesis in neurodevelopment. From controlling axonal pathfinding to regulating synapse formation and synaptic vesicle release, presynaptic protein synthesis clearly plays a crucial role in ensuring the proper formation of functional neural circuitry. In order for these processes to occur,

however, mRNA must be transported into axons and the translation of these transcripts must be regulated by extrinsic cues.

1.2.2. Axonal mRNA Localization and Translation

Axonal transcriptome analysis has been performed on a variety of diverse neurons, including *Xenopus* RGCs, mouse RGCs, rat hippocampal neurons, rat peripheral neurons, and rat sympathetic neurons [140, 152-154]. Each of these transcriptomes has revealed the presence of thousands of different mRNAs in their axons. Many of these identified transcripts are common between different cell types, such as those encoding cytoskeletal proteins, protein synthesis machinery and mitochondrial proteins. However, some transcripts appear to be enriched in specific cell types. As an example, *Impa1* transcripts are present in axons of peripheral neurons but not central neurons [154]. Axonal transcriptomes dynamically change throughout development, which suggests that axons have the ability to recruit specific and functionally relevant transcripts from the cell body in response to a stimulus via anterograde transport [140]. In the case of *Impa1*, an element within the 3'UTR directs the transcript into axons of developing sympathetic neurons, specifically in response to NGF stimulation [154]. This form of extrinsic regulation of *cis*-acting elements to recruit distinct mRNAs into axons occurs in developing peripheral neurons in response to a variety of extracellular growth cues, including NGF, BDNF, NT-3, SEMA3A and myelin-associated glycoprotein (MAG) [155].

Transcripts contain *cis*-acting localization elements that can bind to RNA binding proteins (RBPs) to direct their localization, such as ZBP1, which binds to

the β-actin zipcode sequence within the 3'UTR and directs it to subcellular compartments [128-131]. Together with their target mRNAs, RBPs form transport ribonucleoprotein particles (RNPs), which directly associate with molecular motors, thereby enabling axonal localization via microtubules [156]. During transport, mRNAs are translationally repressed until they are released for translation [157]. Post-translational modifications (PTMs) of RBPs can regulate their interaction with molecular motors, as is seen with the RBP, La [158]. Unmodified La binds to kinesin motors to regulate anterograde RNP transport, whereas sumoylation of La switches its association to dynein, resulting in retrograde RNP transport.

Once a transcript is localized to an axon, it has the potential to be translated when induced by certain cues. The translation of these transcripts can be regulated in numerous ways. Extrinsic cues can activate global translation via the PI3K-AKT-mTOR pathway. mTOR, a master regulator of cellular homeostasis, activates the initiation of translation by phosphorylating its two substrates, 4E-BP1 and S6K [159, 160]. However, increased global translation does not necessarily mean that all axonally localized transcripts will be translated. In fact, some cues, while increasing global protein synthesis, can induce the repression of some mRNAs, such as *Hsp70* transcripts in retinal axons in response to EN1 application [161]. Extrinsic cues can achieve this level of specificity via several different mechanisms. Transcripts can be recruited into or released from RNA granules in response to cues, thereby regulating their transport, stability and translation [162]. Components of RNA granules, such as

RBPs, can also be regulated by extracellular cues. Such is the case for *β-actin*, which undergoes translational de-repression via BDNF-induced ZBP1 phosphorylation [163]. Additionally, extracellular cues can regulate transcript stability via microRNAs and non-sense mediated decay of intron-retaining mRNAs [164]. Furthermore, ribosomal interactions with receptors can regulate mRNA translation specificity. The transmembrane receptor, DCC, colocalizes with multiple translation components, which disassociate with the receptor upon netrin binding, thereby inducing translation [165].

Developing axons employ myriad mechanisms to regulate the translation of transcripts in response to extrinsic cues, and this axonal protein synthesis is crucial for the accurate development of the nervous system. Mature axons in adult brains still retain localized transcripts, but these transcripts are largely believed to be translationally silent as axonal protein synthesis is no longer necessary. However, the translation of these transcripts is significantly upregulated in response to an injurious event, and this axonal protein synthesis event serves as a critical regulator of cell survival and regeneration.

1.2.3. Axonal Protein Synthesis in Mature Axons

Once an axon matures into adulthood and functional neural circuitry has been formed, the need for local protein synthesis subsides in comparison to developing axons. Because of this, it was long believed that axons do not maintain the ability to synthesize proteins after development. The first evidence indicating that mature axons may maintain protein synthesis capacity came from the field of axotomy. After an injury, an axon must regenerate itself in order for

the neuron to survive, and this process necessitates proteins. In order to determine whether this regeneration process required localized protein synthesis, Alvarez and colleagues crushed rat peroneal nerves in the presence and absence of the protein synthesis inhibitor, cycloheximide (CHX) [166]. Surprisingly, axons incubated with CHX showed reduced regenerative elongation and less frequent axonal sprouting, indicating that regenerating axons require a local source of proteins. Moreover, a study by Verma and colleagues demonstrated in adult DRGs that axonal protein synthesis underlies growth cone initiation after axotomy, a process that is required for axonal regeneration [167]. Interestingly, growth cone initiation after axotomy has also been shown to be Ca^{2+}-dependent, which suggests that Ca^{2+} may be acting to promote injury-induced axonal protein synthesis [168].

These studies clearly demonstrated that local protein synthesis is important for the efficient regeneration of the axon following injury. However, the question of which proteins were being translated in axons still remained. Previous studies had demonstrated that axonal injury in *Aplysia* neurons induces the axonal accumulation of proteins containing a nuclear localization sequence (NLS), which provides access to the retrograde transport/nuclear import pathway [169, 170]. Based on these findings, the Fainzilber lab proposed that importin-β, a nuclear import protein containing an NLS, is synthesized in axons and retrogradely transported to the nucleus in response to injury [171]. Using crushed L4/L5 DRG axons, they demonstrated that importin-β is indeed immediately synthesized in axons following injury and that its inhibition resulted in delayed

regenerative outgrowth. This phenomenon was also confirmed in injured human embryonic stem cell (hESC)-derived neurons and injured hippocampal neurons [172, 173]. To investigate the possibility of a *cis*-acting element within mRNAs encoding importin-β, transcripts from cell bodies and axons were compared. This led to the identification of a long 3'UTR variant that directed the localization of transcripts encoding importin-β into axons [174]. Subcellular knockout of this transcript was achieved by targeting the long 3'UTR variant, and delayed functional recovery was confirmed *in vivo* after injury without axonal importin-β. Interestingly, in the absence of locally synthesized importin-β after injury, 60% of genes that are normally activated after axotomy were affected. This suggests that although axonal importin-β plays a major role in regulating injury-dependent gene expression, almost one-third of the injury-induced transcriptional response is likely associated with other signaling pathways. Together, these studies provided convincing evidence that adult axons do retain the capacity to synthesize proteins and that this process is necessary for efficient neuronal recovery and regeneration after injury.

Further investigation into the injury-induced local synthesis response by Fainzilber and colleagues revealed the identities of other axonally synthesized proteins in response to axotomy. Vimentin, a type III intermediate filament protein, is also locally synthesized after axotomy, and it signals retrogradely to the cell body [175]. Axonally synthesized vimentin acts as a scaffolding protein between importin-β and activated ERK, a MAP kinase. Vimentin's binding to phosphorylated ERK allows for protection of the phosphorylated site from

phosphatases, which enables the activated signal to travel efficiently from axons to cell bodies. Once in the cell body, phosphorylated ERK activates the transcription factor, Elk-1, via phosphorylation.

In addition to vimentin, the Fainzilber lab also identified the proteins RANBP1 and STAT3 to be locally synthesized in response to injury [176, 177]. Prior to injury, importin-α is bound to dynein, and upon the dimerization of importin-α and importin-β, the complex and any associated proteins are retrogradely transported [176]. Axonal synthesis of RANBP1, a RanGTPase, acts to promote the dissociation of RanGTP from importins α and β, which stimulates their dimerization and subsequent retrograde transport. STAT3 is a transcription factor that is locally synthesized and activated in axons following injury [177]. Similar to importin-β and vimentin, activated STAT3 signals retrogradely to the nucleus, and there it induces pro-regenerative gene expression. The activation and retrograde signaling of STAT3 after injury has been demonstrated previously [178]. However, this study from the Fainzilber lab was the first to demonstrate that STAT3 was synthesized itself at the site of injury [177]. Without locally synthesized STAT3, there is increased neuronal apoptosis in response to injury, which illuminates the role of axonally synthesized STAT3 in promoting cell survival after a peripheral injurious event.

Together, these studies demonstrate the importance of axonal protein synthesis for cell survival and regeneration after axotomy. Though the majority of axonally localized transcripts remain translationally silenced within the adult brain, they can be immediately synthesized in response to injury to form a

retrograde injury-signaling complex that informs the cell body of the peripheral insult and thereby enables pro-regenerative gene expression to occur. This presents the intriguing hypothesis that other peripheral insults beyond axotomy, such as a degenerative stimulus, could similarly induce axonal protein synthesis and retrograde signaling to the nucleus.

Part 2

Chapter 2. Local Protein Synthesis in $A\beta_{1-42}$-Dependent Toxicity

2.1. Introduction

Our lab decided to investigate whether the neurodegenerative stimulus, $A\beta_{1-42}$, could induce axonal protein synthesis events, and we hypothesized that $A\beta_{1-42}$-induced local protein synthesis enabled retrograde signaling to the nucleus, which mediates $A\beta_{1-42}$-dependent cell death. We chose $A\beta_{1-42}$ as a degenerative stimulus based on the *in vivo* studies demonstrating that axonal exposure to $A\beta_{1-42}$ alone is sufficient to induce neurodegeneration and apoptosis of the entire neuron [92, 93]. Furthermore, studies from the field of AD have demonstrated that axonal dysfunction precedes death of the neuron, which underscores the importance of axonal dysfunction in disease pathogenesis [80]. If our data supported our hypothesis, this could provide convincing evidence that $A\beta_{1-42}$-induced axonal protein synthesis could be among the earliest pathological signaling events in AD and could serve as one of the precipitating events in pathogenesis.

This project is fully complete and was published in 2014 in *Cell* [179]. The lead investigator on this project was a postdoctoral fellow in our lab, Jimena Baleriola. I worked alongside her to help with the *in vitro* studies using primary hippocampal neurons cultured in microfluidic chambers. Based on my contribution to this project I am the second author on the publication, and our published findings serve as Part 2 of my thesis. Specifically, I performed experiments from Figures 3E, 3F, S3C, S4A and S4C. I also performed, processed and analyzed experiments from Figures 2B, 3A, 3B, 3C, 3D and 3I.

Additionally, I helped to analyze and interpret data from Figure 7. Though not shown in the paper, I also utilized qRT-PCR to confirm significant increases in ten axonal transcripts chosen from our sequencing data.

2.2. Manuscript

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Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions

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SUMMARY

In Alzheimer's disease (AD) brain exposure of axons to Aβ causes pathogenic changes that spread retrogradely by unknown mechanisms affecting the entire neuron. We found that locally applied $A\beta_{1–42}$ initiates axonal synthesis of a defined set of proteins including the transcription factor ATF4. Inhibition of local translation and retrograde transport or knockdown of axonal *Atf4* mRNA abolished Aβ-induced ATF4 transcriptional activity and cell loss. $A\beta_{1–42}$ injection into the dentate gyrus (DG) of mice caused loss of forebrain neurons whose axons project to the DG. Protein synthesis and *Atf4* mRNA were upregulated in these axons, and co-injection of *Atf4* siRNA into the DG reduced the effects of $A\beta_{1–42}$ in the forebrain. ATF4 protein and transcripts were found with greater frequency in axons in the brain of AD patients. These results reveal an active role for intra-axonal translation in neurodegeneration and identify ATF4 as a mediator for the spread of AD pathology.

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AUTHOR CONTRIBUTIONS

J.B. with C.A.W. performed and analyzed most experiments. C.M.T proposed and established the *in vivo* model, Y.Y.J. performed the $A\beta_{1–42}$ injections. P.L.N. and J.B. performed the RNA-sequencing. J.F.C. and J.B. performed and analyzed the experiments on human samples. U.H. conceived the project; U.H. and J.B. designed the experiments, analyzed the RNA sequencing data with assistance from P.L.N., and wrote the manuscript.

INTRODUCTION

β-amyloid pathology is a central component of Alzheimer's disease (AD) and $A\beta_{1–42}$ is considered causative for most neurodegenerative alterations in AD (Hardy and Selkoe, 2002). Accumulation of soluble oligomeric forms of $A\beta_{1–42}$ is positively correlated with the onset of cognitive decline in AD brain, and it elicits neurodegeneration in primary neurons. As axons and dendrites are generally much larger than their cell bodies and project over long distances in the brain, elevated $A\beta_{1–42}$ levels will first be sensed by neurites. Consequently, pathogenic signaling mechanisms will initially be triggered within neurites. Several aspects of AD pathogenesis such as tau hyperphosphorylation or impaired transport are first apparent in axons (Iqbal et al., 2009; Perlson et al., 2010), and local application of $A\beta_{1–42}$ is sufficient to induce neurite degeneration (Ivins et al., 1998) and to interfere with retrograde axonal trafficking (Poon et al., 2013). Indeed, pathogenic changes within axons may be primary events driving the development of the classical pathological changes (Krstic and Knuesel, 2013). For example, in AD brains with amyloid plaques restricted to the cortex, subcortical neurons with cortical projections degenerate suggesting that axonal exposure to $A\beta_{1–42}$ is sufficient to induce neurodegeneration over long distances (Liu et al., 2008). Similarly, in AD patients' brains monoaminergic neurodegeneration occurs in the locus coeruleus in the absence of local Aβ pathology (Marcyniuk et al., 1986). Therefore, in order to understand the pathogenesis of AD it is crucial to investigate the intra-axonal signaling pathways triggered by $A\beta_{1–42}$ separately from its effects on soma and dendrites.

Compartmentalized signaling is especially important for neurons, the most morphologically polarized cells. In order to react to stimuli in a spatially and temporally acute manner, axons are able to synthesize a subset of proteins locally (Jung et al., 2014). During development intra-axonal protein synthesis is crucial for growth cone behavior, axonal pathfinding, axon maintenance, and retrograde signaling (Jung et al., 2014). After the developmental period, the composition of the axonally localized transcriptome changes (Gumy et al., 2011), overall levels of mRNAs and ribosomes are lower (Kleiman et al., 1994), and mature axons have long been thought to be incapable of protein synthesis. However, recent evidence shows that protein synthesis persists in post-developmental CNS axons *in vivo* (Dubacq et al., 2009; Kar et al., 2014; Willis et al., 2011; Yoon et al., 2012). Additionally, upon injury of mature axons, a specific set of mRNAs and translation machinery are rapidly recruited into axons, and proteins are locally synthesized within mature axons (Rishal and Fainzilber, 2014). In contrast to its well-established role during development and regeneration, the role of intra-axonal protein synthesis in the context of neurodegenerative disorders remains unexamined.

Here, we asked whether intra-axonal protein synthesis was activated in response to $A\beta_{1–42}$ and functionally relevant for the retrograde transmission of neurodegenerative signals across brain regions. We report that axonal translation is activated in response to $A\beta_{1–42}$. Axonal ATF4 synthesis is required for the retrograde spread of $A\beta_{1–42}$-induced neurodegeneration, and axons in brains of AD patients show more frequent localization of ATF4 protein and mRNA.

RESULTS

Local exposure to $A\beta_{1–42}$ oligomers induces intra-axonal protein synthesis in hippocampal neurons

To investigate whether central nervous system (CNS) neurons locally synthesize proteins in axons in response to oligomeric $A\beta_{1–42}$, rat embryonic hippocampal neurons were grown in tripartite microfluidic chambers which allow for the fluidic isolation of axons from cell bodies and dendrites (Figures 1A and 1B) (Hengst et al., 2009; Taylor et al., 2005). The small culture volume and the hydrophobicity of microfluidic chambers influences the effective concentrations of peptides (Toepke and Beebe, 2006). We used an $A\beta_{1–42}$ concentration (3 μM) that is equivalent to ~250 nM in regular cultures (Figure S1A). Aβ concentrations in normal aging and AD brain range from ~2 pM to 2 μM, respectively (Wang et al., 1999). First we determined the axonal abundance of two molecular markers of translation: p-4EBP1 and p-S6. Levels for 4EBP1 and p-4EBP1 were non-significantly elevated, whereas S6 and p-S6 levels were significantly increased in axons upon $A\beta_{1–42}$ treatment (Figure 1C). Cell body levels of 4EBP1 and p-4EBP1 did not change and levels of S6 and p-S6 were slightly reduced (Figures 1C). $A\beta_{1–42}$ selectively applied to the cell body compartment caused an increase in both 4EBP1 and p-4EBP1 levels in the soma that did not propagate to the axonal compartment (Figures S1B and S1C). Next, we used bioorthogonal noncanonical amino acid tagging to detect newly synthesized proteins (Figure S1D). No local protein synthesis was detected in axons treated for 24 h with vehicle, a scrambled $A\beta_{1–42}$ peptide or soluble oligomeric $A\beta_{1–40}$ while $A\beta_{1–42}$-treated axons exhibited a significant increase in L-azidohomoalanine (AHA) incorporation (Figure 1D). Protein synthesis was detected in axons exposed to $A\beta_{1–42}$ for 48 h but not in axons treated for 24 h with $A\beta_{1–42}$ followed by a 24 h recovery period, indicating that local protein synthesis does not persist after removal of $A\beta_{1–42}$ (Figure 1E). AHA incorporation was prevented in the presence of the protein synthesis inhibitors anisomycin and emetine in the axonal but not the cell body compartment (Figures 1F and S1E). These results establish that axonally applied $A\beta_{1–42}$ activates local protein synthesis within 24 h.

Intra-axonal protein synthesis and retrograde transport are sequentially required for neurodegeneration triggered by axonal exposure to $A\beta_{1–42}$

Application of $A\beta_{1–42}$ to axons did not increase axonal fragmentation or cell death within 24 h, and after 48 h of $A\beta_{1–42}$ exposure the number of TUNEL-positive neurons was significantly greater while axonal fragmentation was not induced (Figure 2A). This effect was specific for $A\beta_{1–42}$ as neither the scrambled peptide nor $A\beta_{1–40}$ had any effect on cell death (Figure 2B). The observed neurodegeneration at 48 h is the result of pathogenic changes originating in the axons as only extremely little $A\beta_{1–42}$ was detected in the soma (Figure 2C).

To test whether $A\beta_{1–42}$-induced intra-axonal protein synthesis was required for the induction of cell death, axons were treated with vehicle or $A\beta_{1–42}$ for 24 h in the absence or presence of anisomycin or emetine. To minimize toxic side effects of the protein synthesis inhibitors, axons were exposed to them only during the last 6 h of the $A\beta_{1–42}$ treatment period. A significant increase in TUNEL-positive and corresponding decrease in Calcein-positive

neurons were observed upon treatment of axons with $A\beta_{1–42}$ (Figures 2D and S2A). Inhibition of intra-axonal protein synthesis completely abolished the effect of axonally applied $A\beta_{1–42}$, demonstrating that intra-axonal protein synthesis is required for $A\beta_{1–42}$-induced cell death.

To investigate whether transport from axons to soma was required for $A\beta_{1–42}$-induced neurodegeneration we used the retrograde transport inhibitors ciliobrevin A and EHNA. Both inhibitors significantly reduced retrograde movement of axonal lysosomes in microfluidic chambers (Figures S2B). When applied during the last 6 h of the 24 h $A\beta_{1–42}$ treatment period, ciliobrevin A only partially abolished $A\beta_{1–42}$-mediated cell death while EHNA had no effect (Figures 2D and S2A). However, both inhibitors completely abolished $A\beta_{1–42}$-dependent cell death when applied during the last 6 h of the 48 h experiment, while application of anisomycin at this time did not interfere with cell death (Figures 2E and S2C), consistent with our finding that axonal protein synthesis is not persistent after the removal of $A\beta_{1–42}$ (Figure 1E). To ensure that the effect of the inhibitors was not due to alterations in the minute levels of $A\beta_{1–42}$ transported to the cells bodies, axons were treated as before, and cell bodies were immunostained for $A\beta_{1–42}$. No correlation was found between somatic $A\beta_{1–42}$ levels and cell death (Figure S2D). These results establish that sequential intra-axonal protein synthesis and retrograde transport are required to transmit a neurodegenerative signal to the neuronal cell bodies in response to axonal $A\beta_{1–42}$ application.

The transcription factor ATF4 is locally synthesized in axons exposed to $A\beta_{1–42}$

To identify proteins that might transmit the neurodegenerative signal from axons to the soma, we performed RNA sequencing on total RNA isolated from vehicle and $A\beta_{1–42}$-treated axons and their cell bodies. Only mRNAs with higher expression levels than previously reported non-axonal transcripts were included in our analysis (Figure S3A). The axonal transcriptomes of control and treated axons show only partial overlap (Figure S3B), indicating that exposure of axons to $A\beta_{1–42}$ triggers the recruitment of a specific cohort of mRNAs (Supplemental Table 1). Among the axonally recruited mRNAs was the transcript coding for activating transcription factor 4 (ATF4). As a transcription factor ATF4 is a prime candidate for a retrogradely transported protein, and it can suppress the transcription of memory related genes and activate the transcription of proapoptotic genes in response to intracellular stress (Ameri and Harris, 2008). Additionally, ATF4 is a key molecule of the unfolded protein response (UPR) pathway (Ron and Harding, 2012), which is activated in many neurodegenerative diseases, possibly including AD (Ma et al., 2013). Comparative analysis of the RNA-seq datasets and quantitative RT-PCR revealed an increase in axonal *Atf4* abundance following $A\beta_{1–42}$ treatment while levels in cell bodies were unchanged, indicating that the upregulation of *Atf4* in axons is likely the result of increased axonal transport rather than transcription (Figures 3A). No increase in *Atf4* was detected in axons treated with vehicle control, $A\beta_{scrambled}$, or $A\beta_{1–40}$ for 18 h (Figure 3B).

Axonal *Atf4* mRNA levels determined by quantitative fluorescent *in situ* hybridization (FISH) were significantly increased following 6 h of $A\beta_{1–42}$ treatment and remained elevated until at least 24 h (Figure 3C). Similarly, ATF4 protein levels were significantly

increased at 6, 12 and 18 h of $A\beta_{1–42}$ treatment but dropped to lower than control levels at 24 h (Figure 3D). The increase in ATF4 at 18 h was abolished by the local application of protein synthesis inhibitors (Figure 3E) that did not affect *Atf4* mRNA localization in $A\beta_{1–42}$-treated axons (Figure S3C). To unambiguously demonstrate local translation of *Atf4* in axons we transfected *Atf4* targeting siRNAs into axons. The RNAi pathway is functional in axons, enabling knockdown of axonal mRNAs without affecting somato-dendritic mRNA levels (Hengst et al., 2006). Neither *Atf4* siRNA significantly altered *Atf4* levels in control axons but both blocked the increase of *Atf4* in $A\beta_{1–42}$-treated axons, with siRNA 1 decreasing *Atf4* levels below control conditions (Figure S3D). In all subsequent experiments siRNA 1 was used. The siRNA's effect was restricted to axons as ATF4 mRNA and protein levels were unchanged in cell bodies (Figures S3E and S3F). Selective knockdown of axonal *Atf4* completely inhibited the increase in axonal ATF4 protein levels following 18 h of exposure to $A\beta_{1–42}$ (Figure 3F).

To test whether the drop in axonal ATF4 abundance at 24 h of $A\beta_{1–42}$ treatment was due to ATF4 transport to the soma, we applied retrograde transport inhibitors locally. Axonal ATF4 levels were significantly increased in axons after 24 h of $A\beta_{1–42}$ treatment when retrograde transport was inhibited (Figure 3G, 3F and S3G), but axonal *Atf4* mRNA levels were unchanged (Figure S3H). Inhibition of intra-axonal but not somatic protein synthesis completely abolished the $A\beta_{1–42}$-dependent increase of axonal ATF4 in the presence of ciliobrevin A (Figure 3G). ATF4 protein levels were significantly decreased in control or *Atf4* siRNA transfected axons exposed to $A\beta_{1–42}$, and the accumulation of ATF4 in ciliobrevin A treated axons in response to Aβ1–42 was completely abolished in *Atf4* siRNA transfected axons (Figure 3H). These results establish that local application of $A\beta_{1–42}$ oligomers induces local ATF4 synthesis and its retrograde transport.

$A\beta_{1–42}$ triggers moderate eIF2a activation

Atf4 belongs to a group of transcripts, whose translation is activated by phosphorylation of the translation initiation factor eIF2α (Ron and Harding, 2012). Total eIF2α levels in axons were significantly increased by 12 h of $A\beta_{1–42}$ treatment but returned to control levels by 24 h (Figure 3I). p-eIF2α levels were significantly increased starting at 6 h, first due to the increase in total eIF2α and starting at 18 h due to an increase in the p-eIF2α/eIF2α ratio (Figure 3I). The increase in p-eIF2α was much lower than the increase in *Atf4* mRNA levels indicating the increase in axonal ATF4 protein might be primarily driven by increased *Atf4* localization. At 24 h, when we had observed strong upregulation of general protein synthesis in axons, we also detected a significant activation of eIF2α. There are four mammalian eIF2α-kinases, including the ER stress activated kinase PERK (Wek et al., 2006). Two activators of ER stress, tunicamycin and thapsigargin, did not trigger axonal recruitment of *Atf4* mRNA at 18 h (Figure 3J), but both efficiently initiated ER-stress in neuronal cell bodies (Figure S4A), suggesting that local ER stress does not phenocopy the effect of $A\beta_{1–42}$ oligomers on *Atf4* mRNA recruitment.

Axonally synthesized ATF4 induces gene expression in cell bodies and mediates retrograde somatic degeneration via CHOP

Next we asked if axonally derived ATF4 would function as a transcription factor in response to local application of $A\beta_{1–42}$ using an ATF4 firefly luciferase reporter gene construct. We also included an ATF6 luciferase reporter (Wang et al., 2000) to investigate whether Aβ1–42 causes local ER stress leading to the activation of the ATF6 arm of the UPR. ATF4- or ATF6-dependent luciferase transcription was efficiently detected upon treatment of cell bodies with tunicamycin or thapsigargin for 24 h (Figure S4A). No firefly luciferase activity of either construct could be detected when axons were treated with vehicle, or following 24 h of $A\beta_{1–42}$ treatment (Figure 4A). However, 48 h after $A\beta_{1–42}$ exposure a significant increase in cell body ATF4 abundance (Figure S4B) and transcriptional activity was observed (Figure 4A, left graph) while ATF6 activity remained undetectable (Figure 4A, right graph). Thus, axonal exposure to $A\beta_{1–42}$ induces ATF4-but not ATF6-dependent transcription. Next we analyzed somatic expression of CHOP, a transcriptional target of ATF4 (Averous et al., 2004), following 48 h of $A\beta_{1–42}$ treatment. CHOP expression was significantly increased in cell bodies in response to axonal $A\beta_{1–42}$ but not $A\beta_{scrambled}$ or $A\beta_{1–40}$ exposure (Figures 4B and S4C).

We then asked whether activation of ATF4-dependent gene expression was mediated by axonally synthesized ATF4. The ATF4 increase in cell bodies after $A\beta_{1–42}$ exposure was fully blocked by axonally applied anisomycin and partially blocked by ciliobrevin A (Figure S4D). Thus, we treated axons with $A\beta_{1–42}$ for 48 h, adding ciliobrevin A 6 h prior to sample processing and assessed ATF4 activity via luciferase and CHOP expression assays. In both assays inhibition of retrograde transport completely abolished the effect of axonal $A\beta_{1–42}$ (Figures 4C and 4D), and knockdown of axonal *Atf4* prevented $A\beta_{1–42}$-dependent transcription of luciferase, CHOP expression, or increase of ATF4 in cell bodies (Figures 4E, 4F and S4E), demonstrating that axonally synthesized ATF4 is required for ATF4-dependent gene expression after axonal $A\beta_{1–42}$ treatment.

Prolonged CHOP expression leads to cell death (Zinszner et al., 1998), and therefore, we asked if $A\beta_{1–42}$-dependent neurodegeneration was mediated by axonally synthesized ATF4. A significant induction of apoptosis and corresponding decrease in Calcein staining was found when control siRNA transfected axons were treated with $A\beta_{1–42}$, whereas depletion of axonal *Atf4* mRNA fully rescued the cells (Figures 4G and S4F). Additionally, $A\beta_{1–42}$ significantly increased the amount of TUNEL-positive nuclei in cell bodies transfected with control siRNA, but *Chop* knockdown blocked $A\beta_{1–42}$-mediated neurodegeneration (Figure 4H).

These results reveal that local application of $A\beta_{1–42}$ triggers the intra-axonal synthesis and retrograde transport of ATF4, and these events are required for ATF4-dependent transcription leading to CHOP-dependent cell loss.

Atf4 is locally translated in cholinergic axons in the mouse brain in response to Aβ

Next, we used a mouse model of semi-acute amyloidopathy by intra-hippocampal injection of $A\beta_{1–42}$ oligomers to analyze the *in vivo* relevance of our *in vitro* findings (Sotthibundhu

et al., 2008). In contrast to the more widely used transgenic mouse models for $A\beta_{1–42}$ amyloidopathy, this model allows the spatially restricted and temporally acute exposure of axons to elevated $A\beta_{1–42}$ levels. Intra-hippocampal injection of oligomeric $A\beta_{1–42}$ induces neurodegeneration of basal forebrain cholinergic neurons (BFCNs) within 2 weeks post-injection (Sotthibundhu et al., 2008). BFCNs project their axons ipsi-laterally to the hippocampus (Leranth and Frotscher, 1989), allowing the contra-lateral injection of vehicle to be utilized as a control in the same animal. Also, with the exception of very few cholinergic neuronal cell bodies in the dentate hilus, which can easily be avoided, choline acetyltransferase (ChAT) immunoreactivity in the dentate gyrus (DG) is a specific marker for BFCN axons (Leranth and Frotscher, 1989).

We injected $A\beta_{1–42}$ into the DG and analyzed brain sections 2 to 7 days post injection (DPI) at sites adjacent to the injection where the DG layers were intact (Figures 5A, S5A and S5B). First, we confirmed the presence of oligomeric $A\beta_{1–42}$ in these sites at 2, 4 and 7 DPI (Figure 5A). *Atf4* mRNA was readily detectable above background levels in cholinergic axons in all layers of the DG 2, 4 and 7 DPI in $A\beta_{1–42}$- but not vehicle-injected hemispheres (Figures 5B). ChAT staining appeared to be more punctate in the vicinity of cell bodies, especially in the granule cell layer (GCL) and co-localized with synaptophysin staining in control hemispheres (Figure S5B), consistent with the known termination pattern of BFCN axons. Puncta were more evident over time in $A\beta_{1–42}$-injected hemispheres suggesting synaptic/neuritic retraction. *Atf4* granules were frequently found in these puncta, possibly indicating their localization to synaptic terminals and/or retracting synapses. However, no reduction in ChAT-positive features was seen in $A\beta_{1–42}$-injected hemispheres even 7 DPI (Figure S5C). Also, no *Atf4* above background was observed in granule cell bodies under any condition (Figure S5D).

p-S6 and ATF4 levels were significantly increased within ChAT-positive axons in the $A\beta_{1–42}$-injected side 7 DPI (Figures 5C and 5D). In granule cells, a moderate increase in p-S6 and a strong upregulation of ATF4 were detected (Figures S5E and S5F), indicating that both axons and cell bodies respond to $A\beta_{1–42}$ by increasing ATF4 levels. To confirm synthesis of ATF4 within BFCN axons, both hemispheres of the brain were injected with $A\beta_{1–42}$, and either a control siRNA or an *Atf4* siRNA. At 7 DPI, *Atf4* siRNA caused a completed knockdown of axonal *Atf4* mRNA and significant reduction of ATF4 protein (Figures 5E and 5F) without causing axonal loss (Figure S5I). ATF4 protein was significantly reduced, and *Atf4* mRNA remained undetectable in granule cells (Figures S5G and S5H). These results demonstrate that axons in the mature mammalian brain synthesize ATF4 and likely other proteins in response to $A\beta_{1–42}$.

Axonally synthesized ATF4 is required to transmit a neurodegenerative signal from the DG to BFCNs

Next we investigated if ATF4-dependent gene expression was induced in BFCNs. Fluorogold was co-injected into both hemispheres of the brain to define the region of the basal forebrain from which axons close to the injection site originated. The unaffected detection of retrogradely transported fluorogold in the basal forebrains of all mice (Figure S6A and S6B) suggests that cholinergic afferents were functional and capable of retrograde

transport. ATF4 levels in BFCNs were significantly increased at 2 and 4 DPI with a non-significant increase at 7 DPI (Figure 6A, upper panels and left graph). ATF4 induction was evident in cholinergic neurons but not in all neurons present in the basal forebrain (Figures S5C). CHOP positive cholinergic neurons were significantly increased at 7 DPI (Figure 6A, lower panels and right graph), indicating that ATF4-dependent gene expression was induced in the basal forebrain.

Next we quantified the number of ChAT-positive neurons to determine if $A\beta_{1–42}$ injected in the hippocampus was sufficient to induce neurodegeneration of BFCNs at some point between 2 and 7 days. $A\beta_{1–42}$ injection did not change the number of ChAT-positive neurons in the forebrain at 2 or 4 DPI, but caused a significant ~20% reduction at 7 DPI (Figure 6B). Conversely, no overall decrease was seen in NeuN-positive neurons (Figure S6C, right graph) as expected considering that not only BFCNs resides in the basal forebrain. We confirmed these results using stereology as a complimentary approach (Figure S6D and S6E, left graphs). A significant ~24% increase in TUNEL-positive cells was found in the $A\beta_{1–42}$-injected hemisphere compared to the control hemisphere (Figure 6C). These results demonstrate that $A\beta_{1–42}$ injection into the hippocampus induces retrograde degeneration of BFCNs.

We had observed that ATF4 and CHOP induction was uneven across the basal forebrain suggesting a greater response of BFCNs in the nucleus in the diagonal band (NDB) than in the medial septum (MS). Indeed, a significant decrease in BFCNs was apparent only in the NDB, whereas cell death affected both nuclei to a similar extent (Figures 6D and S6D, right graph), suggesting that cells other than BFCNs degenerate in the MS in response to $A\beta_{1–42}$ injection. Next we determined whether ATF4-dependent signaling in the basal forebrain required $A\beta_{1–42}$-dependent *Atf4* synthesis in cholinergic axons in the hippocampus. Consistent with our previous observations that ATF4 protein was not significantly induced in the basal forebrain at 7 DPI, no reduction was detected in *Atf4* siRNA injected hemispheres (Figure 6E, upper panels, left graph). However, $A\beta_{1–42}$-dependent CHOP induction was significantly reduced by *Atf4* siRNA in the NDB (Figure 6E, lower panels, right graph). Thus, synthesis of ATF4 in the hippocampus induces ATF4-dependent signaling in BFCNs.

Finally, we sought to determine whether axonally derived ATF4 was required for the loss of BFCNs. Co-injection of *Atf4* siRNA blocked the decrease in density of BFCNs in the NDB, in contrast to the MS, which remained unaffected (Figures 6F and S6F, Supplemental Table S2). When compared to non-siRNA conditions (dashed lines in Figures 6F and 6G), *Atf4* siRNA reduced the number of TUNEL-positive cells in the NDB by ~63% but restored the number of ChAT-positive neurons to normal levels. This discrepancy indicates that other cells in the forebrain die as well, but only BFCNs die in an Atf4-dependent manner. Additionally axonally synthesized ATF4 might cause a loss of cholinergic phenotype in BFCNs, as is suggested by the fact the number of NeuN positive cells does not decrease significantly in the forebrain upon $A\beta_{1–42}$ injection.

We observed a significant thinning of the GCL and increased cell death in DG exposed to $A\beta_{1–42}$ (Figure S6G), but *Atf4* siRNA had no effect on the thickness of the GCL (Figure

S6H, left graph), and far from rescuing dying cells, *Atf4* siRNA exacerbated cell death in the DG (Figure S6H, right graph). Thus, the decrease in BFCNs was not caused by neurodegeneration in the hippocampus.

Atf4 mRNA granules and ATF4 protein are present in processes in human AD brains

Finally, we analyzed the presence of ATF4 mRNA and protein in post mortem brain samples of 8 AD patients and 8 age-matched controls. Axons and cell bodies containing *Atf4* mRNA granules were found in the hippocampal formation in all cases (Figure 7A). However, AD brains exhibited a higher frequency of *Atf4*-containing axons in the hippocampus, the subiculum, and the entorhinal cortex (Figure 7B). A decrease was observed in *Atf4*-positive cell bodies in the hippocampus of AD brains, but a higher frequency was found in the subiculum and entorhinal cortex (Figure 7C). In AD brains, ATF4-positive processes could be observed in the vicinity of amyloid plaques (Figure 7D). ATF4 was found in relatively intact processes and in beaded neurites (Figure 7D). More ATF4-positive axonal structures were found in the subiculum and the entorhinal cortex but not the hippocampus of AD brains (Figure 7E). ATF4-positive cell bodies (Figure 7D) were generally restricted to the subiculum and entorhinal cortex for both control and AD cases, with a higher frequency in the entorhinal cortex for AD cases (Figure 7F). The increased frequencies of ATF4 mRNA and protein in axons in the subiculum and entorhinal cortex of AD patients are highly suggestive of intra-axonal ATF4 synthesis in those regions of the brain that are especially vulnerable in AD (Khan et al., 2014). The results from human brain samples, although correlative, closely mirror our findings in hippocampal neurons and in the adult mouse brain providing evidence for the pathophysiological significance of our proposed model (Figure S6I).

DISCUSSION

Several prior studies have demonstrated the importance of local translation for axon maintenance (Yoon et al., 2012), mitochondrial function (Kar et al., 2014) and survival (Cox et al., 2008), and suppression of local translation of *lb2* mRNA causes neurodegeneration *in vivo* (Yoon et al., 2012). Here we report another dimension of local protein synthesis: in response to a physiologically relevant neurodegenerative stimulus axonal protein synthesis plays an active role in the transmission of neurodegeneration. Rather than acting solely as a factor in cellular homeostasis, local protein synthesis can be a major component of neuronal dyshomeostasis under pathological conditions.

Our finding that oligomeric $A\beta_{1–42}$ application to distal axons triggers the rapid recruitment and local translation of a distinct set of mRNAs is reminiscent of the activation of local translation upon nerve injury (Rishal and Fainzilber, 2014). However, the changes to the axonal transcriptome appear to be unique to the exposure of distal axons to oligomeric $A\beta_{1–42}$. For example we find that the transcriptome of $A\beta_{1–42}$-treated axons contains mRNAs of many AD related genes, including transcripts for 4 out of the current list of 20 AD susceptibility loci (Lambert et al., 2013): APP, ApoE, Clu, and FERMT2. These proteins function in $A\beta_{1–42}$ production (APP) and metabolism (Apoe, Clu), and have been implicated in tau pathology (FERMT2) (Shulman et al., 2014). The post-transcriptional

regulation of these genes by $A\beta_{1–42}$ suggests that these proteins might function in feedback mechanisms downstream of amyloid-pathology in AD.

Transcriptional changes in AD brain or in response to $A\beta_{1–42}$ have been extensively studied in various experimental settings (Miller and Geschwind, 2010). While these studies have provided valuable insight into the signaling pathways affected in $A\beta_{1–42}$ pathology, many of the mRNAs we identified as regulated by $A\beta_{1–42}$ in axons have never before been described to be changed in response to $A\beta_{1–42}$. This is likely due to the fact that they are post-transcriptionally regulated, rather than by increased promoter activity; in fact we did not observe an overall up- or down-regulation for the vast majority of the axonally localized mRNAs. Our study is thus a demonstration that post-transcriptional mechanisms of gene expression must be taken into account when investigating changes in gene expression. Especially in morphologically polarized cells such as neurons, mRNA localization can be as functionally relevant as transcriptional regulation, and disorders of the nervous system cannot be completely understood without the consideration of translational mechanisms.

We found that the increase of ATF4-positive BFCNs is greater than the observed cell loss, suggesting a model in which ATF4 is not directly leading to the transcription of pro-apoptotic genes but rather triggers the expression of a variety of genes whose functions cause pathogenic changes in the neurons, leading to cell death as a secondary effect. The finding that *Atf4* siRNA is more efficient in rescuing the loss of ChAT-positive BCFNs than in preventing apoptosis supports this model. Our finding that BFCNs in the MS and NDB react differentially to $A\beta_{1–42}$ injection into the DG indicates that the exact transcriptional response to axonally derived ATF4 differs between cell types. In fact, depending on the context, ATF4 in neurons has variously been described as pro-apoptotic, pro-survival, or memory suppressing (Ameri and Harris, 2008). It is possible that in response to low-levels of eIF2α phosphorylation, as has been seen in AD patients' brains and AD model mice (Ma et al., 2013), ATF4 acts mainly in a neuroprotective and memory suppressing manner while upon prolonged exposure to $A\beta_{1–42}$ it can contribute to cell death.

Our study adds to a growing body of evidence that some transcription factors are axonally synthesized (Ji and Jaffrey, 2014). It remains an unanswered question what might be the advantage of synthesizing a transcription factor in axons. In the case of ATF4 an appealing idea is that local synthesis might favor dimerization with an otherwise outcompeted binding partner. ATF4 binds promoter sequences either as a homodimer or a heterodimer (Ameri and Harris, 2008). The relative abundance of potential binding partners in axons could favor the formation of other heterodimers in axons than in cell bodies leading to differential transcriptional activities.

AD progression is characterized by the spread of pathology throughout the brain. Interfering with the spread would be an ideal approach to slow the decline of cognitive function that is characteristic of AD. Our results unravel a mechanism for the spread of disease that is based on the retrograde transport of ATF4. In this model, the exposure of axons to pathological levels of $A\beta_{1–42}$ leads to neuron-wide pathogenic changes due to pathogenic alterations in gene expression. Our finding that siRNA-mediated knockdown of *Atf4* mRNA in axons alone is sufficient to prevent neurodegeneration in response to acutely applied $A\beta_{1–42}$ *in vivo*

indicates a an unexpected target for a future therapy. Indeed, small molecules exist that could be used to repress ATF4 expression in the brain (Moreno et al., 2013; Sidrauski et al., 2013).

In conclusion, we describe a pathway through which a neurodegenerative signal is transmitted from the periphery of neurons to the soma across macroscopic distances in the brain. Our findings provide a mechanistic explanation for the spread of parts of the pathological changes in AD brain and potentially indicate new avenues for the development of therapeutic interventions for AD.

EXPERIMENTAL PROCEDURES

Extended Experimental Procedures can be found in Supplemental Information.

Axon specific treatment *in vitro*

To apply peptides, inhibitors or siRNA specifically to axons, rat embryonic hippocampal neurons were grown in tripartite microfluidic chambers with two 200-μm-long microgrooves barriers (Taylor et al., 2005). Synthetic $A\beta_{1–42}$ peptides were oligomerized (Stine et al., 2003) and applied to the axonal compartment at 3 μM at 9–10 DIV. Whenever stated, the axonal or cell body compartments were treated with 10 μM anisomycin, 500 nM emetine, 30 μM ciliobrevin A, 10 μM EHNA, 10 μg ml^{-1} tunicamycin, or 1 μM thapsigargin, or transfected with siRNA using NeuroPORTER (Genlantis, San Diego, CA).

RNA-seq Analysis

Axons were exposed to $A\beta_{1–42}$ or vehicle for 24 h. Total RNA was purified from the cell bodies and axons using the PrepEase RNA isolation kit (Affymetrix, Santa Clara, CA). cDNA libraries were created using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). Sequencing was performed on an Illumina MiSeq instrument (paired-end, 2× 150 bp) with biological replicates. Reads were aligned to the rat genome (Rn5) and counted using DESeq2.

Real time RT-PCR

Total RNA from the cell bodies and the axonal compartments was isolated as above, reverse transcribed, pre-amplified with the TaqMan PreAmp Kit (Life Technologies, Carlsbad, CA) and real time RT-PCR was performed with TaqMan Gene Expression master mix and the *Atf4* gene expression set (Rn00824644_g1). Gene expression was normalized to input RNA.

Fluorescent *In Situ* Hybridization (FISH)

Atf4 mRNA in hippocampal neurons was detected by quantitative FISH using a mixture of *in vitro* transcribed, digoxigenin-labeled riboprobes following establish protocols (Hengst et al., 2009). FISH on sections of mouse and human brain was performed with RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, Hayward, CA) according to manufacturer's instructions.

Luciferase Assay

Cell bodies of hippocampal neurons were transfected using NeuroPORTER with an ATF4- (Promega) or ATF6-firefly reporter (Addgene, Cambridge, MA) and a Renilla luciferase construct (Promega). Firefly luciferase activity was measured 24 or 48 h after $A\beta_{1–42}$ treatment using the Dual-Luciferase Reporter Assay System (Promega).

$A\beta_{1–42}$ injection experiments

Stereotaxic were performed following Sotthibundhu et al. (2008). 9–12-month-old C57Bl/6J mice were anesthetized, and placed in a stereotaxic frame (Stoelting, Wood Dale, IL). Stereotaxic injections were conducted using convection-enhanced delivery at a rate of 0.5 μl min^{-1} using the Quintessential Stereotaxic Injector (Stoelting) (coordinates from bregma: anterior-posterior, −2.00 mm; medial-lateral, ±1.3 mm; dorsal-ventral, −2.2 mm) resulting in an estimated $A\beta_{1–42}$ concentration in the DG of ~30 nM. Guidelines for the care and use of laboratory animals were followed for all mouse experimentation.

Brain samples

Post mortem brain samples of AD patients and age-matched controls were obtained from the New York Brain Bank. 8 μm paraffin embedded sections were analyzed histochemically for the presence of ATF4 protein or by RNAscope for *Atf4* mRNA.

Statistical Analyses

When comparing multiple groups, one-way ANOVA followed by Bonferroni post-hoc test was performed. To compare two groups, *t*-tests were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Locally applied $A\beta_{1–42}$ triggers recruitment of mRNAs into axons and local translation.
- ATF4 is locally synthesized and retrogradely transported in response to $A\beta_{1–42}$.
- Knockdown of axonal *Atf4* mRNA reduces $A\beta_{1–42}$-induced neurodegeneration *in vivo*.
- ATF4 transcript and protein levels are increased in axons in the brain of AD patients.

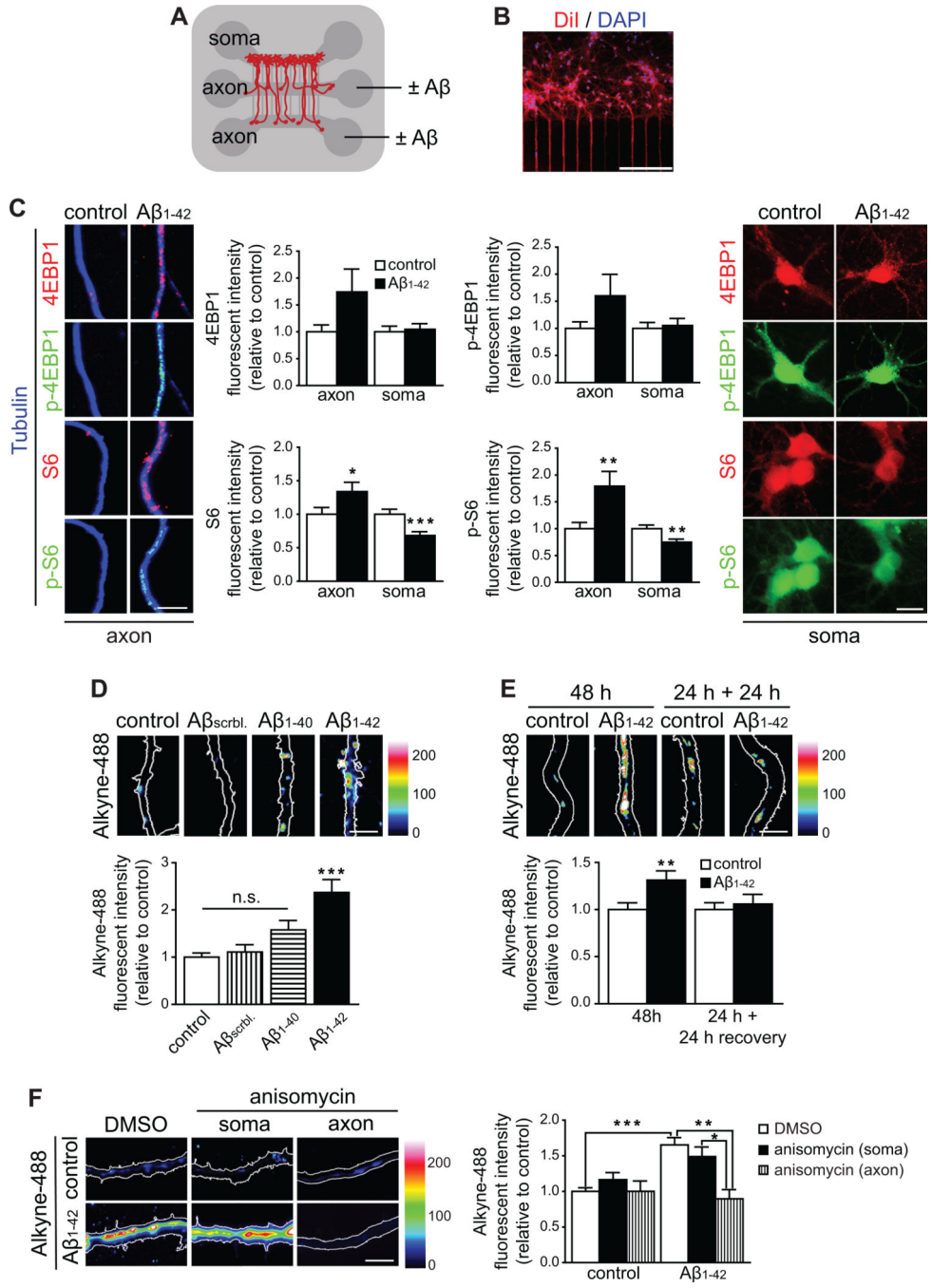

Figure 1. Locally applied $A\beta_{1–42}$ oligomers induce intra-axonal protein synthesis

(A) Scheme of a microfluidic chamber used to isolate axons of hippocampal neurons. Neurons were cultured in the upper compartment. Axons cross through two 200-μm-long microgroove barriers into the axonal compartments.

(B) Neuronal cell bodies were retrogradely labeled by applying DiI selectively to the axons. Typically between 40% (optical fields proximal to the microgrooves) and 30% (distal fields) of neurons were labeled indicating their axons had crossed the microgrooves. Scale bar, 200 μm.

(C) Hippocampal neurons were cultured in microfluidic chambers for 9–10 DIV and axons were treated with vehicle or $A\beta_{1–42}$ for 24 h. Axons (left micrographs) and cell bodies (right micrographs) were immunostained for 4EBP1, p-4EBP1, S6 or p-S6. Mean ±SEM of 23–25 optical fields per condition (n=5 biological replicates per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Scale bars, 5 μm (left micrographs), 20 μm (right micrographs).
(D) Axons were treated with vehicle, $A\beta_{scrambled,}$ $A\beta_{1–40}$ or $A\beta_{1–42}$ for 24 h. 2 h prior to fixation, axons were sequentially incubated with AHA and 488-DIBO. Newly synthesized proteins were detected by the fluorescent signal (represented in pseudo color). Mean ±SEM of 25–35 optical fields per condition (n=5–7 biological replicates per group). *** $p<0.001$. Scale bars, 5 μm.
(E) Axons were treated with vehicle or $A\beta_{1–42}$ for 48 h or for 48 h replacing the oligomer-containing medium with fresh 50% conditioned medium after 24 h. 2 h prior to sample processing axons were treated as in D. Mean ±SEM of 35–45 optical fields per condition (n=7–9 biological replicates per group). ** $p<0.01$. Scale bar, 5 μm.
(F) Axons were treated with vehicle or $A\beta_{1–42}$ for 24 h. 2 h and 30 min prior to fixation, axons were sequentially incubated with anisomycin or vehicle, and with AHA and 488-alkyne. Newly synthesized proteins were detected by their fluorescence signal (represented in pseudo color). Mean ±SEM of 25–65 optical fields per condition (5–13 biological replicates per group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Scale bar, 5 μm.
See also Figure S1.

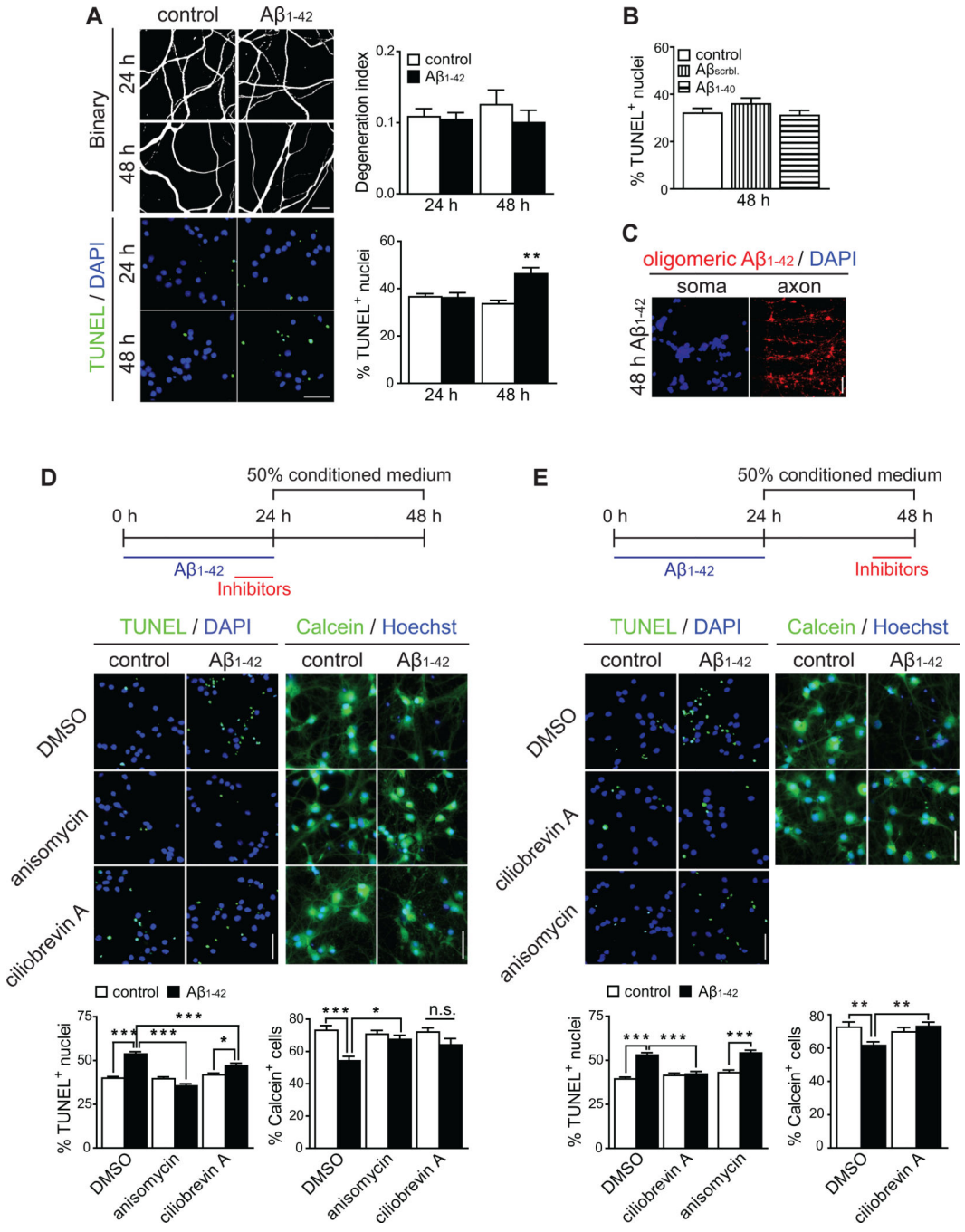

Figure 2. Intra-axonal protein synthesis and retrograde transport are sequentially required for Aβ$_{1–42}$-induced somatic degeneration

(A) Axons were treated with vehicle or Aβ1–42 for 24 or 48 h. Fragmentation of axonal tubulin (upper micrographs) or nuclear TUNEL staining (lower micrographs) were measured. Mean ±SEM of 25–55 axonal fields per condition (upper graph, n=5–11 biological replicates per group) and 50–70 somatic fields per condition (lower graph, n=5–7 biological replicates per group). **p<0.01.

(B) Axons were treated with vehicle, $A\beta_{scrambled}$ or $A\beta_{1–40}$ for 48 h. TUNEL-positive nuclei were quantified. Mean ±SEM of 25–35 optical fields per condition (n=5–7 biological replicates).

(C) Immunostaining for $A\beta_{1–42}$ on axons and cell bodies.

(D) Inhibitors were applied to axons during the last 6 h of the 24 h $A\beta_{1–42}$ treatment period. The culture medium from the axonal compartments was then replaced with 50% conditioned medium and cells were allowed to recover. Cell death (left panels) or survival (right panels), were assessed by TUNEL and Calcein staining, respectively. Mean ±SEM of 50–70 somatic fields stained for TUNEL per condition (left graph) and 25–31 somatic fields stained for Calcein (right graphs) per condition (n=5–7 biological replicates per group). *$p<0.05$; ***$p<0.001$.

(E) Inhibitors were applied to axons during the last 6 h of the 48 h experimental period. Cell death and survival were assessed as before. Mean ±SEM of 50–100 somatic fields stained for TUNEL per condition (left graph) and 30 somatic fields stained for Calcein (right graphs) (n=5–10 biological replicates). *$p<0.05$; ***$p<0.001$.

Scale bars, 50 μm. See also Figure S2.

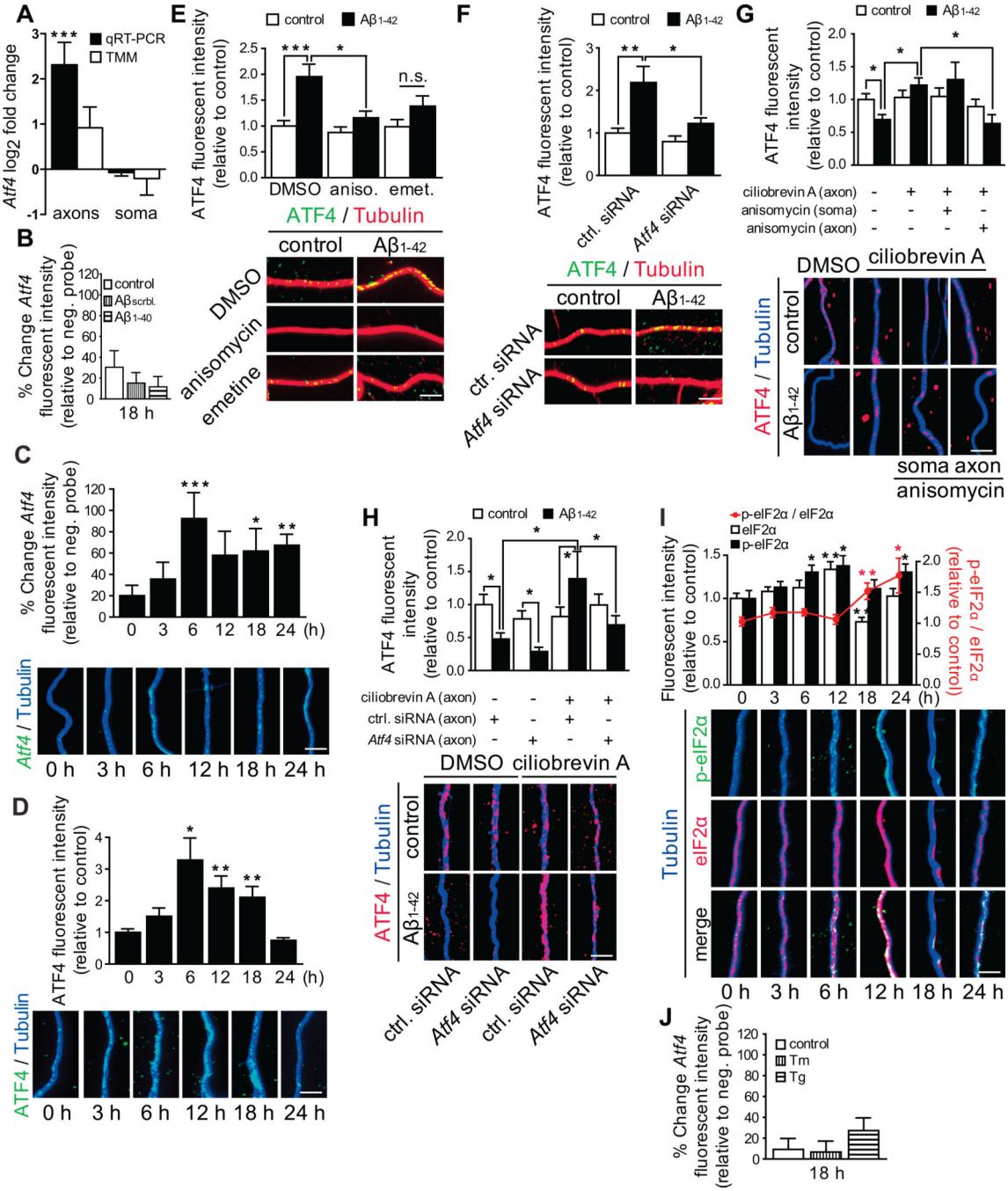

Figure 3. *Atf4*mRNA is recruited into $A\beta_{1–42}$-treated axons, and axonal ATF4 protein is locally synthesized and retrogradely transported

(A) Log_2 fold change *for Atf4* mRNA as determined by real time RT-PCR and DESeq2 (TMM). ***p<0.001.

(B) Hippocampal neurons were cultured in microfluidic chamber for 9–10 DIV, axons were treated with vehicle, $A\beta_{scrambled}$ or $A\beta_{1–40}$ for 18 h, and axonal *Atf4* mRNA levels were measured by quantitative FISH. Mean ±SEM of 25–30 optical fields per condition (n=5–6 biological replicates).

(C) Axons were treated with $A\beta_{1–42}$ for the indicated times, and axonal *Atf4* mRNA levels were measured by quantitative FISH. Mean ±SEM of 25–40 axonal fields per condition (n=5–8 biological replicates per group). The background fluorescence was determined using a non-targeting probe (neg. probe) and set to zero. *p<0.05; **p<0.01; ***p<0.001. Scale bar, 5 μm.

(D) Neurons were cultures and treated as in C. Axonal ATF4 protein levels were measured by quantitative immunofluorescence. Mean ±SEM of 20–40 axonal fields per condition (n=4–8 biological replicates per group). *p<0.05; **p<0.01. Scale bar, 5 μm.

(E) Hippocampal neurons were cultured and treated as in B. 3 h prior to sample processing axons were treated with DMSO, anisomycin or emetine. Axonal ATF4 protein levels were determined by quantitative immunofluorescence. Mean ±SEM of 25–35 axonal fields per condition (n=5–7 biological replicates per group). ***p<0.001: *p<0.05. Scale bar, 5 μm.

(F) Hippocampal neurons were cultured in microfluidic chambers for 8 DIV. Axons were transfected with a control (ctrl.) siRNA or a siRNA targeting *Atf4*. 24 h after transfection axons were treated with vehicle or $A\beta_{1–42}$ for 18 h. ATF4 protein levels were measured by quantitative immunofluorescence. Mean ±SEM of 35–55 axonal fields per condition (n=7–11 biological replicates per group). **p<0.01: *p<0.05.

(G) Axons were treated with vehicle or $A\beta_{1–42}$ for 24h, in the presence or absence of ciliobrevin A for 6h. Anisomycin was added to the cell body or the axonal compartment for 3 h. Axons were immunostained for ATF4 protein. Mean ±SEM of 30–40 axonal fields per condition (n=6–8 biological replicates per group). *p<0.05.

(H) Axons were transfected with a control siRNA or siRNAs targeting *Atf4* mRNA and treated with $A\beta_{1–42}$ and ciliobrevin A as in G. Axons were immunostained for ATF4 protein. Mean ±SEM of 30–40 axonal fields per condition (n=6–8 biological replicates per group). *p<0.05.

(I) Neurons were cultured and treated as in C. eIF2α and p-eIF2α levels were determined by quantitative immunofluorescence. Mean ±SEM of 20–35 axonal fields per condition (n=4–7 biological replicates per group).

(J) Neurons were cultured as in B. Axonal were treated for 18 h with tunicamycin (Tm) or thapsigargin (Tg) and *Atf4* mRNA levels were determined by quantitative FISH. Mean ±SEM of 30 optical fields per condition (n=6 biological replicates).

Scale bars, 5 μm. See also Figure S3 and Supplemental Table S1.

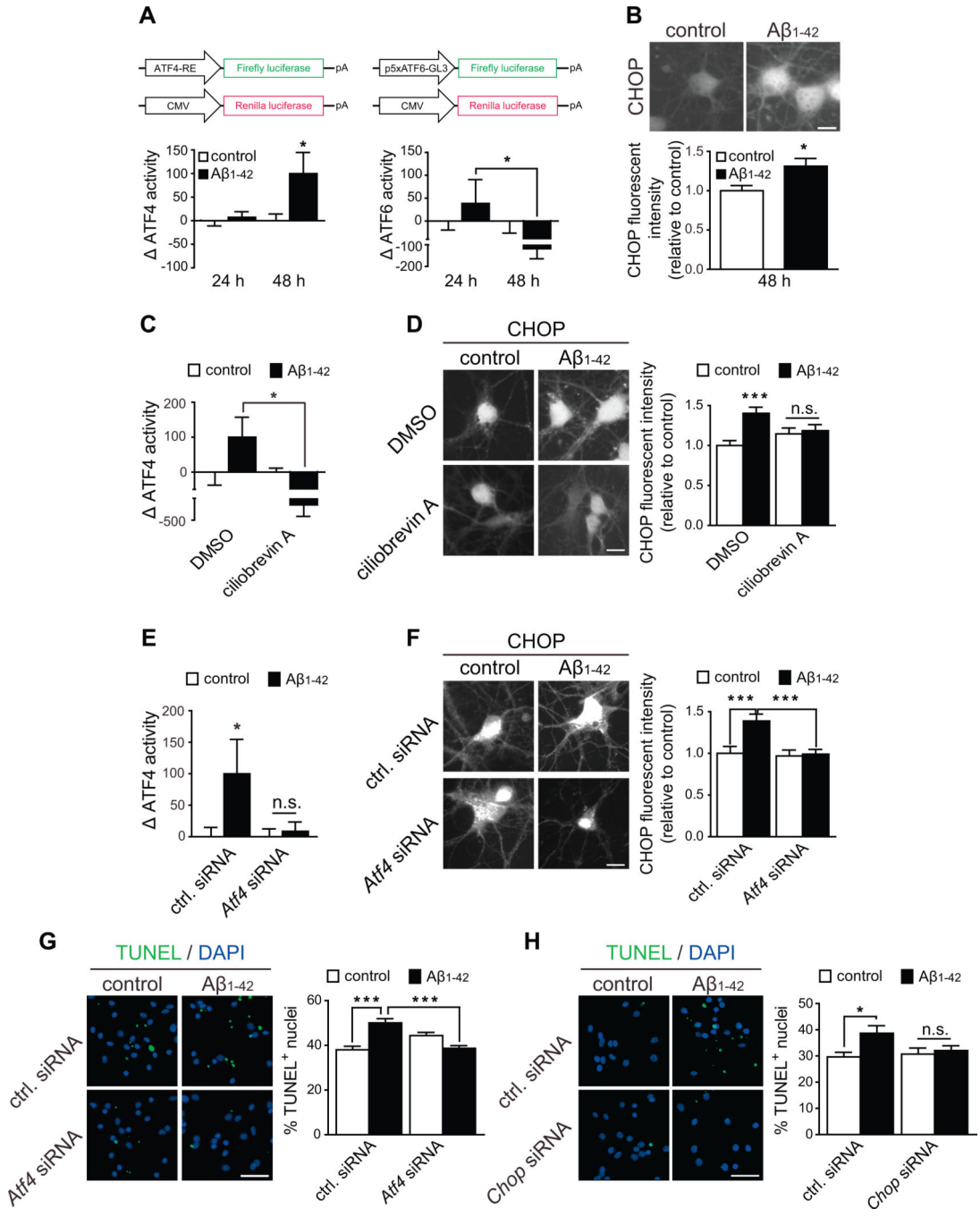

Figure 4. Axonally synthesized ATF4 induces ATF4-dependent gene expression in the nucleus and leads to retrograde somatic degeneration via CHOP

(A) Neurons were grown in microfluidic chambers and cell bodies were transfected with the reporter gene constructs 24 h before local exposure of axons to $A\beta_{1–42}$. Luciferase activities were measured in cell lysates 24 and 48 h after axons had been treated with vehicle or $A\beta_{1–42}$. Data are plotted as the ratio Firefly(RLU)/Renilla(RLU) and normalized to vehicle. The maximum increase in Firefly(RLU) activity per experiment was set to 100%. Mean ±SEM of 7–12 biological replicates per condition. *p<0.05.

(B) CHOP levels were measured in cell bodies by quantitative immunofluorescence after 48 h of local application of Aβ1–42 to axons. Mean ±SEM of 30–40 microscopy fields per condition (n=6–8 biological replicates per group). *p<0.05. Scale bar, 20 μm.

(C) Neurons were cultured as in A and axons were exposed to $A\beta_{1-42}$ oligomers for 48 h. 6 h prior to luciferase measurement axons were exposed to vehicle or ciliobrevin A. Mean ±SEM of 6–10 biological replicates per condition. *p<0.05.

(D) Axons were treated as in C. CHOP levels were measured in cell bodies by quantitative immunofluorescence. Mean ±SEM of 35–45 optical fields per condition (n=7–9 biological replicates per group). ***p<0.001. Scale bar, 20 μm.

(E) Neurons were cultured as in A and axons were transfected with control or *Atf4* siRNA 24 h before $A\beta_{1-42}$ treatment. Luciferase activities were measured and represented as in A. Mean ±SEM of 10–12 biological replicates per condition. *p<0.05.

(F) Axons were treated as in E. CHOP levels in cell bodies were measured by quantitative immunofluorescence. Mean ±SEM of 30–40 microscopy fields (n=6–8 biological replicates per group). ***p<0.001. Scale bar, 20 μm.

(G) Neurons were cultured and treated as in E. Cell bodies were processed for TUNEL staining. Mean ±SEM of 70–90 microscopy fields (n=7–9 biological replicates per group). ***p<0.001. Scale bar, 50 μm.

(H) Neurons were cultured as in A and cell bodies were transfected with control or *Chop* siRNA 24 h before $A\beta_{1-42}$ treatment. Cell bodies were processed for TUNEL staining after 48 h of $A\beta_{1-42}$ application to axons. Mean ±SEM of 60 microscopy fields (n=6 biological replicates per group). *p<0.05. Scale bar, 50 μm. See also Figure S4.

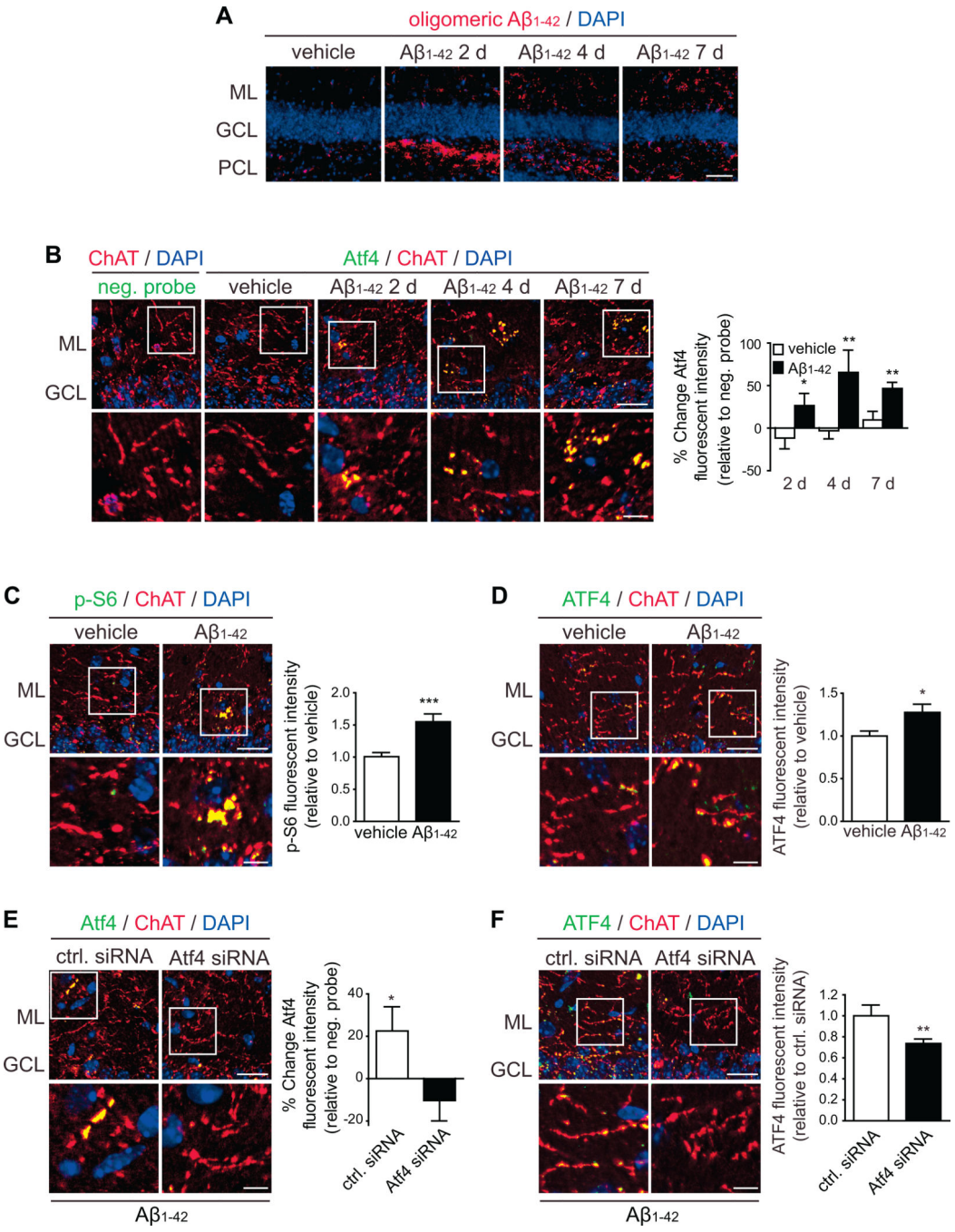

Figure 5. Intra-hippocampal injection of $A\beta_{1–42}$ induces synthesis of ATF4 in BFCN axons

(A) Presence of $A\beta_{1–42}$ in the DG of mice injected with vehicle and Aβ1–42 oligomers 2 to 7 DPI. 4 to 5 mice were analyzed per condition. ML, molecular layer; GCL, granule cell layer; PCL, polymorphic cell layer. Scale bar, 50 μm.

(B) FISH *for Atf4* mRNA in the DG of mice injected with vehicle and $A\beta_{1–42}$. BFCN axons were identified by ChAT immunostaining. Cell bodies were counterstained with DAPI. Mean ±SEM of measurements performed in 3–4 brain slices per mouse (n=4 mice per

group). Background fluorescence was determined non-targeting probe signal and set to zero. *p<0.05; **p<0.01. Scale bars, 20 μm, 5 μm (insets).

(C) Phosphorylation levels of ribosomal protein S6 within ChAT-positive axons were measured by quantitative immunofluorescence on brain sections 7 DPI. Mean ±SEM of measurements typically performed in 4 brain slices per mouse (n=4 mice). ***p<0.001.Scale bars, 20 μm, (insets, 5 μm).

(D) ATF4 protein levels within ChAT-positive axons were measured by quantitative immunofluorescence on brain sections 7 DPI. Mean ±SEM of measurements typically performed in 4 brain slices per mouse (n=4 mice). *p<0.05. Scale bars, 20 μm, (insets, 5 μm).

(E) Mice were injected with $A\beta_{1–42}$ oligomers in both hemispheres of the brain. The left hemisphere was co-injected with a control (ctrl.) siRNA and the right hemisphere with an *Atf4* siRNA. The presence of *Atf4* mRNA within ChAT-positive axons was analyzed by FISH 7 DPI. Mean ±SEM of measurements typically performed in 3 brain slices per mouse (n=3 mice). Background fluorescence was determined non-targeting probe signal and set to zero. *p<0.05. Scale bars, 20 μm, (insets, 5 μm).

(F) Mice were injected as in E. ATF4 protein levels within ChAT-positive axons were measured by quantitative immunofluorescence on brain sections 7 DPI. Mean ±SEM of measurements typically performed in 4 brain slices per animal (n=4 mice). **p<0.01. Scale bars, 20 μm, (insets, 5 μm).

See also Figure S5.

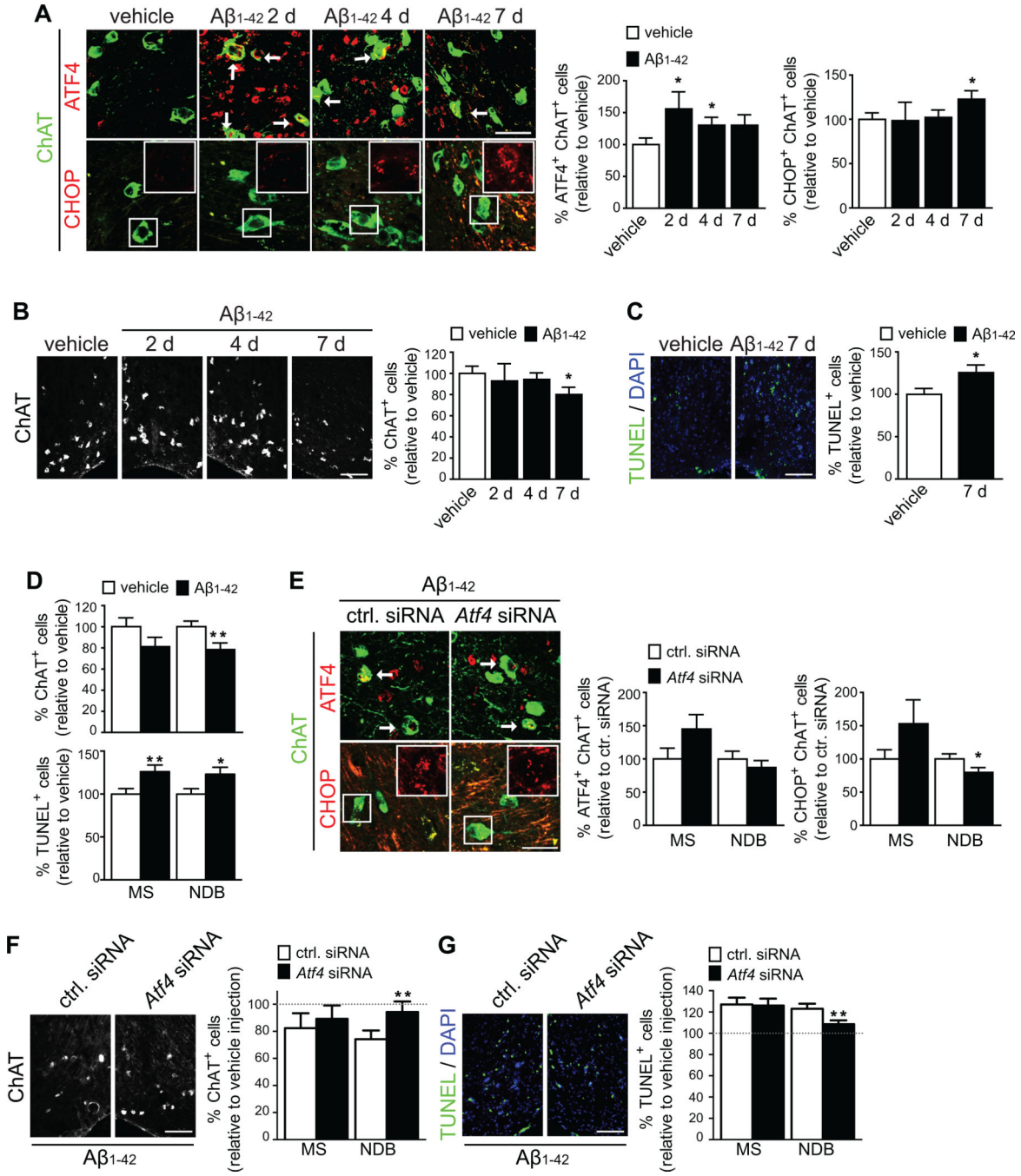

Figure 6. Intra-axonal synthesis of ATF4 leads to neurodegeneration in the adult mouse brain

(A) Mice were injected with vehicle in the left hemisphere of the brain and with $A\beta_{1-42}$ in the contralateral hemisphere. Sections of the basal forebrain were immunostained for ChAT and ATF4 or CHOP 2 to 7 DPI. Mean ±SEM of positive cells relative to vehicle in ~8 brain slices per animal (n=4–5 mice per condition). *p<0.05. Scale bar, 50 μm.

(B) ChAT-positive neurons in the basal forebrain of injected mice. Mean ±SEM of ChAT-positive neurons relative to the vehicle injected side in ~8 brain slices per animal (n=4–5 mice per condition). *p<0.05. Scale bar, 100 μm.

(C) TUNEL-positive cells in the basal forebrain of injected mice 7 DPI. Mean ±SEM of TUNEL-positive cells relative to the vehicle injected side in ~8 brain slices per mouse (n=5 mice). *p<0.05. Scale bar, 100 μm.

(D) Comparison of the effect of $A\beta_{1–42}$ injection on ChAT- and TUNEL-positive cells in the MS and NDB 7 DPI. Mean ±SEM of positive cells in ~8 brain slices per mouse (n=5 mice). *p<0.05; **p<0.01.

(E) $A\beta_{1–42}$ injections were performed in both hemispheres of the brain. A control (ctrl.) siRNA was co-injected into the left hemisphere and an *Atf4* siRNA was co-injected in the right hemisphere. Basal forebrain sections were immunostained for CHAT and ATF4 or CHOP. ATF4- and CHOP-positive cholinergic neurons were quantified in the MS and NDB. Mean ±SEM of double-positive cells relative to ctrl. siRNA in ~8 brain sections per animal (n=5 mice). *p<0.05. Scale bar, 50 μm.

(F) Mice were injected as in E. ChAT-positive neurons in the basal forebrain of injected mice were quantified in the MS and NDB. Mean ±SEM of ChAT-positive neurons relative to ctrl. siRNA in ~8 brain slices per animal (n=5 mice per condition). **p<0.01. Scale bar, 100 μm.

(G) TUNEL-positive cells in the forebrain of injected mice. Mean ±SEM of TUNEL-positive cells relative to ctrl. siRNA in ~8 brain slices per mouse (n=5 mice). **p<0.01. Scale bar, 100 μm.

See also Figure S6 and Supplemental Table S2.

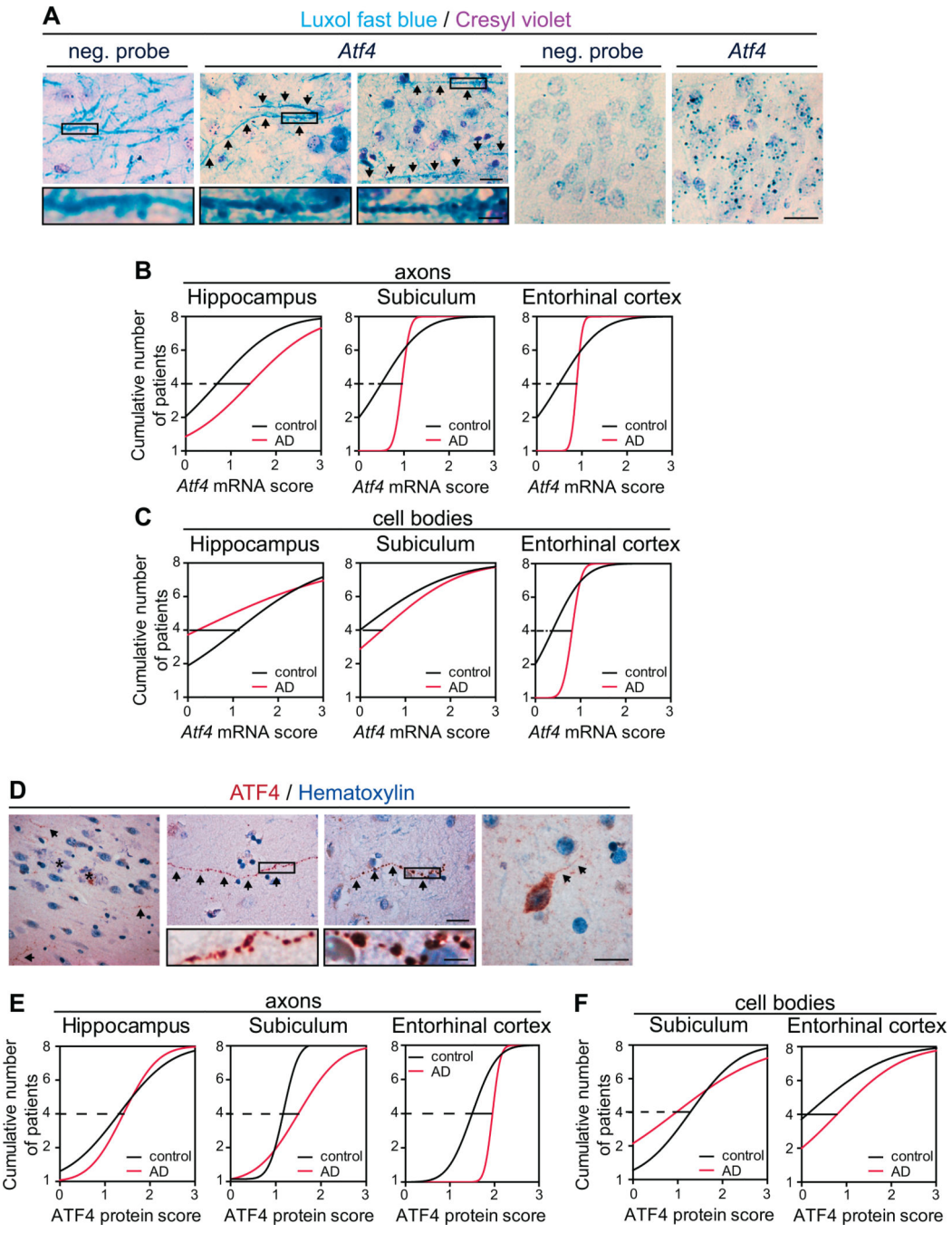

Figure 7. Presence of *Atf4* mRNA granules and ATF4 protein in axons and axonal-like structures in the AD brain

(A) Representative micrographs of *Atf4* mRNA granules in axons and cell bodies in human brain samples. Panels 1–3: axons stained with luxol fast blue and a negative probe or an *Atf4*-targeting probe. *Atf4*-containing axons are indicated with arrows. Panels 4–5: examples of granule cells stained with cresyl violet and a negative or *Atf4*-targeting probe. Scale bars, 20 μm (Insets, 5 μm).

(B) Cumulative frequency distributions of *Atf4*-containing axons in the hippocampus, the subiculum, and the entorhinal cortex of control and AD cases (n=8 brains per condition).
(C) Cumulative frequency distributions of *Atf4*-containing cell bodies in the hippocampus, the subiculum, and the entorhinal cortex of control and AD cases (n=8 brains per condition).
(D) Representative micrographs of ATF4 protein in processes and cell bodies in human brain samples. First panel: an ATF4-positive process (arrows) in the vicinity of amyloid plaques (asterisks). Second panel: a relatively intact ATF4-positive process. Third panel: a beaded process. Fourth panel: A positive cell body and neurite (arrows). Scale bars, 20 μm (insets, 5 μm).
(E) Cumulative frequency distributions of ATF4-positive processes axons in the hippocampus, the subiculum, and the entorhinal cortex of control and AD cases (n=8 brains per condition).
(F) Cumulative frequency distributions of ATF4-positive cell bodies in the subiculum and the entorhinal cortex of control and AD cases (n=8 brains per condition).

2.3. Discussion

In line with our hypothesis, we determined that axonal application of $A\beta_{1-42}$ leads to the recruitment of a distinct subset of functionally relevant transcripts, including *Atf4*. By studying the temporal regulation of *Atf4* transcripts, ATF4 protein and it's upstream regulator, p-eIF2α, I showed that $A\beta_{1-42}$ induces the localization and translation of *Atf4* fairly rapidly into axons, within six hours of $A\beta_{1-42}$ treatment. Upon its synthesis in axons, ATF4 is retrogradely transported to the cell body, where it induces pro-apoptotic gene expression, which is required for $A\beta_{1-42}$-induced cell death. This was confirmed *in vitro* and *in vivo*, and studies in post-mortem human brains of neurologically healthy and AD patients demonstrated increased prevalence of *Atf4* transcripts and ATF4 proteins in axons of AD brains. This study illuminates axonal protein synthesis, specifically of ATF4, as a potential pathogenic pathway that is activated very early on in AD progression in response to oligomeric $A\beta_{1-42}$, likely prior to the appearance of symptoms in patients.

ATF4 is a particularly interesting protein in the context of neurodegeneration as studies have demonstrated that inhibition of neuronal ATF4 expression leads to increased neuronal protection from deleterious agents such as stroke and ER stress, suggesting a pro-apoptotic role of ATF4 [180, 181]. Moreover, increased levels of phosphorylated eIF2α, a modulator of the endoplasmic reticulum stress (ERS) pathway and upstream activator of ATF4, have been found in the substantia nigra (SN) of PD patients compared to controls [182]. Similarly, we found that $A\beta_{1-42}$ induces axonal eIF2α

phosphorylation in parallel with axonal ATF4 expression. Intriguingly, ATF4 can act pro- or anti-apoptotically, depending on the context. In line with a pro-survival role of ATF4, ATF4-null mice are more sensitive to DNA-damaging agents, and mutations within ATF4 that confer activation reduce glutamate toxicity [181, 183]. Furthermore, ATF4 protects neurons from apoptosis in cellular PD models [184]. Our study demonstrates that in the context of AD and $A\beta_{1-42}$-induced neurotoxicity, ATF4 acts pro-apoptotically, which induces expression of apoptotic genes, such as *CHOP*, and acts upstream of cell death.

Our study illustrates the ability of $A\beta_{1-42}$ to influence the axonal transcriptome of hippocampal neurons, which have been shown to degenerate very early in AD progression. This is reminiscent of studies from neurodevelopment, which have shown that developmental cues within the brain are capable of rapidly influencing the axonal transcriptome of various neurons [140]. One important question that was beyond the scope of this project was how $A\beta_{1-42}$ is able to influence the localization of transcripts into axons. My *in vitro* studies from this project demonstrated that $A\beta_{1-42}$ is able to recruit *Atf4* into axons fairly quickly, within six hours of axonal $A\beta_{1-42}$ application. Based on published rates of retrograde transport and anterograde RNP signaling, axonal $A\beta_{1-42}$ must be acting rapidly to apprise the cell body of the peripheral insult [132, 185, 186]. I found this finding to be particularly interesting as it suggests that immediate intra-axonal signaling mediates a somatic response to axonal $A\beta_{1-42}$, which acts upstream of *Atf4* localization to axons. Rapid, stimulus-induced protein synthesis has been demonstrated previously in injured axons, which rapidly synthesize

proteins including importin-β, vimentin, RANBP1 and STAT3 [171, 175-177]. These proteins act to form a functional retrograde injury signaling complex that informs the nucleus of the injury and mediates pro-regenerative and pro-survival gene expression, which is required for axonal regeneration and cell survival following the insult. This led me to hypothesize that similarly, axons exposed to $A\beta_{1-42}$ respond rapidly to induce synthesis of proteins that allow for $A\beta_{1-42}$-induced localization of *Atf4* into axons. Based on the finding that axonal protein synthesis is required for DRG growth cone initiation after injury, which has also been shown to be Ca^{2+}-dependent, I hypothesized that this proposed immediate $A\beta_{1-42}$-dependent axonal protein synthesis is mediated by $A\beta_{1-42}$-induced Ca^{2+} signaling [168].

There is a wealth of literature from the AD field that demonstrates that $A\beta_{1-42}$ oligomers induce intracellular Ca^{2+} dyshomeostasis [187-191]. $A\beta_{1-42}$-dependent Ca^{2+} dysregulation occurs via several different mechanisms. $A\beta_{1-42}$ can form amyloid ion channels made up of $A\beta_{1-42}$ oligomers, which mediates $A\beta_{1-42}$ neurotoxicity [192-195]. Oligomeric $A\beta_{1-42}$ also leads to a release of Ca^{2+} from the ER [41, 196]. Intracellular Ca^{2+} dyshomeostasis can also be induced by $A\beta_{1-42}$ via receptors, such as ionotropic glutamate receptors and NMDA receptors [197, 198]. While I was not particularly interested in the precise mechanism by which axonal $A\beta_{1-42}$ induces Ca^{2+} dyshomeostasis, I proposed that increased intracellular Ca^{2+} levels could regulate immediate-early $A\beta_{1-42}$-induced axonal protein synthesis, thereby regulating the downstream axonal *Atf4* signaling events that we have previously published. This hypothesis served as the basis of

Part 3 of my thesis, which is not yet published but is in preparation for submission.

Part 3.

Chapter 3. Immediate $A\beta_{1-42}$-dependent axonal protein synthesis

3.1. Introduction

As described in Part 2 of my dissertation, our lab found that $A\beta_{1-42}$ leads to the localization of numerous transcripts into axons, including *Atf4*, though the mechanism by which this occurs was unknown. One possible mechanism that could explain this quick localization event is immediate $A\beta_{1-42}$-induced protein synthesis and retrograde transport. There are several molecules that are immediately synthesized in axons following axotomy, which signal retrogradely and act upstream of a nuclear transcriptional response [171, 175-177]. Based on our findings and the axotomy literature, I hypothesized that axons exposed to $A\beta_{1-42}$ similarly induce immediate axonal protein synthesis and that these proteins travel retrogradely to stimulate a nuclear response to the degenerative stimulus, which leads to axonal *Atf4* localization and translation.

There are several different available methods for investigating mRNA translation and protein synthesis *in vitro*. One of the most well-described pathways regulating protein synthesis is the mTOR pathway, which acts upstream of two proteins, S6 and 4EBP1, to promote increased mRNA translation [159, 160]. Phosphorylation of either protein is indicative of an increased ribosomal capacity to carry out mRNA translation. An alternative, more direct method to investigate immediate protein synthesis is to measure newly synthesized proteins. There are several different techniques available that utilize “pulse-chase” techniques to visualize and measure nascent protein synthesis *in vitro*, such as AHA and puromycin [199, 200]. Both of these molecules act as

analogs of native molecules that act during the translational process and get incorporated into the nascent polypeptide. AHA is a methionine analog, which gets incorporated into every newly synthesized protein at least once, but likely several times. Following fixation, cells are processed using a catalytic reaction that reacts with the incorporated AHA molecules and allows for fluorescent detection of any proteins containing an AHA residue. The challenge, however, to using AHA is that it necessitates the depletion of methionine from the culture medium followed by addition of the AHA molecule, which requires several hours of treatment and incubation. This is not an ideal method for investigating immediate responses in axons as the methionine depletion is likely stressful and therefore may induce stress signaling in axons. Puromycin mimics tRNA molecules, and during protein synthesis, the puromycin molecule gets incorporated into the nascent peptide chain and causes premature release of the puromycylated chain from the ribosome. Using an antibody against puromycin, newly synthesized proteins can be measured via immunocytochemistry. This method is better suited for studying immediate protein synthesis in axons because it can be added along with or even after $A\beta_{1\text{-}42}$ treatment, thereby avoiding the stress that methionine depletion is likely to induce using the AHA method.

Though it is not entirely clear what is mediating rapid immediate protein synthesis in injured axons, it has been proposed that this may be a Ca^{2+}-dependent event [168]. There is myriad evidence that $A\beta_{1\text{-}42}$ oligomers induce increased intracellular Ca^{2+} via various mechanisms, providing a possible link

between extracellular $A\beta_{1-42}$ application and rapid intra-axonal protein synthesis [41, 187-198]. I tested this hypothesis using the intracellular calcium chelator, BAPTA-AM.

In order for a transcript to be immediately translated in axons, it must be already axonally localized. Transcriptomes from healthy, adult axons show that even fully developed axons contain thousands of transcripts [140, 152-154]. Transcripts encoding importin-β, vimentin, RANBP1, and STAT3 are immediately translated in axons in response to injury, and the rapid translation of these transcripts is necessary for the cell body to mount a regenerative response to axotomy [171, 175-177]. If $A\beta_{1-42}$ does indeed lead to immediate axonal protein synthesis, it is possible that this immediate intra-axonal response is identical to that of injured axons, which would suggest a conserved axonal response to general peripheral insults. Alternatively, it is plausible that axons exposed to $A\beta_{1-42}$ immediately synthesize different molecules, which would allow for specificity to be encoded regarding the specific insult. I experimentally tested this possibility using quantitative immunocytochemistry in conjunction with axonally applied siRNAs [201].

Following axotomy, immediately synthesized proteins function to form a retrograde injury-signaling complex, which acts upstream of pro-regenerative gene expression. Based on our findings that axonal $A\beta_{1-42}$ leads to a distinct, functionally relevant axonal transcriptome within 24 hours of treatment, it is highly likely that transcription plays a role in regulating the approximately 2000 transcripts, including *Atf4*, that are recruited into axons. I tested this hypothesis

using a transcriptional inhibitor and observed whether $A\beta_{1-42}$ can still induce axonal *Atf4* translation. I also investigated whether somatic levels of *Atf4* are changed rapidly after $A\beta_{1-42}$ application using qRT-PCR.

Here, I tested my overarching hypothesis that axonal $A\beta_{1-42}$ leads to the immediate, Ca^{2+}-dependent translation of pre-localized transcripts in axons. I also tested the specificity of immediately synthesized molecules between axotomy and $A\beta_{1-42}$ treatment, and I examined the contribution of transcription to downstream axonal *Atf4* translation.

3.2. Immediate axonal mRNA translation in response to $A\beta_{1-42}$

3.2.1. Immediate activation of translational machinery in response to $A\beta_{1-42}$

To determine whether axonal exposure to oligomeric $A\beta_{1-42}$ induces a rapid activation of mRNA translation in axons, I cultured primary hippocampal neurons in microfluidic chambers, which allow for fluidic isolation of axons from their somatic and dendritic counterparts. I treated axons with 3 μM oligomeric $A\beta_{1-42}$, which our lab previously found to be equivalent to approximately 250-500 nM. (Due to the hydrophobic nature of the microfluidic chambers, the oligomers must be provided in excess compared to standard dissociated cultures to reach the effective concentration required to induce cell death.) I treated axons with $A\beta_{1-42}$ for 15, 30 and 60 minutes and subsequently fixed and immunostained for standard markers indicative of increased mRNA translation: phosphorylated S6 and phosphorylated 4EBP1 (Figures 3-1 and 3-2). Details regarding experimental methodologies and statistical analyses performed are provided in the Appendix. Immunofluorescence levels were measured for both the phosphorylated and total

forms of S6 and 4EBP1, and the ratios of phosphorylated to total levels for each protein were determined. Within fifteen minutes there were detectable increases in the ratios of phosphorylated to total S6 and 4EBP1, indicative of an enhanced mRNA translational capacity in axons. Thirty minutes after $A\beta_{1\text{-}42}$ treatment, the ratio of phosphorylated to total S6 further increased above those seen at fifteen minutes. By one hour, the ratio returned to baseline, suggesting not only a rapid but also transient activation of mRNA translation. This transient activation was also observed with 4EBP1 as the levels of phosphorylated 4EBP1 returned to baseline by 30 minutes of $A\beta_{1\text{-}42}$ treatment.

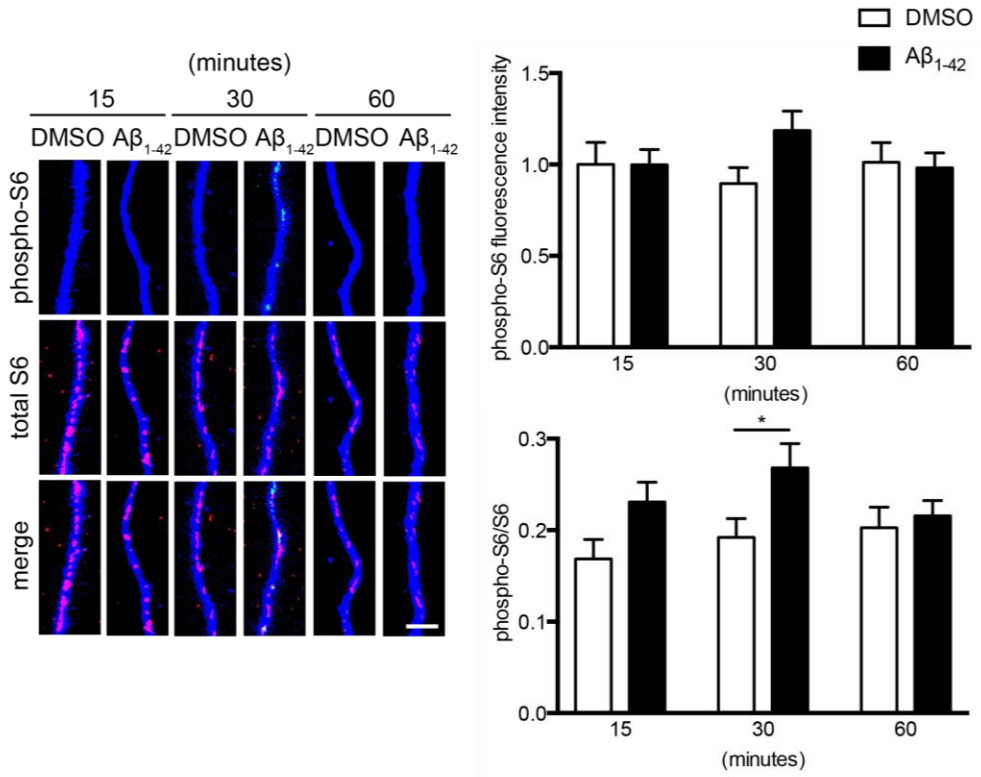

Figure 3-1. Locally applied $A\beta_{1-42}$ rapidly induces S6 phosphorylation
Hippocampal neurons were cultured in microfluidic chambers for 11-12 DIV and axons were treated with vehicle or $A\beta_{1-42}$ for 15, 30 or 60 min. Axons were immunostained for phospho-S6, S6, and β-III-tubulin. Mean ± SEM of 27-40 optical fields per condition (n = 6-8 biological replicates per group). * $p < 0.05$. The scale bar represents 5 μm.

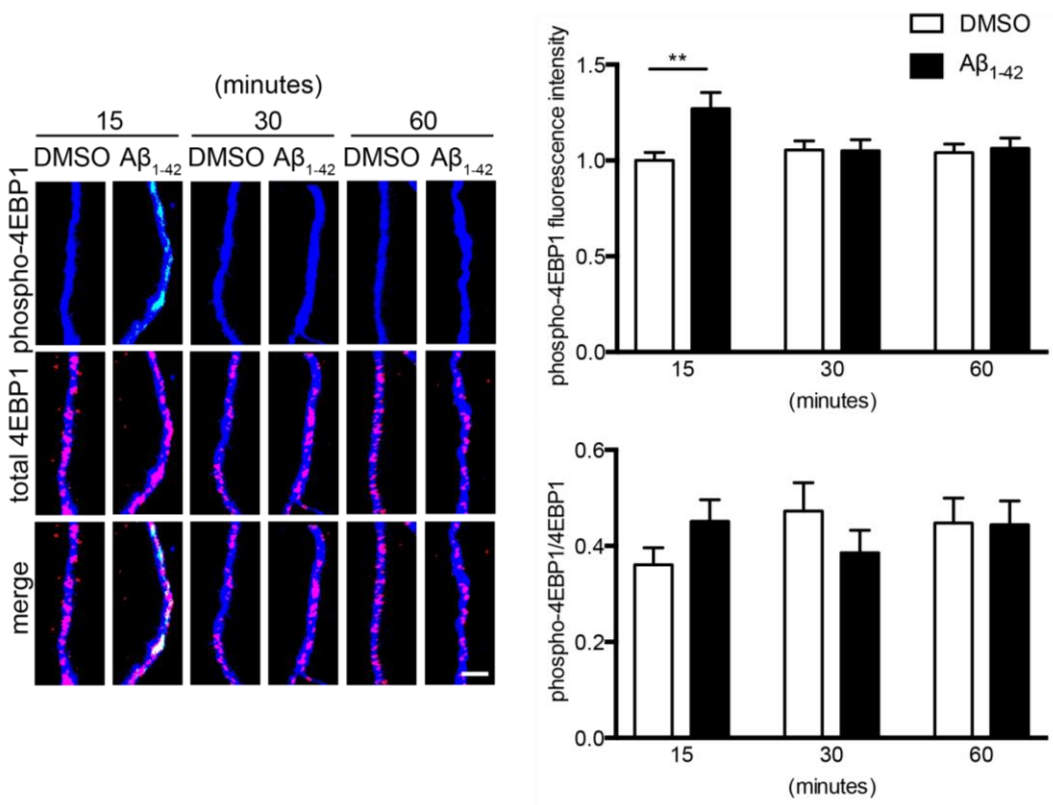

Figure 3-2. Locally applied $A\beta_{1-42}$ rapidly induces 4EBP1 phosphorylation
Axons were treated with vehicle or $A\beta_{1-42}$ for 15, 30 or 60 min. Axons were immunostained for phospho-4EBP1, 4EBP1, and β-III-tubulin. Mean ± SEM of 38-40 opticalfields per condition (n = 8 biological replicates per group). ** $p < 0.01$. The scale bar represents 5 µm.

3.2.2. Immediate protein synthesis in response to $A\beta_{1-42}$

Although the increased phosphorylation of S6 and 4EBP1 suggested that $A\beta_{1-42}$ oligomers activate the protein synthesis machinery, these are not direct indicators of increased protein synthesis itself. Puromycylation is an ideal method for investigating changes in nascent protein synthesis within short treatment periods as it has been demonstrated to achieve very high levels of resolution within minutes of treatment.

I first wanted to investigate the level of specificity of the puromycin signal, so I treated dissociated neurons cultured at low densities with puromycin for 10 minutes, and I compared puromycin levels in these neurons to levels from neurons that had not been exposed to puromycin. Using quantitative immunocytochemistry I confirmed that the presence of puromycin allows for the detection of a very specific signal (Figure 3-3). I further confirmed that the detected signal was truly due to protein synthesis by incubating neurons with the protein synthesis inhibitor, anisomycin. The presence of anisomycin was sufficient to reduce puromycin levels to levels indistinguishable from non-puromycin-treated neurons, suggesting that any detected puromycin signal is highly specific to newly synthesized proteins.

After confirming the specificity of the puromycin signal, I investigated changes in nascent axonal protein synthesis during the first 30 minutes of $A\beta_{1-42}$ treatment, during which I had observed increases in the phosphorylation of both 4EBP1 and S6. To investigate levels of newly synthesized proteins during the 20 to 30 minute window of $A\beta_{1-42}$ treatment, I added puromycin to axons 20 minutes

after $A\beta_{1\text{-}42}$ treatment (Figure 3-4). Using quantitative immunocytochemistry, I confirmed that there was indeed increased nascent protein synthesis in axons rapidly after $A\beta_{1\text{-}42}$ treatment. To investigate whether proteins were being newly synthesized at an even earlier time point, I co-incubated axons with vehicle or $A\beta_{1\text{-}42}$ along with puromycin, and I fixed cells 15 minutes afterwards (Figure 3-5). Once again I found that there was immediate protein synthesis in $A\beta_{1\text{-}42}$-treated axons during the first 15 minutes of treatment.

I further investigated whether this immediate $A\beta_{1\text{-}42}$-dependent protein synthesis event was specific to axons or if I would observe similar changes in whole cells that were treated with $A\beta_{1\text{-}42}$. In order to investigate this possibility, I incubated low-density, dissociated neurons with vehicle and $A\beta_{1\text{-}42}$ for 20 minutes, then added puromycin for an additional 10 minutes (Figure 3-6). Unlike as was observed in axons, there were no changes observed in newly synthesized proteins in response to $A\beta_{1\text{-}42}$, suggesting that the effect of $A\beta_{1\text{-}42}$ on immediate protein synthesis is indeed specific to axons.

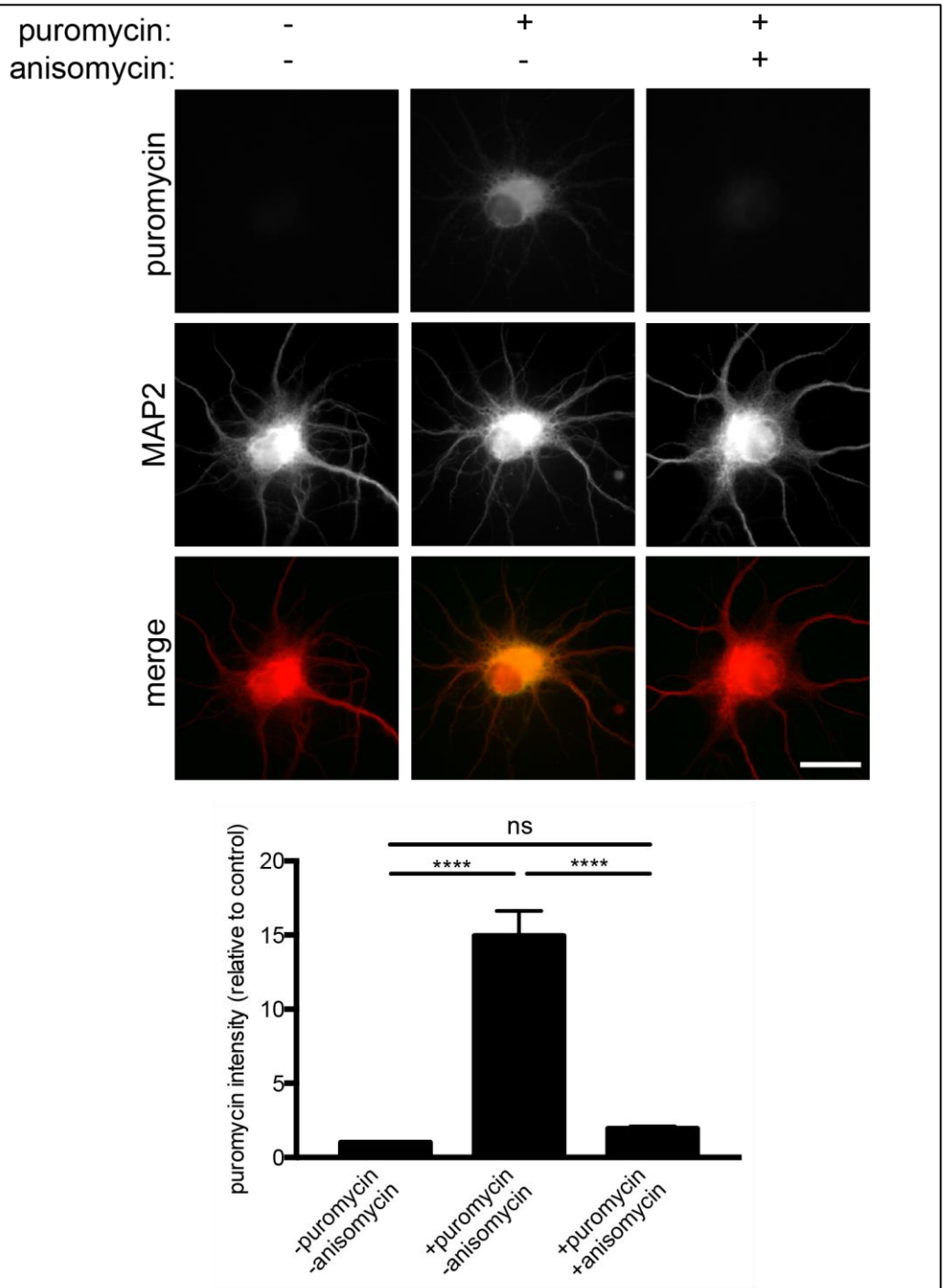

Figure 3-3. Puromycylation provides a highly specific signal
Dissociated hippocampal neurons were cultured at a low density for 11-12 DIV and were incubated in the presence or absence of puromycin (2 μM) for 10 min. Puromycin-treated cells were co-incubated with vehicle or anisomycin (10 μM). Neurons were immunostained for puromycin and MAP2. Mean ± SEM of 15-20 optical fields per condition (n = 3-4 biological replicates per group). **** $p < 0.0001$; ns, not significant. The scale bar represents 5 μm.

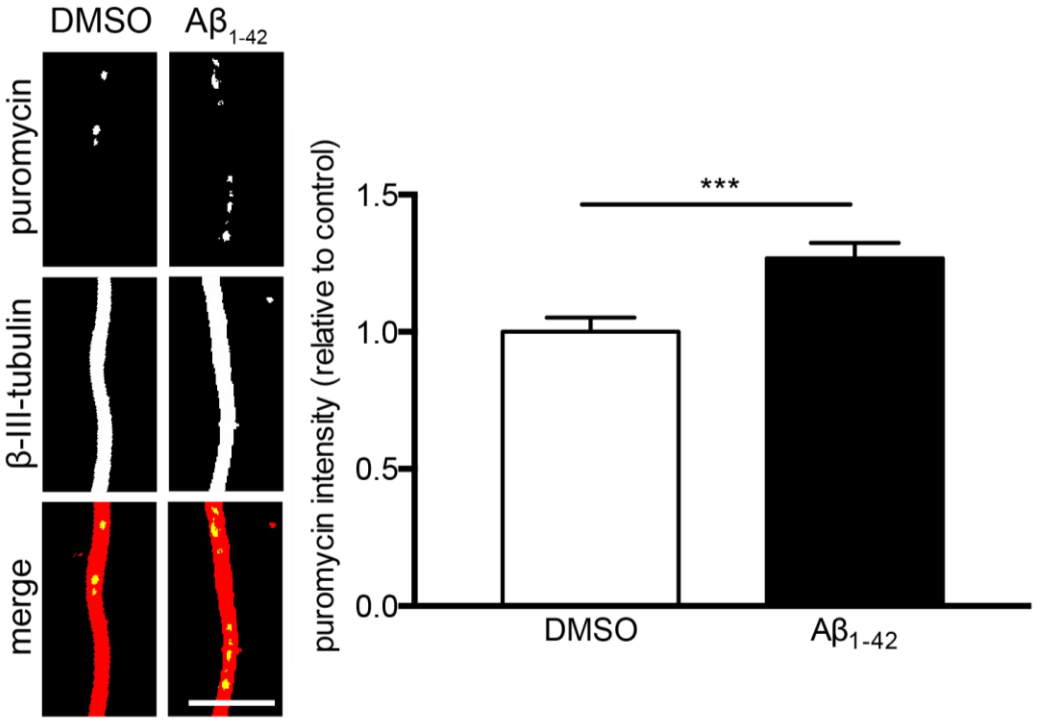

Figure 3-4. $A\beta_{1-42}$ maintains rapid axonal synthesis for 30 minutes
Axons were treated with vehicle or $A\beta_{1-42}$ for 30 min. 10 min prior to fixation, axons were treated with puromycin (2 μM). Axons were immunostained for puromycin and β-III-tubulin. Mean ± SEM of 29 optical fields per condition (n = 6 biological replicates per group). *** $p < 0.001$. The scale bar represents 5 μm.

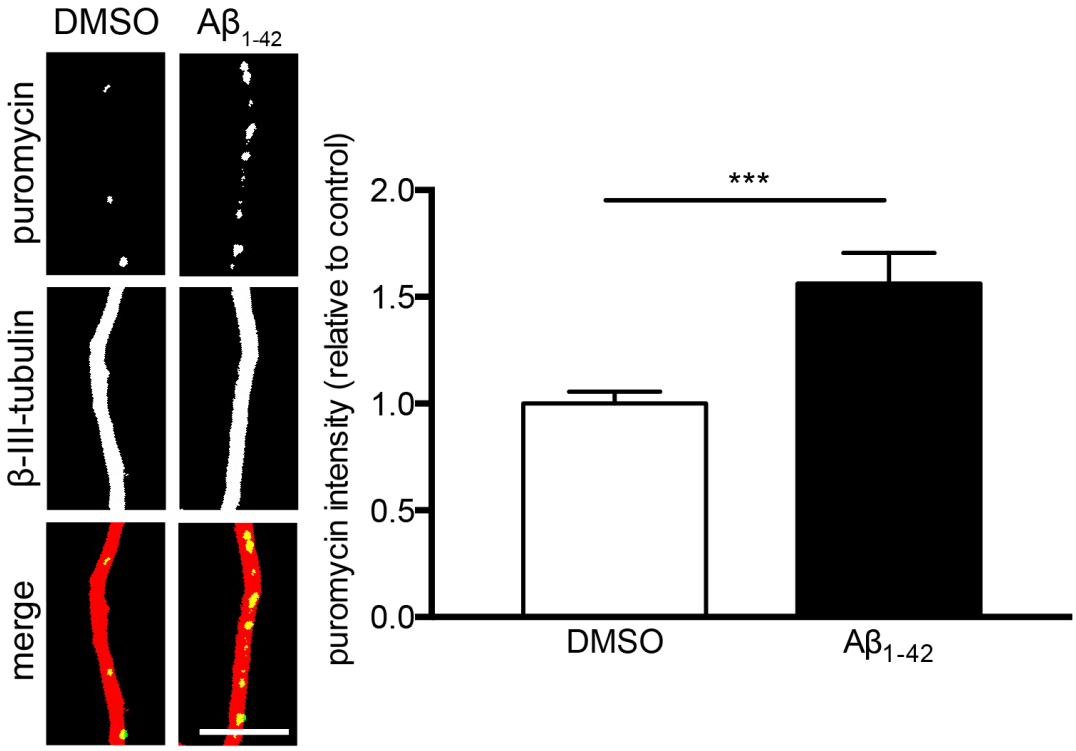

Figure 3-5. $A\beta_{1-42}$ induces rapid axonal protein synthesis
Axons were treated with puromycin (2 μM) along with vehicle or $A\beta_{1-42}$ for 15 min. Axons were immunostained for puromycin and β-III-tubulin. Mean ± SEM of 29-30 optical fields per condition (n = 6 biological replicates per group). *** $p < 0.001$. The scale bar represents 5 μm.

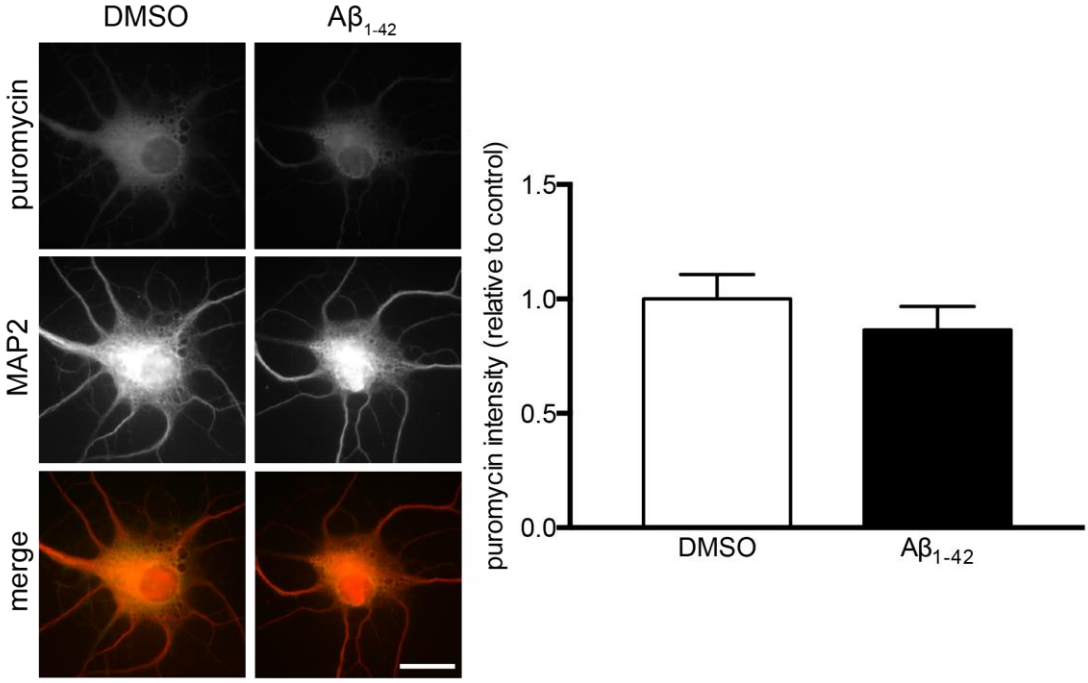

Figure 3-6. $A\beta_{1-42}$ does not induce rapid protein synthesis in dissociated neurons
Dissociated hippocampal neurons were cultured at a low density for 11-12 DIV and were treated with vehicle or $A\beta_{1-42}$ for 30 min. 10 min prior to fixation, axons were treated with puromycin (2 μM). Neurons were immunostained for puromycin and MAP2. Mean ± SEM of 18-20 optical fields per condition (n = 4 biological replicates per group).The scale bar represents 5 μm.

3.2.3. Calcium chelation blocks immediate translational activation

Numerous studies have demonstrated changes in Ca^{2+} signaling in response to oligomeric $A\beta_{1-42}$, and Ca^{2+} signaling has also been suggested to regulate immediate-early protein synthesis in axons following axotomy [41, 168, 187-198]. This suggests that Ca^{2+} signaling may be playing a crucial role in regulating $A\beta_{1-42}$-induced immediate-early axonal mRNA translation. Ca^{2+} has been shown to regulate the activation of S6 [202-204], so I investigated total and phosphorylated S6 levels in the presence and absence of a cell-permeable calcium chelator, BAPTA-AM (Figure 3-7). When axons were pre-treated with BAPTA-AM, the activation of S6 was inhibited as compared to the vehicle-treated condition. I also investigated the ability of BAPTA-AM to inhibit increased 4EBP1 phosphorylation after 15 minutes of $A\beta_{1-42}$ treatment (Figure 3-8). As expected, I detected a significant increase in levels of phosphorylated 4EBP1 in response to $A\beta_{1-42}$ in vehicle-treated axons, and this effect was completely ablated when axons were pre-treated with BAPTA-AM. This suggests that $A\beta_{1-42}$ regulates immediate-early axonal translation via Ca^{2+} signaling.

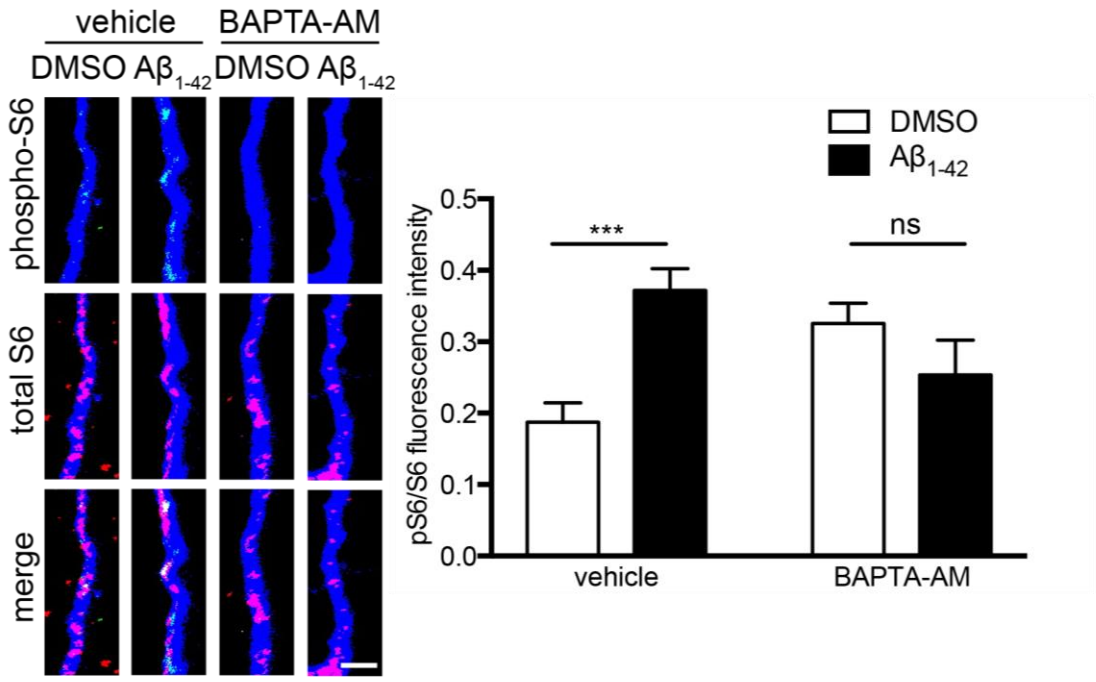

Figure 3-7. Immediate $A\beta_{1-42}$-dependent S6 activation requires Ca^{2+} signaling
Axons were treated with vehicle or BAPTA-AM (10 μM) for 30 minutes. Axons were then treated with vehicle or $A\beta_{1-42}$ for 30 min. Axons were immunostained for phospho-S6, total S6 and β-III-tubulin. Mean ± SEM of 25 optical fields per condition (n = 5 biological replicates). *** $p < 0.001$, ns, not significant. The scale bar represents 5 μm.

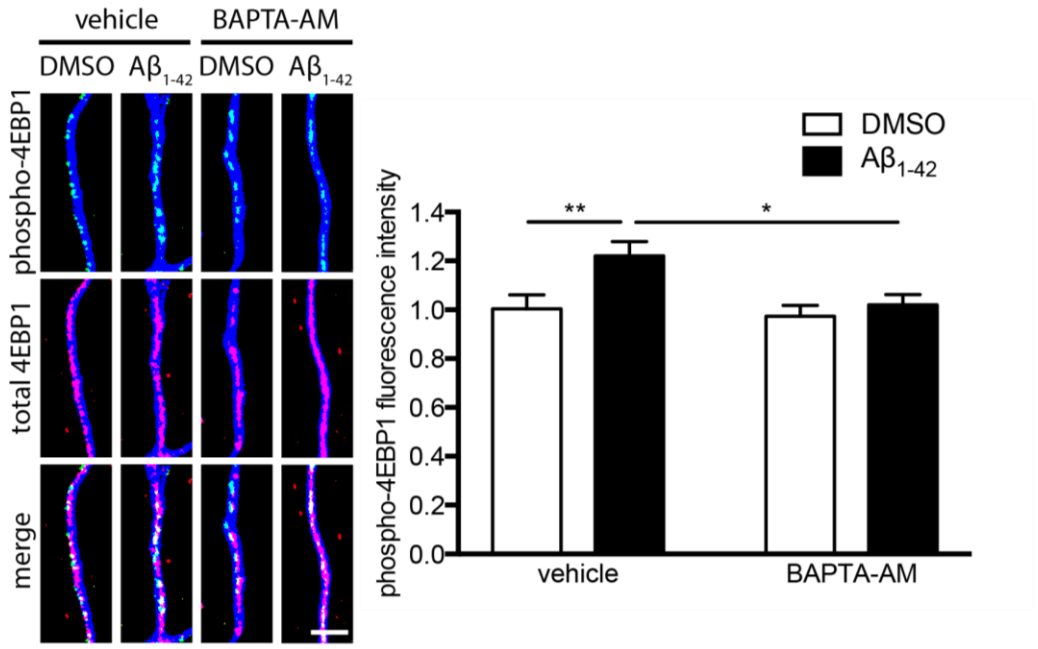

Figure 3-8. Immediate $A\beta_{1-42}$-dependent 4EBP1 activation requires Ca^{2+} signaling Axons were treated with vehicle or BAPTA-AM (10 μM) for 30 minutes. Axons were then treated with vehicle or $A\beta_{1-42}$ for 30 min. Axons were immunostained for phospho-4EBP1, total 4EBP1 and β-III-tubulin. Mean ± SEM of 19-20 optical fields per condition (n = 4 biological replicates). * $p < 0.05$, ** $p < 0.01$. The scale bar represents 5 μm.

3.3. *Atf4* localization and immediate intra-axonal signaling

3.3.1. Axonal Aβ$_{1-42}$ induces rapid localization of Atf4 to axons

To confirm increased axonal *Atf4* levels in response to Aβ$_{1-42}$, as our sequencing results suggested, I measured axonal and somatic *Atf4* levels in vehicle- and Aβ$_{1-42}$-treated axons using qRT-PCR (Figure 3-9). Using FISH, I measured *Atf4* mRNA levels 3, 6, 12, 18 and 24 hours following Aβ$_{1-42}$ treatment (Figure 3-10). *Atf4* transcript levels significantly increased in axons within 6 hours of Aβ$_{1-42}$ treatment and persisted even after 24 hours of treatment, matching what we observed with qRT-PCR and sequencing technologies.

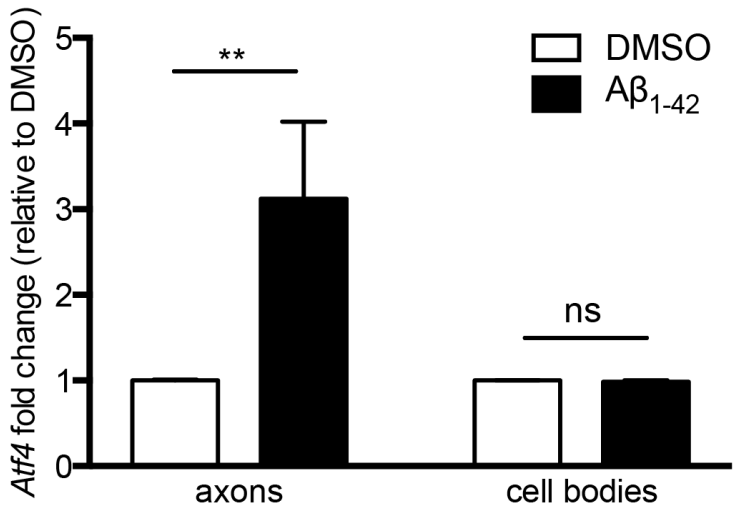

Figure 3-9. Locally applied Aβ$_{1-42}$ induces axonal *Atf4* recruitment Hippocampal neurons were cultured in microfluidic chambers for 11-12 DIV and axons were treated with vehicle or Aβ$_{1-42}$ for 24 hours. RNA was harvested from axons and cell bodies, and *Atf4* levels were measured using qRT-PCR normalized to input. Mean ± SEM of 8-9 technical replicates per condition (n = 3 biological replicates per group). ** $p < 0.01$, n.s., not significant.

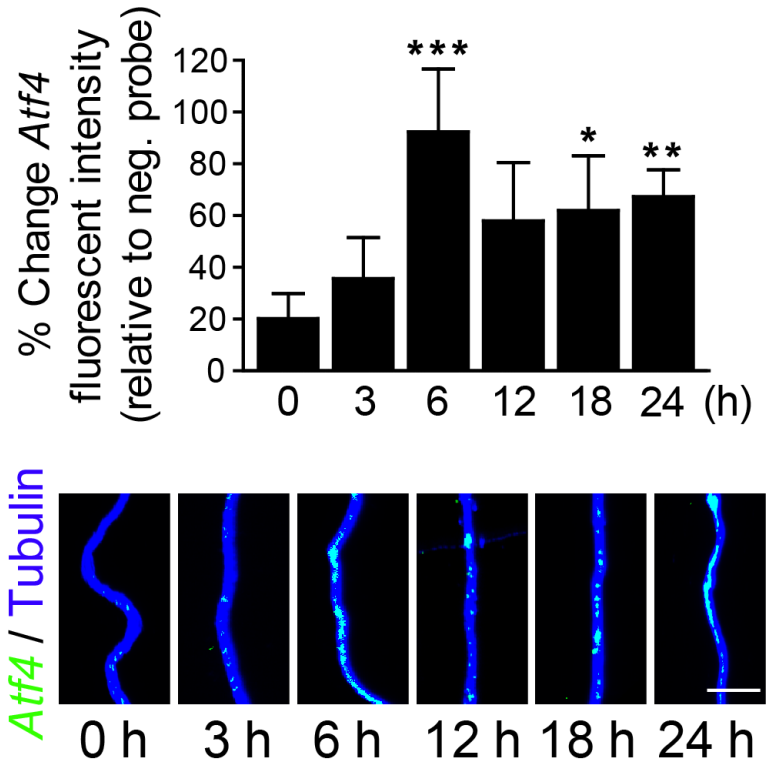

Figure 3-10. Axonal $A\beta_{1-42}$ induces rapid *Atf4* localization
Axons were treated with vehicle or $A\beta_{1-42}$ for the indicated times, and axonal *Atf4* mRNA levels were measured by quantitative FISH. Mean ± SEM of 25-40 axonal fields per condition (n = 5-8 biological replicates per group). The background fluorescence was determined using a nontargeting probe (neg. probe) and set to zero. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3.2. Axonal $A\beta_{1-42}$ induces translation of axonally localized Atf4 transcripts

The $A\beta_{-42}$-dependent localization of *Atf4* transcripts to axons suggests that ATF4 protein synthesis is induced in axons. Using immunocytochemistry, I investigated ATF4 protein levels 3, 6, 12, 18 and 24 hours following $A\beta_{1-42}$ treatment (Figure 3-11). Mirroring what was observed for transcript levels, axonal ATF4 levels significantly increased 6 hours after $A\beta_{1-42}$ treatment. This temporal regulation of *Atf4* transcripts is also in line with observed increases in eIF2α phosphorylation, an established upstream regulator of *Atf4* translation (Figure 3-12). To confirm that the increased levels of ATF4 protein at this time point were dependent upon axonal protein synthesis, I transfected axons with a scrambled siRNA or an *Atf4*-targeting siRNA prior to $A\beta_{1-42}$ treatment. I fixed cells 6 hours after $A\beta_{1-42}$ treatment and measured ATF4 protein levels using quantitative immunofluorescence (Figure 3-13). Whereas when axons treated with scrambled siRNA showed increased ATF4 levels in response to $A\beta_{1-42}$, si*Atf4*-treated axons showed no increase in ATF4 protein levels. This indicates that the $A\beta_{1-42}$-dependent increase in axonal ATF4 levels at 6 hours is due to axonal translation of localized *Atf4* transcripts.

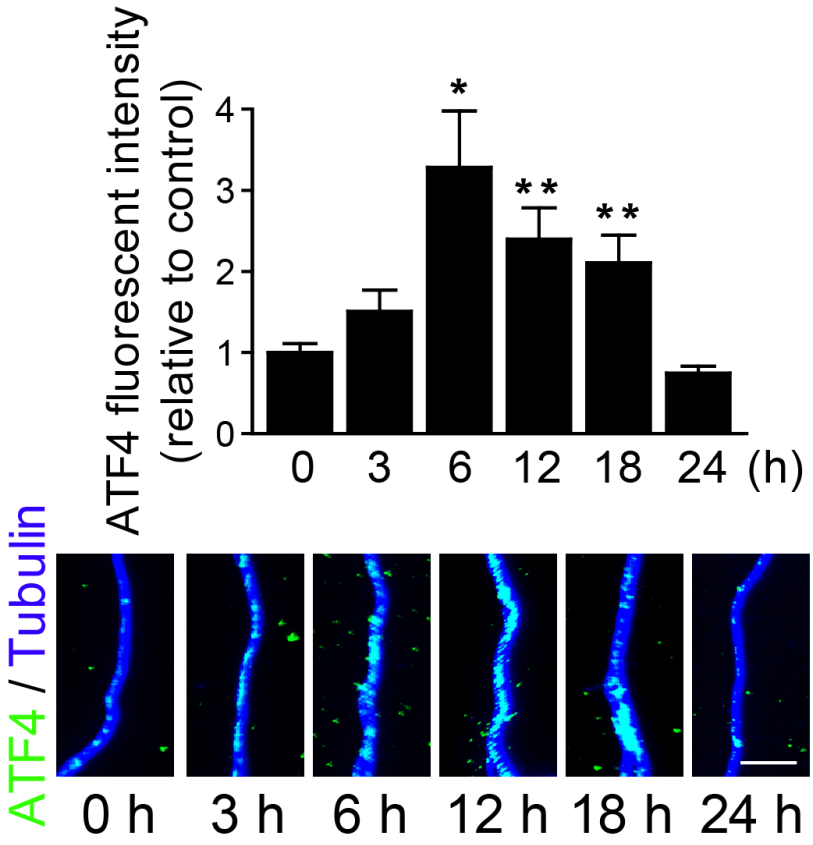

Figure 3-11. Axonal $A\beta_{1-42}$ induces rapid ATF4 synthesis
Axons were treated with vehicle or $A\beta_{1-42}$ for the indicated times, and axonal ATF4 levels were measured by quantitative ICC. Mean ± SEM of 20-40 axonal fields per condition (n = 4-8 biological replicates per group). * $p < 0.05$; ** $p < 0.01$.

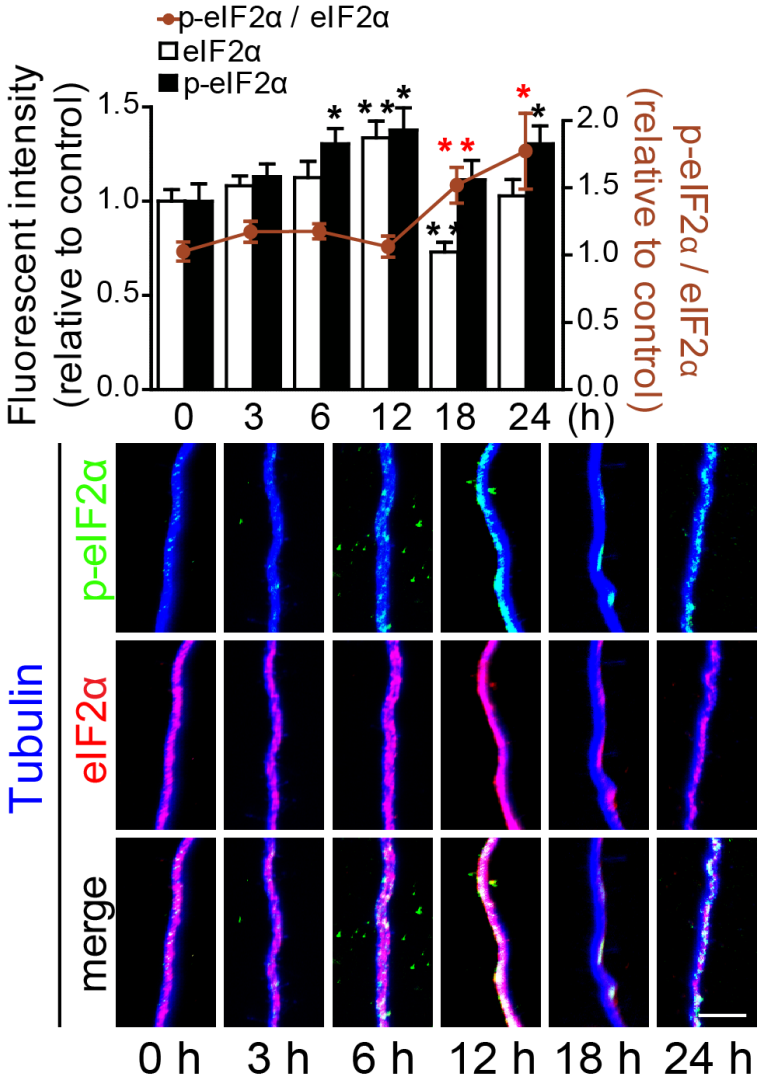

Figure 3-12. Axonal $A\beta_{1-42}$ induces eIF2α phosphorylation
Axons were treated with vehicle or $A\beta_{1-42}$ for the indicated times, and axonal eIF2α and p-eIF2α levels were measured by quantitative ICC. Mean ± SEM of 20-35 axonal fields per condition (n = 4-7 biological replicates per group). * $p < 0.05$; ** $p < 0.01$.

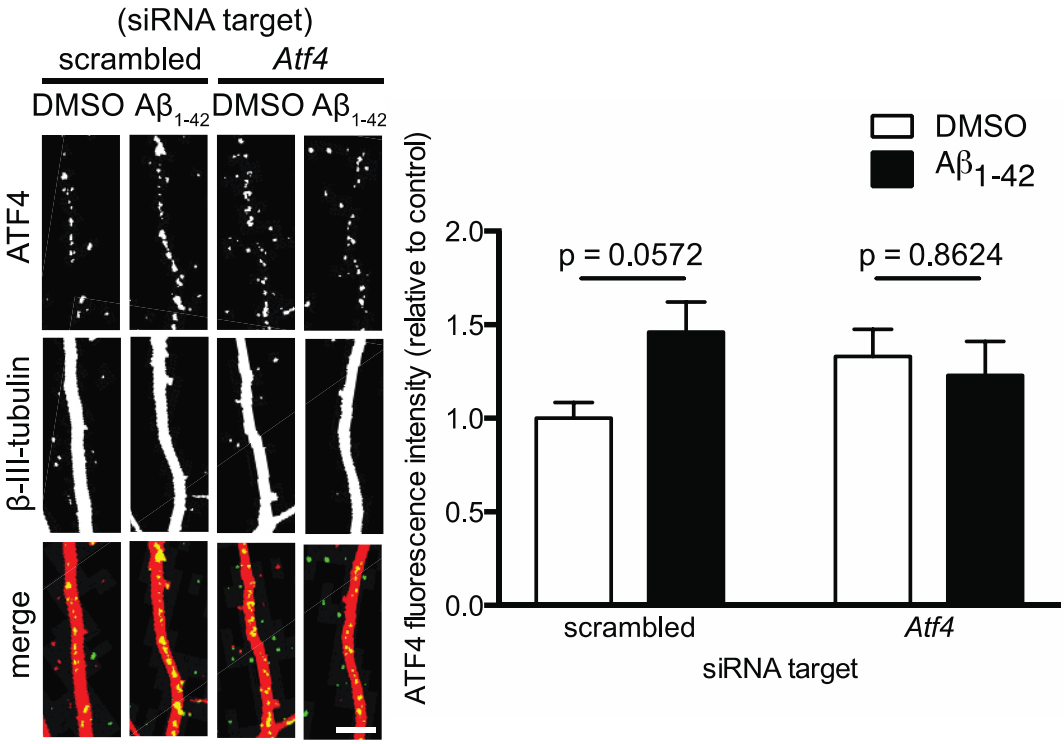

Figure 3-13. Axonal $A\beta_{1-42}$ leads to local translation of *Atf4* within 6 hours
Hippocampal neurons were cultured in microfluidic chambers for 10-11 DIV and axons were transfected with scrambled or *Atf4*-targeting siRNA. Twenty-four hours after transfection, axons were treated with vehicle or $A\beta_{1-42}$ for 6 hours. Axons were immunostained for ATF4 and β-III-tubulin. Mean ± SEM of 30 optical fields per condition (n = 6 biological replicates per group). The scale bar represents 5 μm.

3.3.3. Atf4 localization requires immediate axonal signaling

I observed that oligomeric $A\beta_{1-42}$ treatment led to increased *Atf4* levels in axons within 6 hours, and this led to increased axonal synthesis of ATF4. This poses the question regarding how $A\beta_{1-42}$ leads to increased *Atf4* levels 6 hours following treatment. The delayed localization of a transcript into axons suggests that there is a communication event between axons and somata, which regulates the recruitment of *Atf4* transcripts to axons. I hypothesized that immediate-early protein synthesis of a retrograde signaling complex could coordinate communication between $A\beta_{1-42}$-treated axons and their respective cell bodies. To determine whether my hypothesis was physically possible, I turned to the literature and referenced the speeds at which retrograde signaling complexes and anterograde RNP granules travel. The maximum length that axons can travel using our microfluidic culturing devices is 4.5 mm. At a speed of 1 μm/s [185], a retrograde signaling complex would take approximately 30-75 minutes to reach the somatic compartment. Anterograde RNPs have been estimated to travel at approximately 0.2-1 μm/s [132, 186], so it could take anywhere from about 1 to 6 hours for RNPs to travel from cell bodies to the tips of the longest axons. Given that the immediate axonal protein synthesis that I described previously lasts roughly 30 minutes, these three events could reasonably occur within 6 hours with a little time to spare for a somatic response to the incoming retrograde signaling complex, such as a transcription or RNP assembly. To determine whether immediate-early synthesis of a retrograde signaling complex did indeed regulate the subsequent localization of *Atf4* transcripts into axons, I treated

axons with pharmacological inhibitors prior to $A\beta_{1-42}$ treatment. To inhibit axonal protein synthesis, I treated axons with emetine, a protein synthesis inhibitor. I then treated axons with $A\beta_{1-42}$ for 6 hours. Cells were then subjected to FISH against *Atf4* and quantified. Pre-treatment with emetine was sufficient to block the recruitment of *Atf4* to axons, as was observed in the vehicle-treated condition (Figure 3-14). To inhibit retrograde transport, I treated axons with ciliobrevin A, a specific dynein inhibitor. Using FISH again as a readout for *Atf4* mRNA levels, retrograde transport inhibition via ciliobrevin A was determined to be sufficient to block Aβ-induced *Atf4* localization to axons (Figure 3-14).

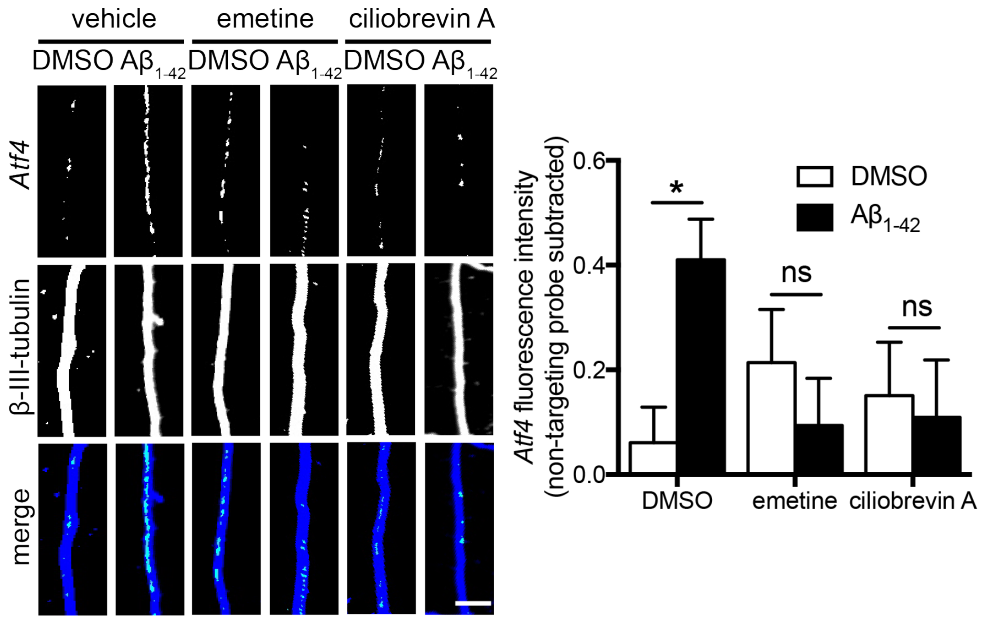

Figure 3-14. $A\beta_{1-42}$-dependent *Atf4* localization requires immediate axonal signaling
Hippocampal neurons were cultured in microfluidic chambers for 11-12 DIV and axons were treated with vehicle, emetine (100 nM) or ciliobrevin A (5 μM) for 45 min. Axons were then treated with vehicle or $A\beta_{1-42}$ for 6 hours. Neurons were fixed and *Atf4* transcript levels were measured using quantitative FISH. The background fluorescence was determined using a nontargeting probe (neg. probe) and set to zero. Mean ± SEM of 26-30 optical fields per condition (n = 6 biological replicates per group). * $p < 0.05$; ns, not significant. The scale bar represents 5 μm.

3.4. Immediate axonal vimentin synthesis

3.4.1. Immediate vimentin synthesis is required for Atf4 translation

Table 3-1. Injury-related transcripts in mature axons

Encoded Protein	Function in injury signaling	Citation	Rat Hippocampal	Mouse DRGs	Xenopus RGCs	Mouse Cortical	Mouse Motoneurons
			Baleriola (2014)	Gumy (2011)	Zivraj (2010)	Taylor (2009)	Saal (2014)
			adult	adult	Stage 32	adult	adult
importin-β	nuclear import	Hanz (2003) *Neuron*	X				X
STAT3	transcription	Ben-Yaakov (2012) *EMBO J.*	X	X	X		
vimentin	scaffolding	Perlson (2005) *Neuron*	X	X	X		X

Upon demonstrating immediate-early protein synthesis and establishing its role in regulating $A\beta_{1-42}$-induced *Atf4* localization to axons, the next logical question was to determine the identity of one or more of likely several $A\beta_{1-42}$-induced immediately synthesized proteins. Based upon the published literature, I compiled a list of proteins that have been described to play a role in retrograde injury-signaling as these proteins are likely candidates for immediate $A\beta_{1-42}$-induced retrograde signaling. Next, I narrowed down this list by cross-referencing published axonal transcriptomes from adult or mature neurons, including our own transcriptome from non-$A\beta_{1-42}$-treated axons. Three of these transcripts were found not only in our published transcriptome but also in at least one other published transcriptome (Table 3-1). I chose to investigate *Vim*, which encodes vimentin, for several reasons. First of all, it was found most consistently in published axonal transcriptomes. Second, it was found at significantly higher levels in our own transcriptome compared to *Stat3* and *Kpnb1*, which encode for

STAT3 and importin-β, respectively. Lastly, a neuropathological study shows that AD patients have significantly higher neuronal vimentin expression levels as compared to age-matched controls [205].

To determine whether local synthesis of vimentin played a role in regulating the recruitment or translation of *Atf4* mRNA in axons, I transfected axons with either scrambled or *Vim*-targeting siRNAs prior to $A\beta_{1\text{-}42}$ treatment. Axons only were treated with oligomeric $A\beta_{1\text{-}42}$ and were fixed 6 hours later and subsequently immunostained for ATF4. ATF4 protein levels were then measured using quantitative ICC (Figure 3-15). Axons transfected with a scrambled, non-targeting siRNA showed a significant increase in ATF4 levels between vehicle- and $A\beta_{1\text{-}42}$-treated axons, as we have seen previously. However, when axons were transfected with siRNA targeting *Vim*, axons no longer showed significant increases in ATF4 protein in response to $A\beta_{1\text{-}42}$. This suggests that the $A\beta_{1\text{-}42}$-induced immediate translation of vimentin is required for the translation of *Atf4* transcripts in axons.

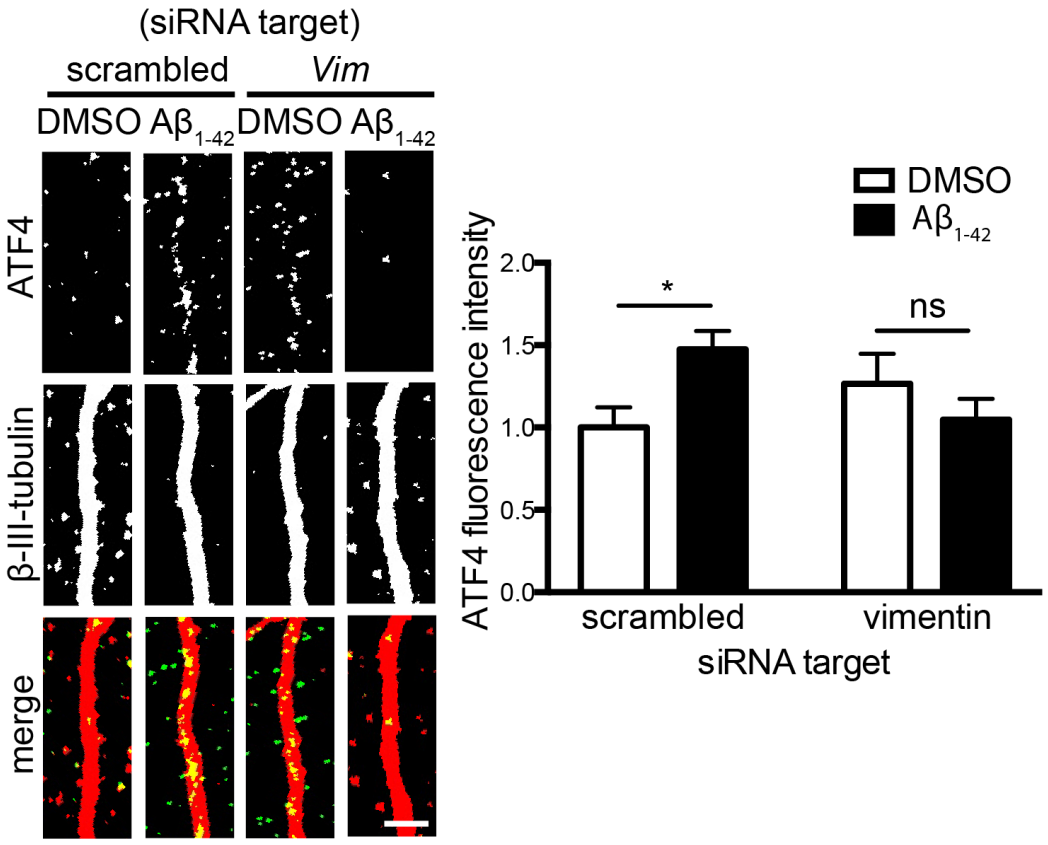

Figure 3-15. $A\beta_{1-42}$-dependent axonal ATF4 synthesis requires immediate axonal *Vim* translation

Hippocampal neurons were cultured in microfluidic chambers for 10-11 DIV and axons were transfected with scrambled or *Vim*-targeting siRNA. Twenty-four hours after transfection, axons were treated with vehicle or $A\beta_{1-42}$ for 6 hours. Axons were immunostained for ATF4 and β-III-tubulin. Mean ± SEM of 30-35 optical fields per condition (n = 6-7 biological replicates per group). * $p < 0.05$. The scale bar represents 5 μm.

3.4.2. Vimentin is immediately synthesized in axons in response to Aβ$_{1-42}$

To more directly confirm that vimentin is indeed immediately synthesized in axons following exposure to oligomeric Aβ$_{1-42}$, I investigated vimentin protein levels 30 minutes after Aβ$_{1-42}$ treatment (Figure 3-16). Interestingly, I observed a significant decrease in axonal vimentin levels compared to vehicle-treated axons. This result was not unexpected as our evidence suggests that the locally synthesized proteins that are immediately synthesized in response to Aβ$_{1-42}$ travel retrogradely to signal to the nucleus. If this is the case for vimentin, it may not be possible to detect any Aβ$_{1-42}$-induced changes due to the fact that it is being retrogradely transported to the cell body immediately following its synthesis. In order to investigate this possibility, I pre-treated axons with the dynein inhibitor, ciliobrevin A, which inhibits retrograde transport and should allow for locally synthesized vimentin to accumulate in axons. Additionally, I transfected axons with scrambled and *Vim*-targeting siRNAs to confirm that any observed changes in vimentin levels were indeed due to local translation of *Vim*. Following ciliobrevin A treatment, I treated axons with vehicle and Aβ$_{1-42}$ for 1 hour and immunostained axons for vimentin (Figure 3-17). When axons were transfected with scrambled siRNA, I observed increased levels of axonal vimentin in response to Aβ$_{1-42}$. However, axons that were transfected with *Vim*-targeting siRNA showed no increase in vimentin levels, suggesting that vimentin was indeed locally synthesized in axons in an Aβ$_{1-42}$-dependent manner. Together, this data suggests that upon its Aβ$_{1-42}$-induced axonal synthesis, vimentin is retrogradely trafficked to the cell body, as is seen in injured axons.

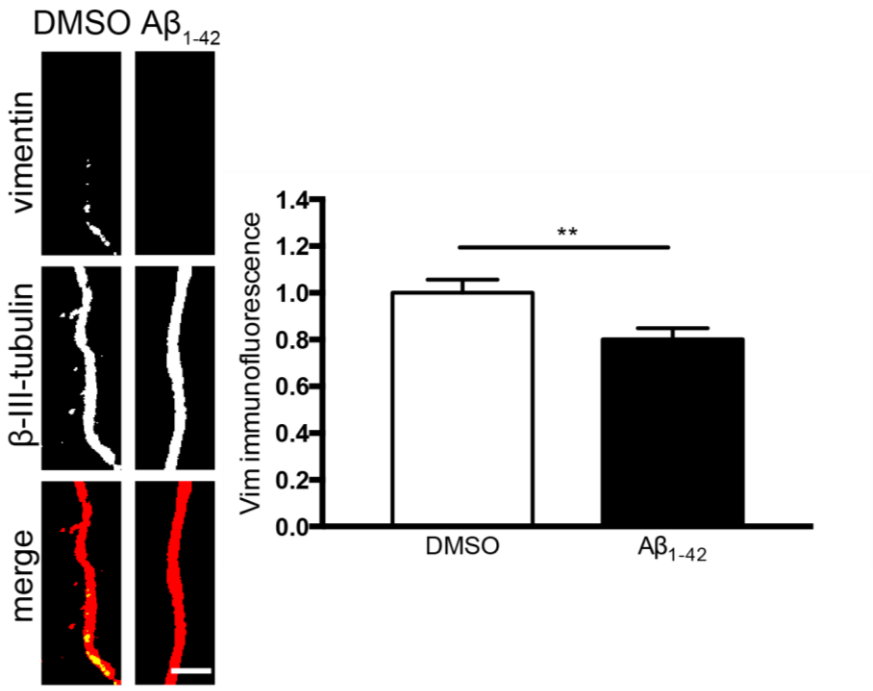

Figure 3-16. Local $A\beta_{1-42}$ leads to rapidly decreased axonal vimentin
Axons were treated with vehicle or $A\beta_{1-42}$ for 30 min. Axons were immunostained for vimentin and β-III-tubulin. Mean ± SEM of 30 optical fields per condition (n = 6 biological replicates per group). ** $p < 0.01$. The scale bar represents 5 μm.

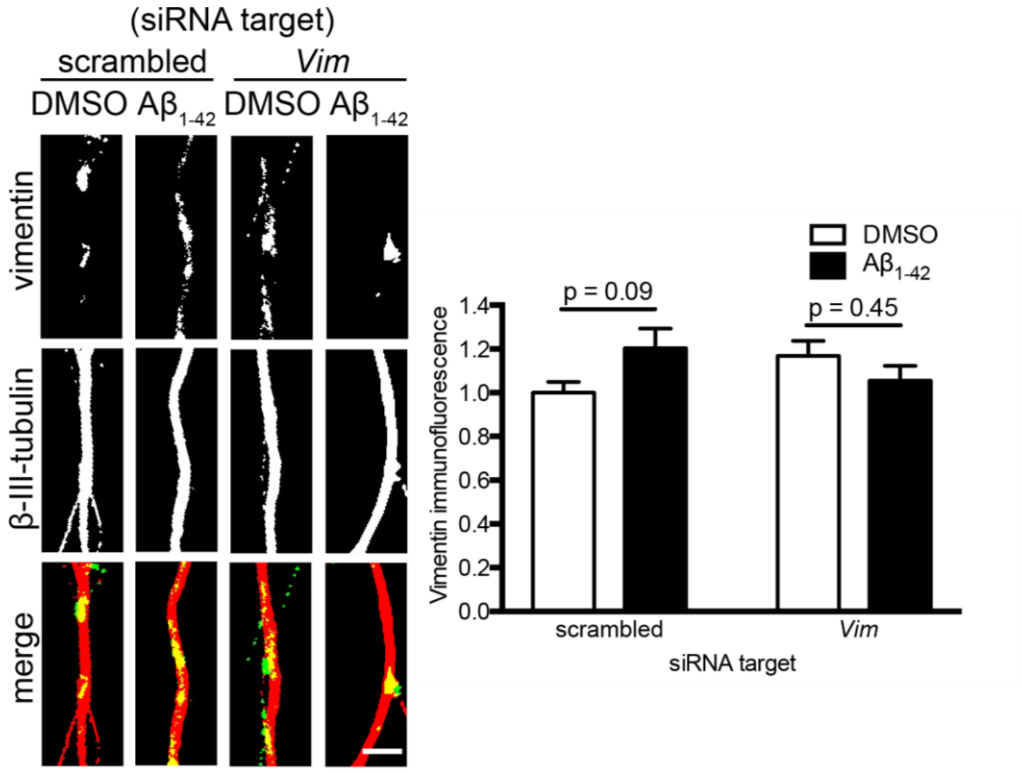

Figure 3-17. $A\beta_{1-42}$ induces immediate axonal vimentin synthesis
Hippocampal neurons were cultured in microfluidic chambers for 10-11 DIV and axons were transfected with scrambled or *Vim*-targeting siRNA. Twenty-four hours after transfection, axons were treated with ciliobrevin A (5 μM) for 45 min followed by vehicle or $A\beta_{1-42}$ for 1 hour. Axons were immunostained for vimentin and β-III-tubulin. Mean ± SEM of 43-45 optical fields per condition (n = 9 biological replicates per group). The scale bar represents 5 μm.

3.5. Immediate-early axonal signaling and transcription

3.5.1. Transcription is partially required for Atf4 translation

To test whether transcription is required for $A\beta_{1-42}$-induced axonal *Atf4* translation to occur, cell bodies were treated with the transcriptional inhibitor, actinomycin D, simultaneously with axonal $A\beta_{1-42}$ treatment. After 6 hours of $A\beta_{1-42}$ treatment, cells were fixed and stained for ATF4 protein. Relative protein levels were determined using quantitative immunofluorescence (Figure 3-18). As expected, $A\beta_{1-42}$-treated axons showed significantly higher levels of ATF4 protein in control, vehicle-treated cells. Cells treated with actinomycin D also showed increased axonal ATF4 levels, but to a lower, non-significant degree. This partial inhibition of $A\beta_{1-42}$-induced ATF4 synthesis suggests that while transcription does play a role in regulating axonal *Atf4* translation, there are other factors beyond transcription that act in response to $A\beta_{1-42}$ to regulate axonal *Atf4*.

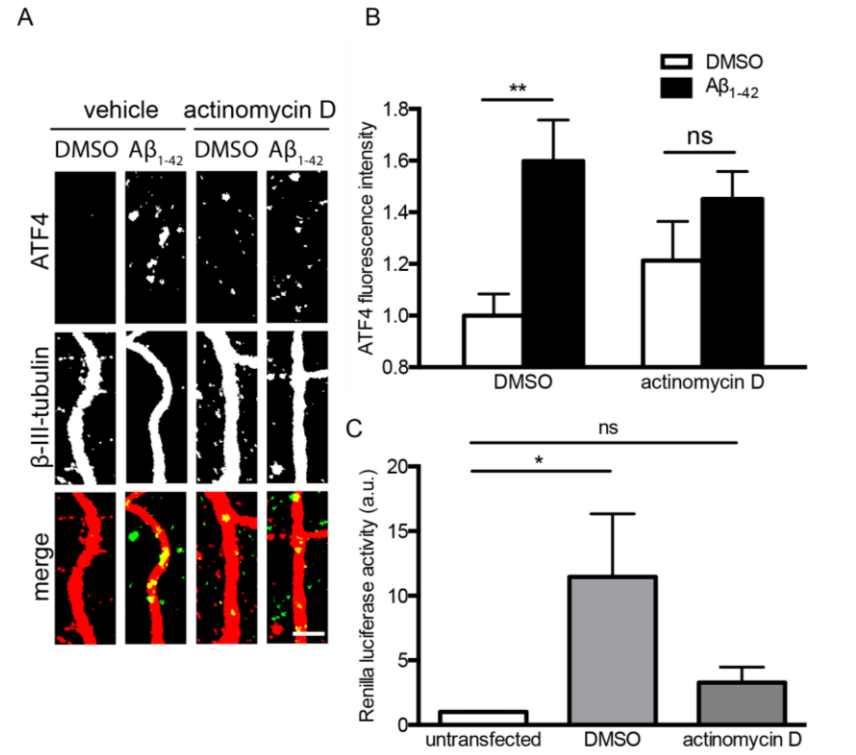

Figure 3-18. Transcription is partially required for $A\beta_{1-42}$-dependent axonal ATF4 synthesis
(A-B) Cell bodies were treated with actinomycin D (40 μM) and axons were subsequently treated with vehicle or $A\beta_{1-42}$ for 6 hours. Axons were immunostained for ATF4 and β-III-tubulin. Mean ± SEM of 39-40 optical fields per condition (n = 8 biological replicates). ** $p < 0.01$, ns, not significant. The scale bar represents 5 μm.
(C) Hippocampal neurons were cultured in microfluidic devices for 10-11 DIV. Cell bodies were transfected with *Renilla* luciferase plasmid. Twenty-four hours after transfection, cell bodies were treated with actinomycin (40 μM) for 6 hours. Cell bodies were lysed and luciferase activity was measured using a luciferase assay. Mean ± SEM of 18 technical replicates (n = 6 biological replicates). * $p < 0.05$, ns, not significant.

3.5.2. Rapid Aβ$_{1-42}$-dependent increases in somatic Atf4

To determine whether *Atf4* itself was being transcribed, I measured *Atf4* levels in cell bodies using qRT-PCR after 1 and 2 hours of axonal Aβ$_{1-42}$ treatment, which is a time frame in which I expected for transcriptional changes to occur (Figure 3-19). Within one hour, *Atf4* levels in cell bodies of Aβ$_{1-42}$-treated axons were significantly increased compared to controls, suggesting a rapid nuclear response to locally applied Aβ$_{1-42}$. This Aβ$_{1-42}$-dependent effect was not only rapid but also transient as *Atf4* levels returned to baseline within 2 hours of Aβ$_{1-42}$ treatment.

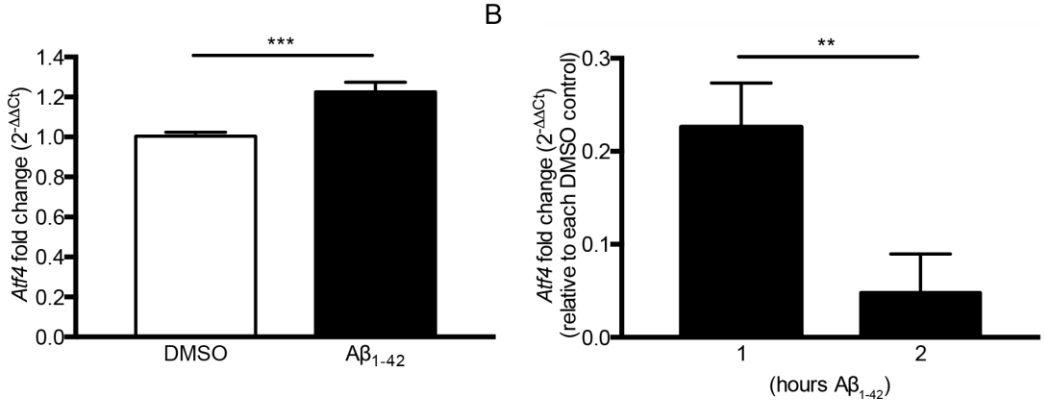

Figure 3-19. Axonal Aβ$_{1-42}$ induces rapid and transient increased somatic *Atf4*
(A) Axons were treated with Aβ$_{1-42}$ for 1 hour. Cell bodies were lysed and RNA was purified. *Atf4* transcript levels were measured using qRT-PCR. Levels were normalized to *Gapdh* transcript levels. Mean ± SEM of 18 technical replicates (n = 6 biological replicates). *** $p < 0.001$.
(B) Axons were treated with Aβ$_{1-42}$ for 1or 2 hours. Cell bodies were lysed and RNA was purified. *Atf4* transcript levels were measured using qRT-PCR. Levels were normalized to *Gapdh* transcript levels. Mean ± SEM of 18 technical replicates (n = 6 biological replicates). ** $p < 0.01$.

3.6. Local $A\beta_{1-42}$-induced STAT3 signaling

3.6.1. STAT3 is locally activated but not synthesized in response to $A\beta_{1-42}$

My results so far suggest that a transcriptional event plays a partial role in the downstream axonal *Atf4* translation that occurs in response to $A\beta_{1-42}$. It has been demonstrated that the transcription factor, STAT3, is immediately synthesized and activated in injured axons and is retrogradely trafficked to the nucleus, where it induces regenerative gene expression [177]. To determine whether this was also occurring in response to local application of $A\beta_{1-42}$, I investigated STAT3 levels in axons 30 and 60 minutes after $A\beta_{1-42}$ treatment using quantitative ICC (Figure 3-20). Interestingly, while there did not appear to be any changes in total STAT3 levels during the first hour of $A\beta_{1-42}$ treatment, I did observe a significant increase in phosphorylated STAT3. This data does not dispute the possibility that STAT3 is locally synthesized in response to $A\beta_{1-42}$ as I have demonstrated that it is necessary to block retrograde transport with a dynein inhibitor in order to observe $A\beta_{1-42}$-dependent increases in local vimentin synthesis.

To investigate whether STAT3 may also be locally synthesized and trafficked back to the cell body in a similar manner, I utilized the same experimental paradigm as previously described in Chapter 3.4.2 (Figure 3-21). Even in the presence in ciliobrevin A, there were no observed changes in STAT3 levels in axons in response to $A\beta_{1-42}$, suggesting that, unlike injured axons, $A\beta_{1-42}$-treated axons do not synthesize STAT3 but rather activate STAT3 via phosphorylation.

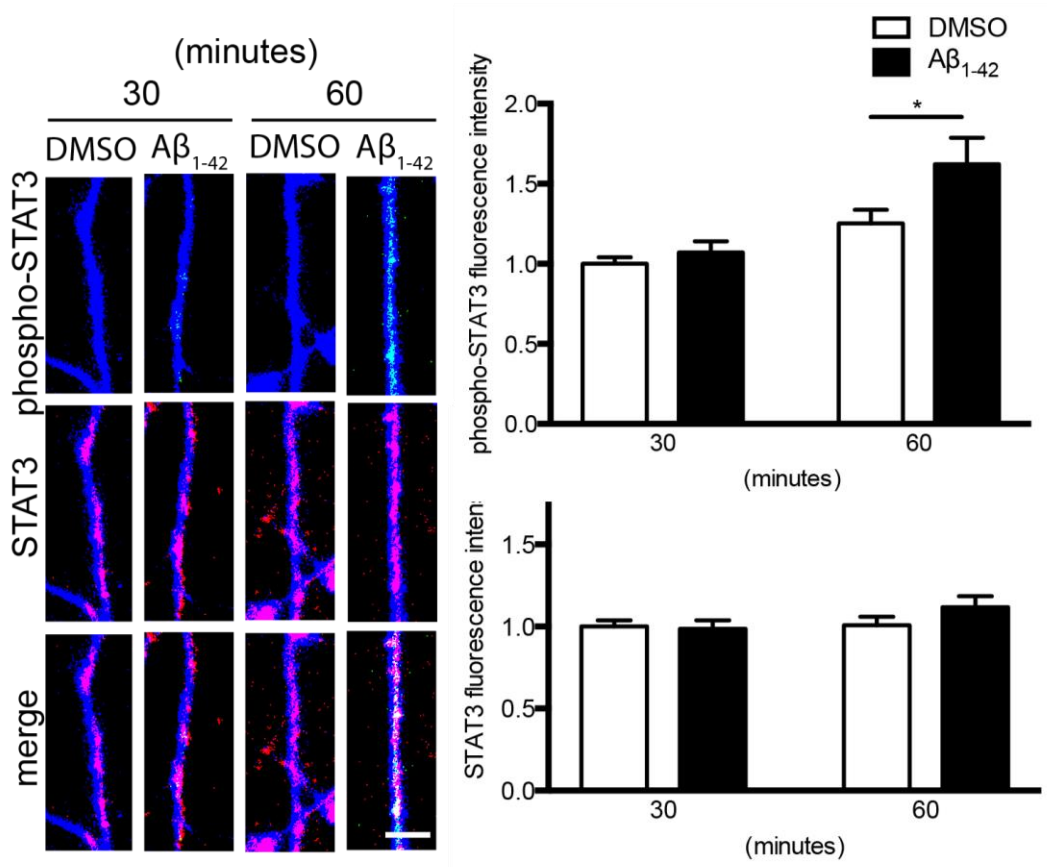

Figure 3-20. $A\beta_{1-42}$ induces local activation of STAT3
(A-B) Axons were treated with vehicle or $A\beta_{1-42}$ for 30 or 60 min. Axons were immunostained for phospho-STAT3, STAT3 and β-III-tubulin. Mean ± SEM of 39-40 optical fields (n = 8 biological replicates). * $p < 0.05$. The scale bar represents 5 μm.

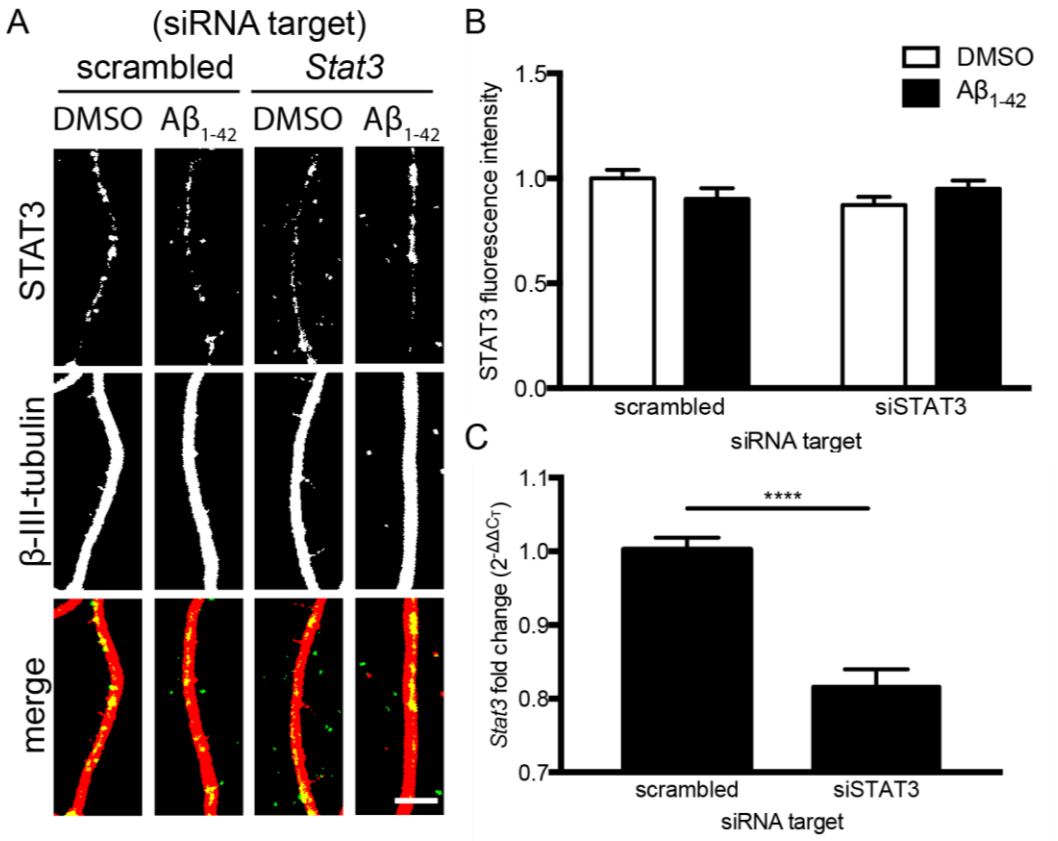

Figure 3-21. $A\beta_{1-42}$ does not induce local translation of *Stat3*
(A-B) Axons were transfected with scrambled or *Stat3*-targeting siRNA. Twenty-four hours after transfection, axons were treated with ciliobrevin A (5 μM) for 45 min and were then treated with vehicle or $A\beta_{1-42}$ for 1 hr. Axons were immunostained for STAT3 and β-III-tubulin. Mean ± SEM of 40 optical fields per condition (n = 8 biological replicates). The scale bar represents 5 μm.
(C) Dissociated hippocampal neurons were cultured for 11-12 DIV. Neurons were transfected with scrambled or *Stat3*-targeting siRNA. Forty-eight hours after transfection, neurons were lysed and RNA was purified. Stat3 transcript levels were quantified using qRT-PCR. Mean ± SEM of 18 technical reads (n = 6 biological replicates). **** $p < 0.0001$.

3.6.2. Locally activated STAT3 does not translocate to the cell body

Canonically, phosphorylated STAT3 is transported to the nucleus where it induces gene expression [206]. To investigate whether locally activated STAT3 is trafficked to the nucleus, I measured nuclear levels of total and phosphorylated STAT3 1.5 and 2 hours after axonal $A\beta_{1-42}$ treatment (Figure 3-22). Interestingly, there were no observed changes in nuclear STAT3 or phosphorylated STAT3 levels in response to $A\beta_{1-42}$, suggesting that axonally activated STAT3 functions outside of the nucleus.

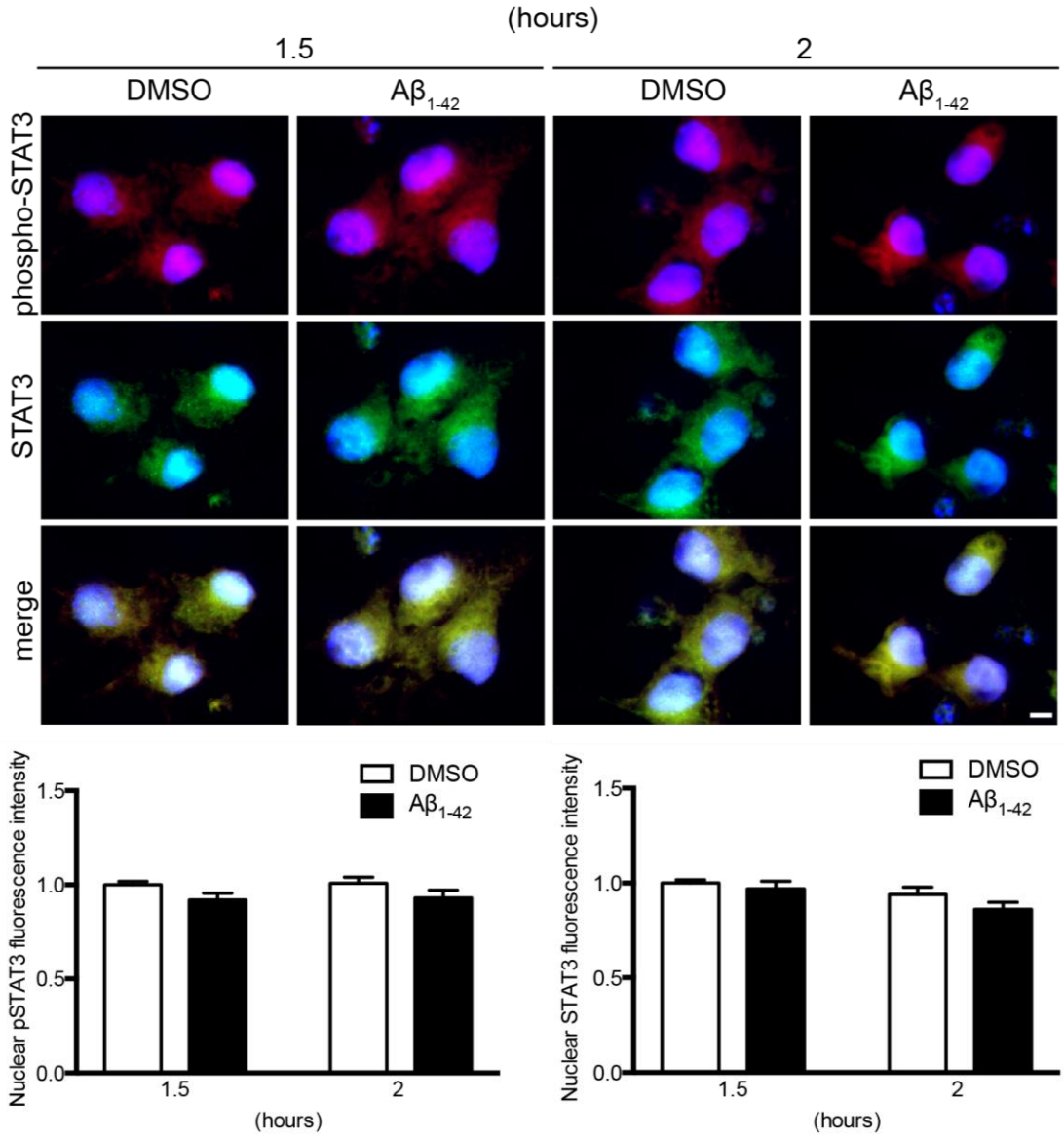

Figure 3-22. Locally activated STAT3 is not retrogradely transported to nuclei
Axons were treated with vehicle or $A\beta_{1-42}$ for 1 or 1.5 hours. Neurons were immuno-stained for pSTAT3 and STAT3 and nuclei were stained with DAPI. Mean ± SEM for 39-50 optical fields (n = 8-10 biological replicates). Scale bar represents 5 μm.

3.7. Discussion

Together, my findings show that the treatment of axons with oligomeric $A\beta_{1-42}$ leads to the rapid translation of at least one, but likely numerous, axonally localized transcripts into protein. This is a surprising finding given that mature axons are largely considered to be translationally silent, especially within the CNS. Holt and colleagues have demonstrated that the local synthesis of the intermediate filament protein, lamin B2, occurs in adult RGCs in the absence of an initiating stimulus, which regulates axonal maintenance by promoting mitochondrial function [161]. More recently, work from the Holt lab has shown that RGC axons contain many transcripts that are actively translated during adulthood, as evidenced by the presence of these transcripts in axonal ribosomes [207]. In addition to promoting mitochondrial function, axonal protein synthesis in adult neurons plays an important role in neurotransmitter release during long-term potentiation [208]. However, these studies are the only existing evidence that ongoing local protein synthesis occurs in the naïve, adult CNS. There are many studies that demonstrate that mature axons of the PNS, particularly DRG axons, retain the ability to synthesize proteins, and this is particularly relevant in the context of axotomy. Several different proteins are synthesized rapidly after injury, and the product of this local synthesis event allows for communication to the cell body and thereby enables axonal regeneration and cell survival. The ability of these axons to rapidly synthesize proteins relies on the pre-localization of transcripts into axons. It has been found in numerous neuronal subtypes, including CNS neurons such as cortical neurons

and RGCs, that adult axons contain thousands of localized transcripts. We found this to also be the case in hippocampal axons. These studies, along with our findings, provide evidence that while mature CNS axons remain largely translationally silent, they do retain the ability to synthesize proteins in response to external challenges, such as injuries or degenerative stimuli.

Though axonal protein synthesis only appears to occur in very specific circumstances in adult neurons, local translation is very active and is absolutely essential throughout neurodevelopment. The significant upregulation of protein synthesis that we demonstrate to occur in axons challenged with the neurodegenerative stimulus, $A\beta_{1-42}$, is very intriguing as it suggests that $A\beta_{1-42}$-challenged axons employ a mechanism that is most commonly observed during the development of the nervous system. This local translation event could represent a reversion of mature axons back to an embryonic state, which is particularly relevant in the context of neurodegeneration.

Cholinergic neurons of the basal forebrain, which innervate regions such as the cortex and hippocampus, are consistently lost throughout the progression of AD [209, 210]. In fact, the degeneration of cholinergic neurons within the basal forebrain is one of the earliest pathological events observed in AD [211-215]. Furthermore, cholinergic deficits in the basal forebrain positively correlate with cognitive impairment in AD patients [216-218]. Interestingly, axotomy of cholinergic neurons leads to decreased expression of cholinergic markers, such as ChAT, but not a corresponding decrease in overall neuron number [219, 220]. We observed a similar phenomenon in our *in vivo* injection model of acute

amyloidosis, where $A\beta_{1-42}$ injection into the hippocampus led to decreased basal forebrain ChAT expression to a greater extent than overall neuron loss. Together, these data suggest that the loss of cholinergic neurons in response to axotomy or $A\beta_{1-42}$ may be due to a loss of cholinergic phenotypic expression rather than death of cholinergic neurons. This proposed loss of phenotype could represent a reversion of these affected neurons from a mature, differentiated state back to a more embryonic state. This possibility is further underscored by my observation that $A\beta_{1-42}$-treated axons act similarly to embryonic axons in that they carry out rapid local protein synthesis. It would be interesting to investigate whether this immediate $A\beta_{1-42}$-induced protein synthesis event plays a role in mediating a loss of cholinergic phenotype in response to amyloid.

The majority of the findings that I present herein have come from experiments using an *in vitro* model of amyloidosis. While this microfluidic approach allows for the investigation of axonal signaling mechanisms, there are also shortcomings to using this culturing paradigm. Most significantly, the culturing of primary neurons in microfluidic devices creates an artificial and unphysiological environment. Due to the nature of the chambers, which enables isolation of axons from cell bodies and dendrites, neurons do not form synapses as they do within the brain. Additionally, axons in chambers grow in the absence of neuronal cell bodies and glia. We isolate hippocampal neurons from brains of rat embryos and culture them until they have reached the highest achievable level of maturity. However, even after nearly two weeks in culture, these neurons may still retain embryonic features, and it cannot be assumed that these axons

act identically to those in the adult brain. Together, these factors must be taken into consideration when extending conclusions from my studies into what may be occurring in the aging adult brain. Though important insights can still be gleaned from my studies, it can be assumed that this compartmentalized *in vitro* environment does not perfectly mimic the *in vivo* setting.

Another important factor that must be considered is that the culturing paradigm that we use to study potential pathogenic mechanisms in AD exclusively uses $A\beta_{1\text{-}42}$ and not hyperphosphorylated tau, which is also consistently observed in the AD brain. Though the amyloid cascade hypothesis proposes that the primary pathogenic agent in AD is $A\beta_{1\text{-}42}$, this is merely a hypothesis, and it should not be assumed that tau does not play a role in pathogenesis. Additionally, the treatment of axons with a bolus of $A\beta_{1\text{-}42}$ differs from the accumulation and deposition of $A\beta_{1\text{-}42}$, which occurs very slowly in the AD brain throughout disease progression. My data shows that $A\beta_{1\text{-}42}$-induced immediate axonal protein synthesis acts upstream of an *Atf4*-dependent pro-apoptotic signaling mechanism that results in cell death within 48 hours of $A\beta_{1\text{-}42}$ exposure. This mechanism, however, is a reaction to an acute $A\beta_{1\text{-}42}$ treatment rather than a slow accumulation of $A\beta_{1\text{-}42}$ over time. As cell death occurs much later than the appearance of oligomeric $A\beta_{1\text{-}42}$ during AD progression, it can be assumed that the mechanism that I describe is not happening in AD brains exactly as it is in our *in vitro* paradigm, particularly temporally. There are a couple of different possible explanations for this phenomenon. First, it is probable that axons do not synthesize proteins in response to $A\beta_{1\text{-}42}$ unless a higher, toxic

threshold is reached. If this is the case, one would expect that axons would remain translationally silent until later stages of AD when $A\beta_{1-42}$ concentrations are significantly higher than in earlier disease stages. This could be addressed experimentally by carrying out a dose-response puromycylation experiment to determine if there is a threshold that must be reached in order to induce immediate axonal protein synthesis or if lower, sub-lethal concentrations of $A\beta_{1-42}$ still induce local synthesis. If the latter scenario is true, it is possible that axons always synthesize proteins in response to oligomeric $A\beta_{1-42}$ but that the specific signaling pathways triggered by axonal protein synthesis differ depending on several different variables. These distinctive variables could potentially lead to the synthesis of entirely different molecules or could induce differential regulation of the same newly synthesized molecules. Some variables that could influence the specific axonal reaction to $A\beta_{1-42}$ include the concentration of $A\beta_{1-42}$ and the amount of time that axons are exposed to $A\beta_{1-42}$.

At higher, toxic concentrations of $A\beta_{1-42}$, my data shows that axons immediately synthesize proteins that act to regulate cell death, which differs significantly from the mechanisms that occur via immediate axonal protein synthesis in axotomized DRGs. In this context, injured axons of the PNS utilize local protein synthesis to promote axon regeneration and cell survival. This presents the interesting possibility that CNS axons challenged with lower levels of $A\beta_{1-42}$ may lead to a local protein synthesis event that promotes cell survival rather than cell death. In this possible scenario, axons could activate a variety of signaling pathways via the common mechanism of immediate local protein

synthesis. This possibility is particularly intriguing as ATF4 can act either pro- or anti-apoptotically, depending on several different factors. If axons do indeed react at first to promote cell survival in the early AD brain as opposed to activating pro-apoptotic signaling mechanisms, this could explain why cells do not die until much later stages of AD.

Another variable that could influence the specific axonal protein synthetic pathways downstream of $A\beta_{1-42}$ is the persistence of axonal exposure to $A\beta_{1-42}$ oligomers. In our *in vitro* experimental paradigm, axons are exposed to amyloid persistently. However, as stated previously, axons do not exist in isolation *in vivo* as they do in our microfluidic devices. In the brain, axons are surrounded by neuronal cell bodies and glial cells, both of which can react to $A\beta_{1-42}$ in several ways, such as secretion of amyloid-degrading enzymes. This suggests that axons are more likely to be briefly exposed to $A\beta_{1-42}$ as opposed to persistently exposed, at least during the earlier stages of AD. We have shown in our published findings that 48 hours of axonal $A\beta_{1-42}$ treatment is sufficient to induce neuronal apoptosis. Within 24 hours of $A\beta_{1-42}$ treatment, axonally localized *Atf4* transcripts are translated, and these protein products are transported to the cell body. Once axonally synthesized ATF4 reaches the cell body, the fate of the cell towards apoptosis no longer depends on the presence of $A\beta_{1-42}$ as removal of the axonal culture medium and replacement with conditioned, non-$A\beta_{1-42}$-containing medium is incapable of rescuing neurons from death. I also show that immediate $A\beta_{1-42}$-dependent axonal protein synthesis acts upstream of the localization of *Atf4* transcripts into axons. It would be of interest to utilize similar washout

studies to determine the required amount of time that axons must be exposed to $A\beta_{1-42}$ to induce the axonal localization of *Atf4*. If axons must be challenged with $A\beta_{1-42}$ for an extended amount of time, such as several hours, to induce *Atf4* localization and translation, the presence of surrounding neurons and glia could prevent or delay the activation of this apoptotic pathway via the secretion of amyloid-degrading enzymes. If this is the case, this could also contribute to the significant amount of time that is observed between the appearance of oligomeric $A\beta_{1-42}$ and the death of neurons during AD progression.

One question that my studies do not address is how axonally applied $A\beta_{1-42}$ actually induces immediate local protein synthesis. There are several different ways in which axonal protein synthesis can be stimulated. One of the most well characterized pathways that regulates mRNA translation is the mTOR pathway. My data demonstrates that two established molecules downstream of mTOR, S6 and 4EBP1, are rapidly phosphorylated in response to $A\beta_{1-42}$, suggesting that the immediate $A\beta_{1-42}$-induced axonal protein synthesis that occurs is mTOR-dependent. However, this has not been experimentally confirmed. Interestingly, my findings show that 4EBP1 phosphorylation occurs more rapidly and transiently than S6 phosphorylation, suggesting that they may be acting downstream of different pathways, possibly both mTOR-dependent and -independent pathways. In support of this possibility, both 4EBP1 and S6 phosphorylation can fall downstream of mTOR-independent pathways. S6K, which mediates S6 phosphorylation, can be activated by the master kinase, PDK1, which can phosphorylate S6K in an mTOR-independent manner [221]. In

line with this possibility, $A\beta_{1\text{-}42}$ neurotoxicity requires PDK1 activation [222]. Studies from the cancer field illuminate a PI3K-dependent signaling pathway that regulates 4EBP1 phosphorylation independently of mTOR [223, 224]. Based on this information, it is plausible that $A\beta_{1\text{-}42}$ influences mRNA translation via mTOR-dependent and/or mTOR-independent signaling pathways. It would be interesting to investigate this possibility further by measuring the ability of $A\beta_{1\text{-}42}$ to induce 4EBP1 and S6 phosphorylation in the presence of the standard mTOR inhibitor, rapamycin.

One way in which $A\beta_{1\text{-}42}$ could act to promote the phosphorylation of S6 and 4EBP1 is via Ca^{2+} signaling. I show that these phosphorylation events are indeed Ca^{2+}-dependent, though these experiments do not address whether Ca^{2+} is sufficient or permissive for promoting immediate axonal mRNA translation. There are several ways in which $A\beta_{1\text{-}42}$-induced Ca^{2+} signaling could be influencing the phosphorylation of these molecules. It could be promoting the activation of the mTOR pathway via regulation of the mTOR-containing complex, mTORC1 [225, 226]. Ca^{2+} could also be inducing the activation of the mTOR-independent pathways mentioned previously via the stimulation of PDK1 and PI3K, both of which can be regulated by Ca^{2+} signaling [227, 228]. $A\beta_{1\text{-}42}$ has been shown to promote intracellular Ca^{2+} dysregulation in several different ways, such as via receptors and ion channels, pore formation in the extracellular membrane, and release from intracellular stores. It is unclear which of these mechanisms are occurring in hippocampal axons. One interesting possible mediator to be considered is the group of voltage-gated Ca^{2+} channels, several

of which have been shown to interact with Aβ in the hippocampus [229]. This possibility is particularly intriguing because this class of channels is highly present along axons and is necessary for neurotransmitter release, which a low, sub-lethal concentration of $A\beta_{1\text{-}42}$ is capable of inducing [230].

In addition to promoting global protein synthesis by activating translational regulators, the immediate $A\beta_{1\text{-}42}$-induced axonal protein synthesis that I observe likely also requires the release of transcripts from axonally localized RNPs. Axonally localized transcripts are considered to be largely translationally silent within mature, adult axons, and therefore they likely remain inaccessible to the translational machinery in the absence of a stimulus. My studies show that *Vim* transcripts are immediately translated in response to $A\beta_{1\text{-}42}$ whereas *Stat3* transcripts are not, even though both transcripts are localized to axons. This suggests that $A\beta_{1\text{-}42}$ only promotes the immediate translation of a specific subset of localized transcripts. This specificity could be achieved by the co-packaging of functionally relevant transcripts into RNPs. If this is the case, external stimuli, such as $A\beta_{1\text{-}42}$, must somehow specifically promote the release of these transcripts from RNPs, possibly through the regulation of particular RBPs.

Together, the findings presented in this dissertation describe intriguing $A\beta_{1\text{-}42}$-induced mechanisms that may be playing important roles in AD pathogenesis. This data provides some insight into how the presence of a peripheral degenerative stimulus could convey an informative message to the cell body and allow for the neuron to react as a whole. Several interesting questions arise from the results of these studies, and it may be informative to

further investigate these signaling pathways in order to better understand some of the important pathogenic events in AD and hopefully identify promising drug targets to delay or prevent the progression of AD.

Part 4

Chapter 4. Key Findings: Significance and Future Directions

This dissertation illustrates the importance of axonal protein synthesis in mediating $A\beta_{1-42}$-induced neurotoxicity. This chapter will outline the achievements, significance and future prospects of this project.

4.1. Key Finding 1: $A\beta_{1-42}$-Induced Axonal Synthesis and Transport of ATF4

Significance: Recent studies in the AD field have demonstrated that axonal dysfunction occurs early in disease progression and precedes death of the neuron [80]. Using compartmentalized cultures of hippocampal neurons, we demonstrated that the neurotoxic stimulus believed to be a causative factor in AD, oligomeric $A\beta_{1-42}$, induces the localization of *Atf4* transcripts into axons, which results in its axonal synthesis. ATF4 is subsequently retrogradely transported to the nucleus where it induces gene expression of pro-apoptotic genes, such as *CHOP*. This phenomenon is likely occurring in the AD brain as post-mortem human AD brains displayed increased axonal levels of *Atf4* transcripts and ATF4 protein as compared to neurologically healthy controls. Our findings suggest that inhibition of axonal ATF4 synthesis is a potential therapeutic avenue that could be pursued to reduce $A\beta_{1-42}$-induced neuronal loss in the AD brain.

Future directions: These findings illuminate a signaling mechanism emanating from axons, which can regulate neuronal loss in response to a neurodegenerative stimulus. This is an important finding as it provides a novel pro-apoptotic mechanism that could be targeted in patients with early AD to

hopefully prevent disease spread throughout the brain and halt the progression of AD. One important question that arises from this study is why ATF4 must be synthesized axonally versus somatically. It is clear that the cell body responds to axonal $A\beta_{1-42}$ by localizing transcripts, such as *Atf4*, to axons, which enables its local synthesis. This is quite a complex mechanism that likely requires extensive $A\beta_{1-42}$-dependent signaling, including anterograde RNP transport, and it is not clear why this seemingly energy inefficient event would be occurring. One possible explanation is that axonally synthesized ATF4 is distinct from somatically synthesized ATF4. In order to be activated, ATF4 must undergo dimerization, either via homodimerization with other ATF4 molecules or heterodimerization with members of the AP-1 and C/EBP family of proteins [231]. By binding with different dimerization partners, ATF4 can target different sets of genes. As the axonal environment is unique from the somatic environment, it is possible that axonally synthesized ATF4 interacts with dimerization partners that it may not readily interact with within the cell body. If this is the case, ATF4 dimers emanating from the axon could act uniquely from those in the soma. Based on this intriguing hypothesis, members in our lab are investigating ATF4 heterodimers in axons and their contribution to $A\beta_{1-42}$-induced ATF4-dependent apoptosis. Upon identifying the ATF4 heterodimer that is responsible for $A\beta_{1-42}$-dependent apoptosis, they will utilize chromatin immunoprecipitation (ChIP) to determine the gene targets of this dimer originating in axons.

4.2. Key Finding 2: $A\beta_{1-42}$-Dependent Immediate-Early Local Translation

Significance: Studies from the field of axonal injury demonstrate that mature axons retain the capacity to immediately synthesize proteins in response to a deleterious stimulus. I found that hippocampal axons also retain the ability to immediately translate mRNAs and thereby synthesize proteins in response to $A\beta_{1-42}$. This serves as a way in which axons of the CNS can rapidly inform cell bodies of the presence of degenerative agents detected in the periphery. I showed that this immediate mRNA translation is Ca^{2+}-dependent, which provides some insight into one of the likely many functions that the well-documented $A\beta_{1-42}$-induced intracellular Ca^{2+} dyshomeostasis plays in AD pathogenesis. This effect is specific to axons, which underscores the vulnerability of axons to $A\beta_{1-42}$.

Future Directions: One important set of experiments that could be performed in the future based on these studies is confirming my findings either in an *in vivo* injection model of amyloidosis or in an *ex vivo* hippocampal slice culture treated with $A\beta_{1-42}$. These follow-up experiments would strengthen my findings and provide further support for my hypothesis. Furthermore, it would be interesting to investigate whether the axonal application of other neurodegenerative stimuli, such as α-synuclein, induce similar immediate protein synthesis events in axons.

Additionally, my findings provide important insights into the cell biology of oligomeric $A\beta_{1-42}$. I identified intracellular Ca^{2+} as an important regulator of immediate, $A\beta_{1-42}$-induced axonal mRNA translation, but I did not identify the particular source of intracellular Ca^{2+} that is regulating this process. There is extensive evidence that $A\beta_{1-42}$ oligomers induce intracellular Ca^{2+}

dyshomeostasis, and this can occur in several different ways, such as via the formation of pores in the extracellular membrane, release from the ER and via different receptors [41, 187-198]. By identifying an immediate, Ca^{2+}-dependent readout, the way in which $A\beta_{1-42}$ acts to regulate immediate axonal protein synthesis can be investigated further using pharmacological inhibitors in conjunction with the axonal puromycylation assay that was utilized in this study.

The rapid effect of $A\beta_{1-42}$ on axonal protein synthesis is intriguing in that it closely resembles the way in which cues within the brain induce protein synthesis in growth cones during neurodevelopment to promote attraction or repulsion of the axon, depending on the cue. Though $A\beta_{1-42}$ has been studied extensively in the context of disease, the primary biological function of $A\beta_{1-42}$ is still largely unknown. Oligomeric $A\beta_{1-42}$ has been demonstrated to regulate both LTP and LTD in hippocampal neurons, though the particular cellular mechanisms by which these occur are not fully understood. Interestingly, local translation of dendritic mRNAs is implicated in LTP and LTD, and it has been proposed that axonal mRNA translation may also play an important role in synaptic plasticity [232-234]. Collectively, these findings suggest that the normal biological function of $A\beta_{1-42}$ may be to induce axonal mRNA translation to regulate LTP and LTD during neurodevelopment. Based on this hypothesis, it would be interesting to investigate the effects of oligomeric $A\beta_{1-42}$ on axonal protein synthesis in the hippocampus during neurodevelopment. If there is indeed an effect, one could further investigate the role of $A\beta_{1-42}$-induced protein synthesis in mediating LTP, LTD and other functional readouts, such as synaptic tagging and pruning.

4.3. Key Finding 3: Comparing and Contrasting $A\beta_{1-42}$ and Axotomy

Significance: Injured axons of the peripheral nervous system immediately synthesize proteins that signal retrogradely to the nucleus to enable pro-regenerative gene expression [177]. My data show that although the generalized mechanism of immediate local protein synthesis is common to both injured and $A\beta_{1-42}$-treated axons, the identities of immediately synthesized proteins are distinct between insults. Vimentin is rapidly synthesized in axons in both contexts, but the downstream effectors of axonally synthesized vimentin may not necessarily be the same between perturbations. Moreover, STAT3 is locally activated but not synthesized in axons in response to $A\beta_{1-42}$, and these activated molecules do not travel to the nucleus, suggesting an extranuclear $A\beta_{1-42}$-induced role for activated STAT3 in axons. Though STAT3 does not appear to act transcriptionally in response to $A\beta_{1-42}$ as it does in response to injury, transcription is at least partially required for $A\beta_{1-42}$-dependent *Atf4* translation. Moreover, axonally sensed $A\beta_{1-42}$ induces a rapid and transient increase in somatic *Atf4* levels, which may be transcription-dependent.

Future Directions: These findings provide interesting insight into the ways in which specificity can be encoded in the general mechanism of immediate protein synthesis in response to various peripheral insults. It would be intriguing to further investigate the $A\beta_{1-42}$-induced regulation of these proteins in axons to discern how distinct each response is to the perturbation. For example, injury-induced axonally synthesized vimentin is shown to act as a scaffolding protein between activated ERK and importin-β, thereby enabling its retrograde transport

and somatic localization [175]. Using a proximity-ligation assay (PLA), one could investigate whether $A\beta_{1-42}$ similarly induces an association between activated ERK and vimentin in axons [235]. In addition, axonal co-application of an inhibitor of ERK activation with $A\beta_{1-42}$ followed by ATF4 ICC could help to determine whether axonally synthesized vimentin acts in a similar fashion to injured axons to mediate axonal *Atf4* translation in response to $A\beta_{1-42}$.

Another possible avenue that could be followed up on experimentally is dissecting the $A\beta_{1-42}$-dependent regulation of locally activated STAT3. There have been several studies describing extranuclear roles of STAT3, such as the regulation of microtubule stability via interaction with stathmin and the regulation of ATP synthesis via association with PDC-E1 in mitochondria [236-238]. It would be interesting to utilize pharmacological inhibitors of STAT3 activation to further investigate whether axonal $A\beta_{1-42}$ application results in STAT3-dependent microtubule stabilization and/or STAT3 association with PDC-E1. Relative microtubule stability can be measured using quantitative ICC against tyrosinated and acetylated α-tubulin, which represent dynamic and stabilized microtubules, respectively [239, 240]. STAT3 association with PDC-E1 can be measured in axons using a PLA between the two proteins [235]. These studies could be interesting in the context of my project as both of these possible responses could be playing roles in regulating the immediate intra-axonal retrograde signaling events induced by $A\beta_{1-42}$. Dynein processivity can be enhanced by increased ATP synthesis as well as increased microtubule stability [241, 242]; therefore, both of these regulatory events could increase dynein-dependent transport of

immediately synthesized proteins in response to $A\beta_{1-42}$. If either of these readouts show $A\beta_{1-42}$-dependent changes, it would be enticing to determine whether the STAT3-dependent stabilization of microtubules and/or STAT3 association with PDC-E1 is necessary for the $A\beta_{1-42}$-dependent localization or translation of *Atf4*.

Another open question that arises from this project is what transcription factor(s) are being immediately regulated by axonal $A\beta_{1-42}$ application. My studies show that transcription plays at least a partial role in regulating downstream $A\beta_{1-42}$-dependent *Atf4* translation in axons, but the exact mechanism by which this is occurring is unknown. To gain some insight into this regulatory event, it would be informative to carry out sequencing from cell bodies of vehicle- and $A\beta_{1-42}$-treated axons in the presence and absence of the transcriptional inhibitor, actinomycin D. By determining exactly which genes are transcribed in response to axonal $A\beta_{1-42}$, it may be possible to identify one or more consensus sequences that are over-represented within these genes. This could allow for the identification of a transcription factor that may either be synthesized and retrogradely transported immediately in axons in response to $A\beta_{1-42}$ or that could be downstream of $A\beta_{1-42}$-dependent retrograde trafficking of locally synthesized proteins. This could lead to the identification of a novel signaling pathway downstream of immediate axonal protein synthesis that is specific to axonal $A\beta_{1-42}$ treatment.

4.4. Closing Statement

Collectively, this dissertation emphasizes the critical role of axonal protein synthesis in $A\beta_{1-42}$-induced neurotoxicity. Given the myriad evidence underscoring the significance of axonal dysfunction in the primary phases of AD, our findings provide important insight into some of the earliest events that may be occurring in AD pathogenesis.

We have demonstrated that inhibition of this pathological signaling pathway is a potential therapeutic avenue for the slowing of AD progression and prevention of the devastating symptoms associated with AD. The findings presented herein describe ways in which axons act autonomously to apprise cell bodies of peripherally sensed $A\beta_{1-42}$ and have intriguing implications for pathogenic signaling mechanisms that may be similarly occurring in axons in numerous neurodegenerative diseases.

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Appendix. Materials and Methods

2.1. Rodent Handling

All rodent procedures were approved by Columbia University Institutional Animal Care and Use Committee.

2.1.1. Compartmentalized Neuronal Culture

All reagents were from Thermo Fisher Scientific (Waltham, MA) unless otherwise noted. Microfluidic devices were made using a Dow Corning Sylgard 184 Silicone Encapsulant Clear Kit (Ellsworth Adhesives, Germantown, WI) at a 10:1 mix ratio. Chambers were cured at 70°C for ≥4 hours. Prior to culturing, chambers were washed in ethanol and air-dried. Glass coverslips (25 mm; Carolina Biological Supply Company, Burlington, NC) were coated in 0.01 mg/mL poly-D-lysine (Sigma-Aldrich, St. Louis, MO) followed by 2 µg/mL laminin. Pregnant Sprague Dawley® rats (Charles River, Kingston, NY) were euthanized on embryonic day 18 using carbon dioxide followed by thoracic thoracotomy. Hippocampi were dissected from the brains of embryos and were subsequently incubated in TrypLE™ Express at 37°C. Hippocampi were washed in Hank's Balanced Salt Solution and resuspended in plating medium (10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin-streptomycin, 1 mM sodium pyruvate in Neurobasal®). Hippocampi were dissociated via trituration with a pipette followed by a flame-polished Pasteur pipette. After dissociation, cells were passed through a 40 µm cell strainer (VWR, Radnor, PA) then centrifuged at 800 rpm for 5 minutes at 4°C. Cells were resuspended in plating medium to a final concentration of 5,500,000 cells/mL. Ten µL of cell suspension was added

to each chamber, resulting in 55,000 cells per chamber. Cells were allowed to settle at 37°C, then plating medium was added to the somatic compartments and cells were returned to incubator. On DIV1, plating medium was replaced with growth medium (1X B-27® supplement, 2 mM L-glutamine in Neurobasal®). On DIV4-5, half of the medium was replaced with fresh growth medium containing 20 μM 5-fluorodeoxyuridine and 20 μM uridine to prevent glial proliferation. All cells were cultured 11-12 days before treatment.

2.1.2. Oligomeric $A\beta_{1-42}$ Preparation

Synthetic $A\beta_{1-42}$ peptides (purchased from Dr. David Teplow, UCLA) were dissolved to 1 mM hexafluoroisopropanol and dried using a SpeedVac. The peptides were resuspended to 1 mM in DMSO by bath sonication for 10 minutes. Solution was then aliquoted and stored at -20°C. For oligomer formation, the peptides were diluted to 100 μM in PBS and incubated overnight at 4°C. Oligomerized peptides were diluted to 33 μM in growth medium and were added to axonal compartments for a final concentration of 3 μM.

2.1.3. Inhibitors

To inhibit axonal protein translation, axons were treated with 100 nM emetine (EMD Millipore, Billerica, MA) 45 minutes prior to Aβ treatment. To prevent retrograde transport, axons were treated with 5 μM HPI-4 (Sigma-Aldrich, St. Louis, MO) 45 minutes prior to Aβ treatment. To chelate intracellular calcium, axons were treated with 10 μM BAPTA-AM (Thermo Fisher Scientific, Waltham, MA) 30 minutes prior to Aβ treatment. To inhibit transcription, cell bodies were

treated with 40 μM actinomycin D (Sigma-Aldrich, St. Louis, MO) immediately prior to Aβ treatment.

2.1.4. siRNA Transfection

SiRNAs were transfected to a final concentration of 50 nM using NeuroPORTER Reagent (Genlantis, San Diego, CA) according to manufacturer's instructions 24 hours prior to Aβ treatment. Briefly, siRNAs were incubated in a NeuroPORTER solution in growth medium for 15 minutes at room temperature to form siRNA complexes. Half of the culture medium was removed and complexes were added to cells. Neurons were incubated at 37°C for 2 hours. Axons were supplemented with growth medium containing 2X B-27 and were incubated at 37°C for an additional 22 hours.

scrambled siRNA sequence: 5'-CCCUUCGUUCCUUCCAAUCUGUCCA-3'

Atf4 siRNA sequence: 5'-AACCCAUGAGGUUUGAAGAGCUUGG-3'

Stat3 siRNA sequence: 5'-UUCCAUUGGCUUCUCAAGAUACCUG-3'

Vim siRNA sequence: 5'-CACCUGCGAAGUGGAUGCCCUUAAA-3'

2.2. Histochemistry

2.2.1. Immunofluorescence

Neurons were fixed with 4% paraformaldehyde (PFA) in cytoskeletal preservation buffer (10 mM MES, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 320 mM sucrose, pH 6.1) for 20 minutes at room temperature. Cells were washed with PBS (3 times; 5 minutes each) and blocked for 30 minutes with 3 mg/mL BSA, 100 mM glycine, 0.25% Triton X-100 in 1X PBS. Samples were incubated overnight at 4°C with primary antibodies against 4EBP1, p-4EBP1 (1:500, Cell Signaling

Technology, Danvers, MA), S6, p-S6 (1:1000, Cell Signaling Technology), vimentin (1:250, Abcam, Cambridge, MA), p-STAT3 (1:100, Cell Signaling Technology), STAT3 (1:1600, Cell Signaling Technology), β-III-tubulin (1:500, Covance, Dedham, MA), or ATF4 (1:1000, Abcam). Samples were washed with PBS (3 times; 5 minutes each) and incubated for 1 hour at room temperature with fluorophore-conjugated Alexa secondary antibodies (1:500, Thermo Fisher Scientific). Samples were washed with PBS (3 times; 5 minutes each), dipped in distilled water and air-dried. Samples were mounted with ProLong Diamond antifade reagent (Thermo Fisher Scientific) and imaged using an EC Plan-Neofluar 40x/1.3 objective on an Axio-Observer.Z1 microscope equipped with an AxioCam MRm Rev. 3 camera (Zeiss, Thornwood, NY).

2.2.2. Fluorescent *in situ* hybridization

Antisense riboprobes were transcribed in vitro from sense oligonucleotides using the MEGAshortscript kit with digoxigenin-labeled UTP (Roche Applied Sciences, Indianapolis, IN) according to manufacturer's instructions. Five non-overlapping probes recognizing *Egfp* (negative control) or *Atf4* mRNA were used for FISH staining. All sense oligonucleotides used were transcribed using a T7 promoter site (…GCCCTATAGTGAGTCGTATTAC-3') at the 3' end.

Egfp.1, 5'-GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAA…

Egfp.2, 5'-GACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTA…

Egfp.3, 5'-ACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTAC…

Egfp.4, 5'-AAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGA…

Egfp.5, 5'-AGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG…

Atf4.1, 5'-AGCCCCCTCAGACAGTGAACCCAATTGGCCATCTCCCAGAAAGTGTAATA…

Atf4.2, 5'-GTTAAGCACATTCCTCGATACCAGCAAATCCCTACAACATGACCGAGATG…

Atf4.3, 5’-AAGGAGGAAGACACTCCCTCTGATAGTGACAGTGGCATCTGTATGAGCCC…

Atf4.4, 5’-CTTAGATGACTATCTGGAGGTGGCCAAGCACTTCAAACCTCATGGGTTCT…

Atf4.5, 5’-AACGAGGCTCTGAAAGAGAAGGCAGATTCTCTCGCCAAAGAGATTCAGTA…

T7, 5’-GTAATACGACTCACTATAGGGC-3’

FISH was performed as previously described (Hengst et al., 2009). Cells were fixed with 4% paraformaldehyde (PFA) in cytoskeletal preservation buffer (10 mM MES, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 320 mM sucrose, pH 6.1) for 20 minutes at room temperature. Samples were washed in PBS (3 times; 5 minutes each), permeabilized with 0.5% Triton X-100 in PBS for 30 minutes at room temperature, then washed again (3 times; 5 minutes each). Samples were incubated with a total of 125 ng of digoxigenin-labeled riboprobe (25 ng each) in 25 µL hybridization buffer overnight at 37°C. Cells were then washed in 50% formamide/2X SSC (30 minutes with agitation at 37°C) followed by another wash in 50% formamide/1X SSC (30 minutes with agitation at 37°C). Samples were washed further in 1X SSC (3 times; 15 minutes each) and 0.1% Tween in PBS (3 times; 5 minutes each). Samples were blocked with 3% BSA in PBS-T for 30 minutes at room temperature and were incubated overnight at 4°C in primary antibodies against digoxin (1:500, Sigma-Aldrich) and β-III-tubulin (1:500, Covance). Cells were washed with PBS-T (3 times; 5 minutes each) then further incubated with fluorophore-conjugated Alexa secondary antibodies (1:500, Thermo Fisher Scientific). Samples were washed with PBS-T (3 times; 5 minutes each), dipped in distilled water and air-dried. Samples were mounted with ProLong Diamond antifade reagent (Thermo Fisher Scientific).

2.2.3. Puromycylation Assay

Neurons were incubated with 2 μM puromycin (company) and incubated at 37°C for 10 or 15 minutes. To inhibit translation, neurons were co-incubated with 10 μM anisomycin (Sigma-Aldrich, St. Louis, MO). Medium was aspirated and cells were washed twice with pre-warmed 1X PBS. Cells were fixed with 4% paraformaldehyde (PFA) in cytoskeletal preservation buffer (10 mM MES, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 320 mM sucrose, pH 6.1) for 20 minutes at room temperature. Cells were washed with PBS (3 times; 5 minutes each) and blocked for 30 minutes with 3 mg/mL BSA, 100 mM glycine, 0.25% Triton X-100 in 1X PBS. Samples were washed with PBS (3 times; 5 minutes each) and incubated overnight at 4°C with primary antibodies against puromycin (1:250, EMD Millipore, Billerica, MA) and β-III-tubulin (1:500, Covance, Dedham, MA) or MAP2 (1:500, Abcam). Samples were washed with PBS (3 times; 5 minutes each) and incubated for 1 hour at room temperature with fluorophore-conjugated Alexa secondary antibodies (1:500, Thermo Fisher Scientific). Samples were washed with PBS (3 times; 5 minutes each), dipped in distilled water and air-dried. Samples were mounted with ProLong Diamond antifade reagent (Thermo Fisher Scientific).

2.3. Biochemistry

2.3.1. Quantitative RT-PCR

Total RNA was extracted from somatic compartments and purified using the PrepEase RNA isolation kit (Affymetrix, Santa Clara, CA) and concentrated with RNeasy MinElute Cleanup Kit (QIAGEN, Valencia, CA). Reverse transcription

was performed with 100 ng RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific) according to manufacturer's instructions using the following conditions: 25°C for 10 minutes, 50°C for 30 minutes, 85°C for 5 minutes, and finally 4°C. Quantitative PCR was performed with TaqMan Gene Expression Master Mix in a StepOnePlus Real-Time PCR instrument using the following conditions: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute. *Atf4* gene expression set (Rn00824644_g1) was used. Gene expression was normalized to *Gapdh* gene expression set (Rn01775763_g1).

2.4. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc; La Jolla, CA). When comparing the means of two groups, an unpaired *t*-test was performed. When comparing the means of three independent groups, a one-way analysis of variance (ANOVA) was performed. When comparing the means of multiple groups encompassing two independent variables, a two-way ANOVA was performed.