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Evidence that inhibition of gamma-secretase impairs axonal and dendritic structure and function: a necessary role for APP

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

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by

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DEDICATION

With deepest appreciation, this dissertation is dedicated to the many people that believed in me and my vision. Thank you for guiding me and supporting me. With your limitless faith, I have achieved.

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ABSTRACT OF THE DISSERTATION

Evidence that inhibition of gamma-secretase impairs axonal and dendritic structure and function: a necessary role for APP

by

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Doctor of Philosophy in Neurosciences

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Due to their impact on amyloid precursor protein (APP) processing, gamma secretase inhibitors (GSIs) have recently been considered for treatment of Alzheimer's disease. Recent evidence now indicates that inhibition of gamma-secretase results in cognitive impairment and neuronal dysfunction; however, a mechanism by which this occurs remains elusive. The two most well studied substrates of gamma-secretase, APP and Notch, are proposed to play essential roles in neuronal toxicity experienced, but a direct report of their effects has yet to be performed. Importantly, the role that increased APP CTFs, which GSI treatment directly produces, is only beginning to be

understood in terms of neuronal dysfunction. Therefore, the main body of this dissertation will address the impact that GSI treatment has on wild-type neuronal function as it pertains to the substrate APP. The main output of neuronal function assessed was axonal trafficking and signaling of brain-derived neurotrophic factor (BDNF), a critical neurotrophic factor involved in the maintenance of synaptic connections in the adult central nervous system.

In brief, Chapter 1 of this dissertation will provide an overview of neurotrophic factors and expand their application to include treatment of disorders using neurotrophic factors. Chapter 2 will provide a direct look at APP and APP processing by highlighting unique transport characteristics of various APP and APP CTF positive vesicles. Chapter 3 will bring both APP and neurotrophic factors together in a study of GSI treatment on wild-type neuronal function with an emphasis on BDNF trafficking and signaling mechanisms.

INTRODUCTION

Neurotrophins are critical for development and maintenance of neurons

Over fifty years ago, Rita Levi-Montalcini and her colleagues initiated what has become decades of research devoted to the study of a particular group of growth factors deemed neurotrophins, or neurotrophic factors (Levi-Montalcini and Hamburger, 1951). Originally described for their ability to regulate differentiation and growth as a survival factor during development of the vertebrate nervous system, they have since then been proven to be essential for higher-order functions of the nervous system as well. The known neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3, NT4), each of which initiate signaling cascades through binding to specific tyrosine kinase receptors (Zweifel et al., 2005). Three different tyrosine kinase receptors known as TrkA, TrkB, and TrkC, are expressed differentially in diverse parts of the central and peripheral nervous system leading to particular neurotrophic factor signaling depending on region. In the central nervous system, BDNF is the predominant factor utilized due to the abundant expression of TrkB, while NGF acts more specifically in basal forebrain cholinergic neurons (BFCNs) which express TrkA. In the peripheral nervous system, NGF is the main neurotrophin where it plays multiple roles in survival and function of sympathetic and sensory neurons (Chao, 2003). Heterozygous mouse studies in which NGF and BDNF levels are reduced by

50% have reinforced the importance of these two factors. Beyond traditional roles in neuronal survival, these mice have provided evidence that NGF and BDNF are required for higher order processes such as memory acquisition and retention, neuronal innervations, weight control, synaptic plasticity, mechanosensitivity and neurotransmitter levels (Chao, 2003). In comparison to these two mainly studied neurotrophins, NT3 and NT4 elicit signaling through TrkC and TrkB respectively and have revealed various pro-survival roles through sciatic nerve injury (Thornton et al., 2008) and striatal grafting studies (Perez-Navarro et al., 2000).

Each of the neurotrophins can also bind the receptor p75NTR, albeit at lower affinities than the Trks. There are many emerging proposed functions for p75NTR binding which include either facilitating Trk-mediated signaling (Ceni et al., 2010; Matusica et al., 2013) or working against it by activation of pro-apoptotic signaling pathways (Coulson et al., 2008; Coulson et al., 2000). The spatial and temporal balance achieved between neuronal survival and death depends on the overall level of neurotrophin present and the type of receptors that are expressed (Chao, 2003).

Neurotrophic factor trafficking

Neurons are highly polarized cells with signals initiating both at proximal and distal regions. Therefore, in order for a neurotrophin to elicit a response in a neuron, the signal must be actively transported from the vicinity of internalization towards the nucleus. Although neurotrophic signaling can occur directly at the cell body, it has been shown that NGF applied only to the axon is sufficient for growth and survival of

sympathetic neurons. In fact, if the neuronal body and axon are compartmentalized from each other in Campenot chambers and then NGF is withdrawn from the distal axon side, the neurons degenerate even when NGF is left at the cell body (Campenot, 1977). This, together with evidence which indicates that there is far more TrkA expression in the axons than cell bodies (Campenot, 2003) suggests that NGF must be retrogradely transported from the distal axon to the cell body in order for the neuron to remain viable. Early work in our lab using PC12 cells and dorsal root ganglia (DRG) neurons show that upon NGF induction, TrkA is phosphorylated and endocytosed into the cell through clathrin mediated endocytosis (Grimes et al., 1996). Following this, the NGF/TrkA complex is carried in a specialized early endosome deemed "the signaling endosome" which contains various signaling molecules such as PLC-γ, pERK and the early endosomal protein, Rab5 (Delcroix et al., 2003; Grimes et al., 1997).

Retrograde axonal transport of BDNF has also been shown in several *in vitro* studies to occur through at endocytic compartment both in dendrites and axons (Cohen et al., 2011; Cui et al., 2007; Poon et al., 2011; Zhou et al., 2012). Unlike NGF, the components of the retrograde axonal BDNF signaling endosome and its complete function are still unknown and are under controversy in vivo (Dieni et al., 2012; Nikoletopoulou et al., 2010).

Besides an endocytosed, signaling endosome, alternative methods of passing axonal neurotrophin signals to the cell body may include calcium waves and propagation of signal along the plasma membrane. These methods would provide the

neuron with potentially faster mechanisms to achieve signaling when compared to the transport of a vesicular compartment (Zweifel et al., 2005). Most likely all types of mechanisms are employed to relay both acute and sustained information over the period of neurotrophic factor binding.

Neurotrophic signaling pathways

Binding of neurotrophic factors causes dimerization of the Trk receptors and autophosphorylation at several key sites. Once phosphorylated, Shc and Src adaptor proteins are recruited to the receptor to initiate all downstream signaling events. There are three main pathways which are described for Trk receptor activation: the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinases (PI3K)/ Akt pathway, and the phospholipase C-γ pathway. It is important to note that because neurons are polarized cells, activation of these pathways can serve either a local signaling role or a long-distance role, depending on whether the signaling proteins are endocytosed with the Trk:neurotrophin complex. For example, activation of the MAPK pathway and subsequent activation of the main signaling protein, ERK1/2, have been shown to be important for activation of genes involved in NGF-mediated differentiation of non-polarized cells (Liu et al., 2007; Zhang et al., 2000). It has been shown that ERK1/2 activation is dependent on endocytosis (Zhang et al., 2000) where it may travel with the signaling endosome to the cell body (Grimes et al., 1997; Liu et al., 2007; Wu et al., 2007; Zhang et al., 2000) to elicit changes in gene expression. However, a separate report provides evidence that activation of ERK1/2 in DRG axons using mixed NGF/BDNF applied at the axon acts only locally and it not transported

with the signal to the cell body. Instead the authors report that activation of ERK5, a member of a unique MAPK pathway, is either transported with the signaling endosome or activated once it reaches the cell body (Watson et al., 2001). Activation of ERK5 by axonal application of NGF/BDNF was shown to be critical for downstream activation of nuclear CREB, a transcription factor important for gene expression (Watson et al., 2001). Taken together, these data point to particular, and potentially independent roles, for the components of the MAPK pathway. In addition, these data highlight differences that may be dependent on neuron type or neurotrophic factor added.

On the other hand, activation of PI3K and subsequent activation of the main signaling protein,Akt, has been shown to signal through endocytic mechanisms (Watson et al., 2001; Zhang et al., 2000), even though components of the PI3K pathway have been identified on clathrin coated vesicles from PC12 cells and DRG neurons (Howe et al., 2001). Evidence for a local signaling role for PI3K/Akt has also been supported by other studies which reveal that axonal Akt signaling is important for early Trk complex vesicle formation (Kuruvilla et al., 2000), not long distance signaling events. Activation of the third pathway involving PLC results in very distinct signaling events which are known to involve calcium release and calcium signaling (Segal, 2003). PLCγ has been found on clathrin coated vesicles from PC12 cells and DRG axons suggesting that it can mediate signaling while in transport with the neurotrophin complex (Howe et al., 2001).

APP processing

Amyloid precursor protein (APP) is a type I membrane protein with several proposed functions, some of which may include cell adhesion, trophic signaling, and synaptic modulation (De Strooper and Annaert, 2000; Thinakaran and Koo, 2008). Trafficking and processing of APP have been the focus of investigation for over 20 years due to APP's documented role in Alzheimer's disease (AD) progression (Koo, 2002; Thinakaran and Koo, 2008). AD is one of the most common neurodegenerative disorders, resulting in cognitive impairment and decline in daily functioning (Koo, 2002). One main pathological hallmark of AD is amyloid plaques deposits in regions of the brain responsible for learning and memory. This finding has provided the rationale for the amyloid cascade hypothesis which posits that amyloid beta 40 and 42 (Aβ40, Aβ42), the main components of amyloid plaques, can lead to neuronal dysfunction by means of aggregation or in their oligomeric form (Karran et al., 2011). Pathogenic Aβ is produced from full length APP by sequential cleavage of the protein by a group of enzymes called secretases. β-secretase makes a first cut at the beginning of the Aβ domain, producing a C-terminal fragment known as β-CTF. Subsequentially, γ-secretase liberates the Aβ peptide through multiple cuts, producing varying length fragments (i.e., Aβ42 or Aβ40) APP intracellular domain (AICD) (Takami et al., 2009; Thinakaran and Koo, 2008). Further processing of Aβ region by γ-secretase, which would produce even smaller peptides, is possible; however their impact, if any, on neuronal function is still unknown (Karran et al., 2011).

A non-amyloidogenic processing pathway can also occur through initial cleavage by α-secretase, which cuts internally into the Aβ domain, producing a C-terminal fragment known as α-CTF. Subsequent cleavage by the same γ-secretase liberates a small fragment called P3. For this pathway, γ-secretase cleavage also produces AICD (Thinakaran and Koo, 2008) which is involved in nuclear signaling events.

APP trafficking

Processing of APP through the β-secretase pathway, (the amyloidogenic pathway) seldom occurs in comparison to the non-amyloidogenic pathway driven by α-secretase. Experiments using cell lines such as CHO and HEK293 cells have shown that processing of APP is spatially regulated at specific intracellular locations. β-secretase processing is thought to occur in acidic compartments such as early endosomes or the trans-Golgi-network, while α-secretase activity is thought to be highest at the plasma membrane (Jager et al., 2009). A series of elegant experiments by Haass and colleagues revealed that γ-secretase activity functions predominantly at the plasma membrane and in early endosomes (Kaether et al., 2006), placing it at the sites that β- and α- activity are the highest. Interestingly, both CTFs can inhibit γ-secretase activity with α-CTF being much more efficient at inhibition (Tian et al., 2010). With regard to CTFs, recent data has pointed to independent and potentially harmful roles for these intermediate fragments, leading to the idea of Aβ-independent dysfunction as well (Griffin, 2010; Jiang et al., 2010; Rodrigues et al., 2012). Some of these roles will be discussed later in this dissertation.

In neurons, APP undergoes fast axonal anterograde transport (Koo et al., 1990) and both full length APP and APP CTFs are located in axon terminals (Buxbaum et al., 1998) in addition to other areas of the neuron. Numerous studies have confirmed that APP transport in axons is driven by the anterograde motor kinesin (Ferreira et al., 1993; Inomata et al., 2003; Kamal et al., 2000; Szodorai et al., 2009; Taru et al., 2002); however the mechanism by which APP recruits kinesin remains controversial (Lazarov et al., 2005; Vagnoni et al., 2013). Regardless, axonal APP levels on vesicles are well-correlated with that of a subunit of kinesin-1, kinesin light chain 1 (KLC1) on the same vesicle. Furthermore, reductions in APP cause similar reductions of KLC1, providing further evidence of their interactive capability (Szpankowski et al., 2012).

Polarity of neurons versus that of non-polarized cells has created the possibility that processing of APP by secretases can occur not only at spatially regulated locations, but also en route to specific locations. Indeed, APP has been found to be present in vesicles containing β-secretase and the catalytic subunit of γ-secretase, presenilin-1 (Kamal et al., 2001). While this has been disputed (Lazarov et al., 2005), APP processing and regulation of processing inside distinct types of vesicles has been identified (Steuble et al., 2010) and is still under current investigation.

Anterograde and Retrograde Motor proteins

Both neurotrophic factors and APP have at least one property in common—they both undergo axonal transport, albeit for the most part in separate directions.

Anterograde movement canonically requires kinesin while retrograde movement requires the retrograde motor dynein to be actively at work. Conventional kinesin, kinesin-1, was first discovered in 1985 (Brady, 1985; Vale et al., 1985). Kinesin-1 is composed of two heavy chains and two light chains (Bloom et al., 1988; DeBoer et al., 2008; Hirokawa et al., 1989). The heavy chains can exist in one of three isoforms known as Kif5a, Kif5b, and Kif5c, while the light chains are named as KLC1 and kinesin light chain 2, KLC2 . Since the discovery of kinesin-1, more than 40 other kinesins have been reported with varying cargoes in which they transport (Hirokawa et al., 2009). For example, Kif1a has no light chain involvement (Hirokawa et al., 2009) and has been shown to associate with synaptic vesicle precursors (Okada et al., 1995). Mitochondria (Glater et al., 2006) and even TrkB receptors (Arimura et al., 2009; Huang et al., 2011) have been reported to associate with different variations of kinesin and/or its subunits as well. Optical trap studies and other *in vitro* experiments have provided a multitude of evidence showing that the stepping behavior of kinesin is invariant and therefore very processive in the anterograde direction. Due to this characteristic, kinesin is considered a strong motor, showing stalling and detachment forces only at high 6-7 pN values (Hendricks et al., 2010; Hirokawa et al., 2009; Holzbaur and Goldman, 2010; Soppina et al., 2009; Yildiz et al., 2008).

Dynein, in comparison to kinesin, exists as a single, large cytoplasmic molecule which must carry out transport of multiple cargoes with little variation in structure. However, the way in which dynein functions is much more variable than kinesin. This lends to its ability to have variant stepping behavior, meaning that

dynein is capable of taking shorter steps, longer steps, backwards steps, and side steps (Gennerich et al., 2007; Kardon and Vale, 2009; Mallik et al., 2004). Dynein has been shown to be very responsive to load in which the higher the load, or force experienced, the more reduced the stepping behavior (Mallik et al., 2004) . In force experiments, mammalian dynein stalls at only 1pN, making it weaker than that of kinesin (Mallik et al., 2004; Soppina et al., 2009).

Even though one motor may dictate movement of a cargo in a particular direction, many organelles have been found to contain both the retrograde and anterograde motors (Hendricks et al., 2010; Soppina et al., 2009). As it pertains to neurotrophic factor and APP transport, both APP and Trk transport vesicles have been shown to be able to recruit *both* kinesin and dynein (Arimura et al., 2009; Huang et al., 2011; Szpankowski et al., 2012; Yano et al., 2001) suggesting that there may be regulatory components of bidirectional transport in which one motor can successfully dictate the net direction. Some of these regulatory features include microtubule affinity of motors, detachment forces, mixed motor ensembles, stall forces, and protein interactions of motors with adaptor proteins (Holzbaur and Goldman, 2010). A simplistic model for bidirectional transport of an organelle which contains both kinesin and dynein is best represented by the Tug-of-War model. This model is based on evidence, both from purified motor protein ensembles conjugated to beads and from *in vitro* purified bidirectional transport organelles, which conclude that on an ordinary transport compartment there must be about 6-7 dyneins in order to "compete" with a single kinesin (Hendricks et al., 2010; Holzbaur and Goldman, 2010; Mallik et

al., 2004) (Derr et al., 2012). The tug-of-war model assumes then that any changes in motor composition to favor one motor binding over the other can dramatically alter the directionality of movement solely based on a tug-of-war between motors (Hendricks et al., 2010). Given the well-documented properties of each motor, this model would then predict that increases in kinesin would be detrimental to directionality in the retrograde direction and that dynein would have to increase substantially to influence movement in the anterograde direction.

Axonal transport deficits in AD

In considering the complexity of transport in both directions, proper regulation of movement along the axon becomes increasingly necessary in order for the daily activities of a neuron to be accomplished. Maintained proper axonal transport is critical for the movement organelles and proteins to and from cell bodies and synaptic areas. Considerable evidence points to an early pathological role for disrupted axonal transport in several neurodegenerative diseases including but not limited to AD (Goldstein, 2012; Reddy and Shirendeb, 2012; Salehi et al., 2006; Shirendeb et al., 2011; Ye et al., 2012). In AD, axonal defects can present themselves in several ways, one of which are axonal swellings and organelle accumulations, which has been found to be present in mouse models of AD (Stokin et al., 2005). In addition, defective axonal transport of mitochondria (Calkins et al., 2011) and the synaptic vesicle precursors, synaptophysin and syntaxin, has been reported (Pigino et al., 2003). Axonal transport of BDNF has also found to be disrupted in AD (Ye et al., 2012) and impaired retrograde transport of BDNF has been confirmed in the AD mouse line

Tg2576 (Poon et al., 2011). Taken together, these data implicate APP and altered APP dose or processing in axonal transport deficits of AD. Indeed, several reports have shown that overexpression of APP or addition of Aβ oligomers can recapitulate the axonal transport deficits described above (Gunawardena and Goldstein, 2001; Poon et al., 2011; Rui et al., 2006; Torroja et al., 1999). In addition, these phenotypes are very similar to what has been found in kinesin mutant mice (Karle et al., 2012; Rahman et al., 1999) suggesting a potential role for motor protein alterations in AD progression as well.

Inhibition of γ-secretase as a treatment for AD

Given the evidence surrounding the effect of APP and Abeta toxicity in Alzheimer's disease, small molecule drugs known as gamma secretase inhibitors (GSIs) have been actively pursued over the years. Since treatment with a GSI will inhibit any final cleavages of APP CTFs, all Abeta formation will be eliminated, including the ones thought to be more toxic. Several GSI compounds have now been brought to human trials even though some toxicity that was attributed to failed processing of γ-secretase's other substrates has been noted with their use (Wolfe, 2008a, b). However, in 2010, Lilly corporation announced that they were halting Phase III clinical trials using a GSI because the AD patients that received the GSI showed significant increased cognitive impairment and loss of daily functioning compared to the placebo group (Doody et al., 2013). They also noted that those taking GSI had increased risk of skin cancer, presumably through inhibiting processing of another gamma-secretase substrate called Notch. These findings have since then been

confirmed in treated Tg2576 mice (Mitani et al., 2012) and in slice cultures (Hajos et al., 2013). In these studies it was postulated that the increase of APP CTFs, a direct effect when inhibiting gamma-secretase, was at least partially responsible. Since then alternative methods for attenuating Aβ-mediated toxicity has been under investigation such as γ-secretase modulators (GSMs) that preferentially lower Aβ40/42 species, leaving overall -secretase substrate processing unaffected (Eriksen et al., 2003; Kounnas et al., 2010; Kukar et al., 2005; Mitani et al., 2012; Weggen et al., 2001). Further studies will be necessary in order to determine if alternative anti-amyloid therapies will be beneficial therapies for AD. In addition, it will be important to elucidate the impact that APP CTF accumulation has on a neuron given that these GSI results highly suggest that there are pathological consequences to increases in APP CTF load, apart from those of that are Abeta-dependent.

Contribution of this dissertation work

BDNF trafficking and signaling has been shown to be critical both in development and during maintenance of neuronal connections in adulthood. It is known that APP or altered APP processing can lead to deleterious effects on the traffic of multiple transport compartments as mentioned above, including BDNF, although complete mechanisms of action are unknown. In this dissertation, we examine the role that altered APP processing plays on BDNF trafficking and signaling by using a small molecule GSI which we know directly increases APP CTFs. The majority of experiments to examine APP CTF accumulation on neuronal function have generally been limited to overexpression studies in which one or more of APP's processed

fragments are exogenously expressed or in mouse models of AD, in which APP CTF accumulation is present early and therefore no temporal control of APP processing can be obtained. By using GSI treatment we were able to directly and temporally control APP CTF accumulation and then assess specific aspects of BDNF function.

The overall contribution of this dissertation is two-fold. Firstly, we provide evidence that GSI treatment causes multiple transport deficits, some of which we show are directly linked to APP CTF accumulation. Over time these phenotypes, most likely working in tandem, would eventually lead to deleterious effects on synaptic function and circuitry. Therefore these data may explain the failure of recent clinical trials using GSIs. Secondly, we provide data and a potential model to inform how APP CTF dose can directly alter the trafficking and signaling of a retrograde signal which may be linked to APP's ability to recruit particular motor proteins. Models like this can be experimentally assessed in disorders in which APP CTF or APP gene dose is elevated (i.e. Down syndrome, PS FAD mutations) to provide insight into novel mechanisms related to disease progression.

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CHAPTER 1

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Translational Neurodegeneration

REVIEW Open Access

Current advances in using neurotrophic factors to treat neurodegenerative disorders

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Abstract

Neurotrophic factors are best known for their roles in both development and continued maintenance of the nervous system. Their strong potential to elicit pro-survival and pro-functional responses in neurons of the peripheral and central nervous system make them good drug candidates for treatment of a multitude of neurodegenerative disorders. However, significant obstacles remain and need to be overcome before translating the potential of neurotrophins into the therapeutic arena. This article addresses current efforts and advances in resolving these challenges and provides an overview of roadmaps for future translational research and neurotrophin-based drug developments.

Keywords: Neurotrophin, Neurotrophic factor, BDNF, NGF, Gene delivery, Mimetics

Introduction

Neurotrophic factors or neurotrophins are a group of growth factors which have been classically described for their ability to regulate differentiation and to support growth during development of the vertebrate nervous system. The family of neurotrophins consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4). In order to elicit a survival response, each binds to one member of the tyrosine receptor kinase (Trk) family: NGF binds to TrkA, BDNF and NT4 bind to TrkB, and NT3 binds to TrkC. Each of the neurotrophins can similarly respond through an apoptotic pathway initiated by binding to the 75 kD neurotrophin receptor ($p75^{NTR}$). The spatial and temporal balance achieved between neuronal survival and death depends on the overall level of neurotrophin present and the type of receptors that are expressed [1]. In the peripheral nervous system, NGF is the dominant neurotrophic factor, acting on sympathetic and sensory neurons. However, in the central nervous system, BDNF is the predominant neurotrophin utilized due to the abundant expression of TrkB, with NGF providing trophic support specifically to the basal forebrain cholinergic neurons (BFCNs) which express TrkA. Studies from heterozygous mice expressing reduced levels of NGF and BDNF reveal that these two factors are essential for multiple functions throughout adulthood such as proper memory acquisition, memory retention, long-term potentiation, and cholinergic innervation [2,3].

Since the discovery of NGF in the 1950s [4] a large body of experimental data has pointed to multiple roles for the neurotrophins. Firstly, most neurotrophins are required during development and differentiation, during a time which specific synaptic connections are being made and proper circuits are being formed. Secondly, neurotrophin signaling plays an important role in adulthood at a time in which continued maintenance and modulation of those connections is required for normal brain function.

Neurotrophic signaling pathways

Although different neurotrophins act on different receptors in the brain, both NGF and BDNF elicit pro-survival and pro-functional responses using essentially the same canonical signaling pathways: the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinases (PI3K)/ the protein kinase B (also known as Akt) pathway, and the phospholipase C-γ pathway (Figure 1). Binding of neurotrophic factor causes dimerization and autophosphorylation of the Trk

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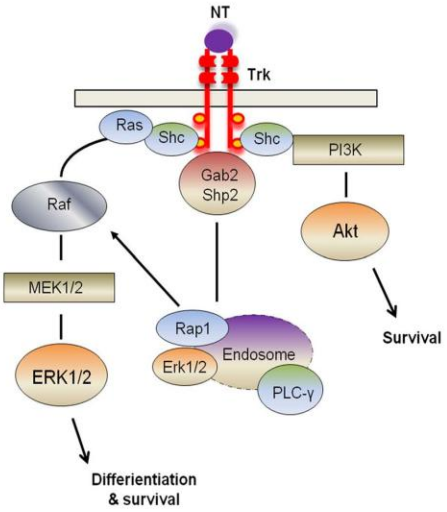

Figure 1 Trk receptor activation by neurotrophic factors. Binding of neurotrophic factor causes dimerization and autophosphorylation of the receptor leading to activation of signaling cascades through Src and Shc adaptor proteins which bind the Trk. Once activated Shc increases the activation of Ras, which leads to MAPK/ERK1/2, causing differentiation and survival through transcriptional events, an event that can also occur in signaling endosomes. Shc activation also leads to increases in Akt through activation of PI3K promoting cell survival by inhibiting apoptotic signaling. NT: neurotrophin; Trk: tyrosine kinase receptor; ERK1/2: extracellular signal-regulated kinase; MEK: mitogen-activated protein kinase; PLC-γ: phospholipase Cγ; rap1: ras-related protein1; Shp2: tyrosine phosphatases.

receptor leading to activation of signaling cascades through Src and Shc adaptor proteins which are recruited to the Trk receptor. Once activated Shc increases the activation of Ras, which leads to MAPK/ERK1/2, causing differentiation and survival through transcriptional events. Activation of this particular pathway is thought to occur as well in a specialized early endosome, the signaling endosome, which has been shown at least for TrkA to contain various signaling molecules such as PLC-γ, pERK1/2, and the early endosomal protein, Rab5 [5,6]. Transport of the signaling endosome from the axon to the cell body is an important means for transmitting trophic signals to the neuronal soma [7,8]. For sustained MAPK activation, Trk activates the scaffolding proteins Gab2/Shp2 and involves the small G protein, Rap1 on the endosome as well [9]. Shc activation also leads to increases in Akt through activation of PI3K promoting cell survival by inhibiting apoptotic signaling, even though this activation is thought to occur more at the cell surface rather than on intracellular endosomes [10]. These different signaling pathways that are activated by neurotrophins work together to support normal neuronal function and to prevent neuronal cellular death.

Neurotrophins and neurodegenerative diseases

Given the critical role played by neurotrophins in regulating neuronal functions, it is not surprising then that a significant number of psychiatric and neurodegenerative disorders is associated with altered NGF and BDNF levels and with changed expression of their receptors. For example, neurodegenerative phenotypes similar to Alzheimer's disease (AD) are observed in a mouse model in which half of the NGF level is neutralized by antibodies [11]. In fact, the brains of AD patients and aged rats show reduced NGF levels in BFCNs [12-14]. Another neurodegenerative disorder, Down's syndrome (DS), exhibits similar NGF signaling deficits in the same region of the brain [15]. Neurotrophins undoubtly have a strong role in preventing cellular death of BFCNs.

The key role for NGF was discovered in early studies on transected fimbria in which administering NGF upon transection was able to markedly reduce cholinergic neuron death, which was typically induced by the procedure [16]. In addition, NGF administration was found to partially reduce cholinergic atrophy in aged rodents [17]. However, to complicate these neurodegenerative disorders further, alterations in BDNF and its receptor are seen in two very important areas that control spatial memory and higher cognitive function: the frontal cortex (FC) and the entorhinal cortex (EC). Alterations in BDNF in these neurons and the overall selective vulnerability of specific areas to degeneration are seen not only in AD, DS, and normal aging, but also other disorders of the brain pointing to multiple roles for BDNF in particular. Both protein and mRNA levels of BDNF are decreased in dopaminergic neurons of the substantia nigra [18], the neurons most vulnerable in Parkinson's disease. BDNF has been shown to have survival role here and alterations in BDNF most likely contribute to the disease [19]. In a similar manner, in Huntington's disease, BDNF transport from cortical to striatal neurons is deficient, contributing to selective loss of striatal neurons and voluntary muscle movements in patients with the disease [20,21]. Moreover, BDNF levels are thought to play an important role in susceptibility of non-neurodegenerative diseases. For various psychiatric disorders like bipolar, depression, anxiety, and schizophrenia, it has been shown that there are abnormal increases and decreases in levels of BDNF throughout the brain and in plasma [22-24]. Strong evidence for the link between these disorders to BDNF specifically originates from data from patients carrying a BDNF variant gene, which contains a methionine mutation in the prodomain. Evidence from these subjects and mouse models carrying the

mutation shows smaller hippocampal volumes, along with decreased activity-dependent BDNF release [22].

While it is still unclear as to why certain areas of the brain are more vulnerable in various disorders over other areas of the brain, one effect that is certain is that the synaptic loss and neuronal dysfunction in these areas lead to detrimental changes to overall synaptic transmission [25-27]. Indeed, vulnerable areas in the brain which are impacted the most in many of the diseases mentioned above show a decrease in neuronal size and number, along with reduced expression of neurotransmitter molecules and receptors in response to the decreased trophic support [28]. It is conceivable that cellular and intracellular changes to neurons, induced by alterations in signaling cascades, can impair neuron's ability to function properly. Alterations in any component along the signaling/survival pathways could potentially exacerbate the deficit in trophic support for neurons, resulting in their dysfunction either locally and/or on a circuit level.

The well documented role for neurotrophic factors to prevent cell death and to maintain cellular function has led scientists to investigate their translational benefit(s). To date, the potential beneficial effect of neurotrophins, NGF and BDNF, in particular, have been explored in light of several neurodegenerative disorders, including but not limited to AD, Amyotrophic lateral sclerosis (ALS) [29], Huntington disease [30], Parkinson's Disease and even obesity [31] (Table 1).

Challenges in neurotrophin-based therapy

Although there are strong rationales suggesting that increasing supply of neurotrophins to degenerating neurons may be a potent way to restore neuronal function in neurodegenerative conditions, delivering neurotrophins into the brain has proven to be a non-trivial matter. Notoriously, CNS diseases are difficult to treat due to the presence of the blood brain barrier (BBB) that makes it almost impossible for large proteins and complex compounds to cross from the blood into the brain. In addition, the cortical and subcortical circuits of the brain are interconnected resulting in crosstalk among multiple regions, so coming up with a treatment strategy that selectively targets affected neurons only, but not those unaffected ones, is a great challenge that has to be carefully considered. To further compound these issues, neurotrophins are relatively large, polar molecules that cannot readily cross BBB and therefore must be administered directly into the central nervous system (CNS). Indeed, all current delivery strategies involve invasive procedures as discussed below.

Infusion of neurotrophins by direct intracerebroventricular (ICV) injection

To bypass the inability of neurotrophins to cross BBB, purified neurotrophins can be directly infused to the brain by intracerebroventricular (ICV) injection. This delivery route is particularly suitable for NGF to treat BFCN degeneration, since BFCNs extend their axons throughout the hippocampus and neocortex. NGF that is infused into the lateral ventricle can act on the TrkA receptor located at the axonal termini to retrogradely transmit trophic support signal for BFCNs. This approach has been proven especially effective in preventing loss of BFCNs in rodents associated with lesions and aging as mentioned above. However, clinical trial with NGF infusion showed that, although long-term NGF administration by ICV injection may cause certain potentially beneficial effects, the intraventricular route of administration is also associated with significant side effects [33], such as hyperinnervation of cerebral blood vessels [34], hypophagia [33,35], Schwann cell hyperplasia with sprouting of sensory and sympathetic neurons [36], and neuropathic pain [33]. As such,

Table 1 Neurotrophic factors that are currently under study for treatment of various disorders

	Neurotrophic factor	Target neurons	Current status
ALS	NGF and BDNF	Motor neurons	Recruiting for Phase 1 and Phase 2
Parkinson's disease	GDNF/neurturin	Striatal neurons	Some Phase 1 complete, ongoing in Phase 1 and Phase 2
Huntington's disease	BDNF	Striatal neurons	Pre-clinical
Alzheimer's disease	NGF and BDNF	Cholinergic neurons, entorhinal neurons	Ongoing in Phase 1
Down Syndrome	NGF	Cholinergic neurons	Pre-clinical
Spinal Cord Injury	BDNF and NT-3	Site of injury	Pre-clinical
Obesity	BDNF	Hypothalamus	Pre-clinical
Lysosomal storage disorders	BDNF	Various in CNS	Pre-clinical
Sensory neuropathies	NGF	Sensory and sympathetic neurons	Phase 2 completed
Supranuclear Palsy	GDNF	Various in CNS	Phase 2 completed

Current status defines Phase trials that have either been completed or are underway [32]. Pre-clinical status was assigned to each if the neurotrophic factor has been shown to rescue neuronal functioning in target neurons in rodent and primate models of disease.

these side effects may cause serious concerns in limiting the dose of infused NGF, thus providing only little therapeutic benefit.

NGF involvement in pain is stemmed from its ability to activate the nociceptive response in sensory neurons [37]. NGF as a therapeutic tool has been particularly impacted by this characteristic, even in attempt to treat peripheral neuropathies such as diabetic and HIV-induced neuropathy, two disorders that do not have the delivery barriers to overcome like those of the CNS. Clinical trials with NGF treatment of these two types of neuropathies have to be terminated due to the fact that severe pains were induced in patients, even though symptoms associated with both disorders were ameliorated in early Phase II studies [38,39]. Even healthy volunteers administered with NGF will begin to feel hyperalgesia at the injection site after 3 hours, with worsening effects over the course of three days [40].

Current efforts with infusion of other neurotrophins have yielded similarly disappointing results. For example, to increase the delivery of BDNF, one study has used intrathecal infusion of N-terminal pegylated BDNF after spinal cord injury. While the authors were able to show that pegylated BDNF was able to reach the spinal cord and induce expression in that area, they saw no improved axonal response or locomotor recovery, suggesting the amount of BDNF that was delivered was still insufficient [41]. In a separate study, although enhanced delivery of BDNF to the CNS was achieved intravenously using a combination of pegylation and conjugation to antibodies targeting the transferrin receptor of BBB, *in vivo* data from this dual approach is still lacking [42]. Intraputamenal infusion of glia-derived neurotrophic factor (GDNF) in Rhesus monkeys has also led to reduced food consumption and weight loss, meningeal thickening and Purkinje cell loss in the cerebellum [43]. More importantly, GDNF infusion provided no significant benefit to human patients with Parkinson's disease [44]. This reoccurring theme of side effects without significant benefit of treatment has also been shown with intrathecal infusion of recombinant BDNF in patients with ALS [29,45].

Neurotrophin-producing cell transplantation

To circumvent the lack of significant therapeutic benefits in combination with serious adverse effects associated with the infusion approach, other methods are designed to achieve more targeted delivery of neurotrophins directly to those populations of neurons affected in disease. This would allow for more controlled and increased dosing while potentially eliminating side effects through avoiding unknown interactions of the neurotrophin. Currently, two approaches of direct delivery of neurotrophic factor into subcortical sites of the brain have been developed and practiced: transfer of cells modified to express neurotrophic factor and delivery of an engineered viral vector encoding the neurotrophic factor protein. The first technique involves establishing cell lines, preferably from the donor host, to express the neurotrophic factor through transfection and selection using vectors containing the gene of interest. Once expression of the gene is assessed and optimized, the cells can be transferred into brain regions to function locally in providing neurotrophic factor (Figure 2). Early proof-of-principle studies for this approach were carried out in 1987 by Gage and colleagues who first established donor rat fibroblast cells to express a prototype HPRT vector then grafted these cells into several regions of the rat brain. They found that HPRT enzymatic activity in the grafted cells remained high for at least 7 weeks after transfer [46]. Following this study, genetically modified fibroblasts secreting NGF were implanted into the brains of rats with fimbria lesions. Not only were the transplanted cells able to survive and produce NGF, they were able to prevent cholinergic loss and cause the surviving neurons to sprout axons towards the direction of NGF-secreting fibroblasts, an indication that the neurons were functioning properly [47]. Similar studies in primate and non-human primates also showed that genetically modified cells from various cell lines (baby hamster kidney and primary cells) were able to rescue cholinergic functioning in injured neurons [48-50]. In addition, this type of gene therapy has been used in both rodent and primate aging models to show that age related reductions in neuronal functioning and memory impairment can be ameliorated through delivery of genetically modified cells that express NGF [51-53]. More importantly, implanted cells sustained NGF production for at least 8 months in the primate brain, and furthermore, administration of NGF in this manner did not elicit those adverse side effects that were seen in infusion studies [52,53], indicating that direct gene delivery could offer a large therapeutic benefit in disease. These findings and ensuing preclinical studies have laid the groundwork that led to the first human clinical trial of NGF gene delivery.

At the beginning of 2001, eight subjects both male and female in early stage AD were enrolled. Primary autologous fibroblasts derived from each subject were genetically modified to produce human NGF using retroviral vectors. Once NGF production was established the cells were injected into the basal forebrain of the subjects either unilaterally or bilaterally. Of those that safely received the NGF delivery, the mean Mini-Mental Status Examination (MMSE) scores showed a mean rate-of-decline reduction of 51 % over a period of 22 months, and an even higher reduction during the 6–18 months when NGF production remained robust. In addition, there were cognitive improvements and increased PET

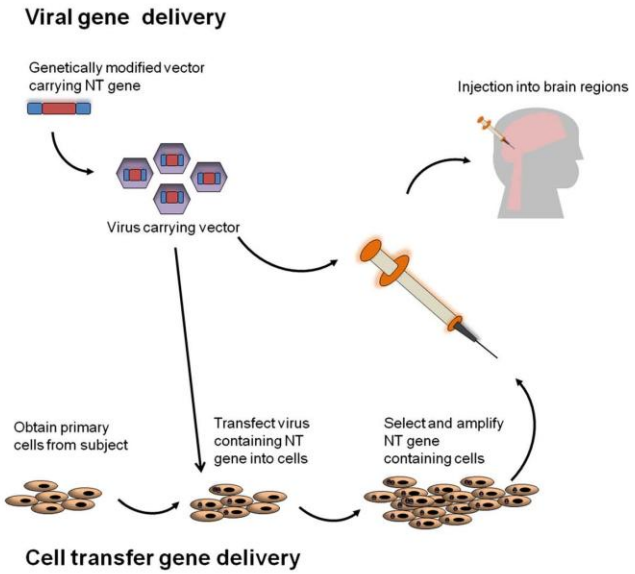

Figure 2 Gene delivery techniques currently being used to deliver neurotrophin to various sites in the nervous system. Direct viral gene delivery of neurotrophin (NT) gene occurs through insertion of the neurotrophin into a viral vector and then placement of the viral vector into a host virus, such as adeno-associated virus or lentivirus. Virus is then directly injected into the brain area(s) through surgical techniques. Cell transfer gene delivery first involves obtaining host cells, preferably from the subject and then transfecting them with virus containing the neurotrophin gene. Once selection and amplification of genetically modified cells is performed, and production of the neurotrophin is confirmed, those cells are then injected or grafted into the brain area(s) through similar surgical techniques.

scan activity in several areas of the brain. Post-mortem analysis of one subject which died 5 weeks after NGF delivery confirmed that there was robust NGF expression in the cell grafts and that cholinergic axons showed sprouting. Overall this study provided the first clinical evidence that directed neurotrophic factor delivery could provide therapeutic benefit without side effects commonly associated with neurotrophic factor infusion. Only hemorrhages in two subjects were observed during injection that may be due to unwanted movements during the procedure. One patient did not recover and died shortly after surgery. General anesthesia has been since adopted to mitigate the problem [54]. Currently, a Phase II clinical trial with this approach is underway.

This technique has also been applied successfully for grafting of BDNF and NT-3 in the treatment of spinal cord injuries. Although not yet performed in human patients, fibroblasts that were modified to express BDNF or GDNF and NT-3 and were grafted into sites of spinal cord injury induced sustained regenerative and sprouting responses into the sites of injury in rats [55,56]. Overall direct gene delivery in the clinical setting may prove to have the most beneficial impact yet, this type of procedure remains an invasive technique. Furthermore, although grafted cells have been shown to be functional for up to one year after implantation, subsequent injections may have to be given over the course of a subjects lifetime in order to sustain benefits from the treatment. Moreover, the long term effect(s) of the presence of the large number of fibroblasts in the brain needs to be fully evaluated.

Viral vector-mediated gene delivery

Due to advances in molecular research in the past decade, viral vector-mediated gene delivery may prove to be a more optimal approach (Figure 2). Delivery of a virus would confer a permanent change in the ability of the neuron to make its own neurotrophic factor, leading to a single injection at the site instead of multiple injections, therefore decreasing the invasiveness. Intrastriatal injection of adeno-associated virus (AAV) vector encoding an enzyme essential in the production dopamine, aromatic L-amino acid decarboxylase (AADC), into MPTP-lesioned non-human primates resulted in expression of the enzyme for at least six years [57]. Even more appealing in viral delivery is that the cumbersome cell preparation associated with the cell transfer technique would be eliminated and that AAV vectors do not express their

own proteins and therefore would not elicit an immune response. To date, viral delivery has been used and evaluated in a number of rodent, primate and human subject studies, particularly for Parkinson's disease (PD). A hallmark of PD is specific dopaminergic loss in the striatum, leading to neuronal and motor dysfunction. Viral gene delivery of AAV encoding AADC was shown to provide eight years of clinical improvement in non-human primates, one of the longest *in vivo* studies that have been performed thus far [58]. Similarly, viral delivery of GDNF by lentivirus reversed motor deficits in MPTP-treated monkeys and prevented nigrostriatal degeneration [59]. AAV-mediated delivery of an analog of GDNF, neurturin, has also been shown to be neuroprotective for dopaminergic neurons in rats [60]. Studies have demonstrated that injection of the neurturin viral vectors is safe, tolerable and could potentially lead to improvements in motor functioning of actual Parkinson's disease patients at 1 year and in rhesus macaques [61,62]. However, as with cell-mediated gene delivery, a small number of human subjects that were given the injection suffered from intracranial hemorrhages [63], indicating that more surgical training and care, perhaps even better injection techniques, need to be adapted to make this type of treatment more applicable.

Viral treatment has also been explored for treating other disorders such as the lysosomal storage disease, late infantile neuronal ceroid lipofuscinosis [64] in which child subjects demonstrated a reduced rate of neurological decline. In addition, administration of a lentiviral construct expressing BDNF into rodent and primate models of AD showed improved cell signaling and a restoration of learning and memory, while reversing synaptic loss [65]. Lentiviral NGF gene delivery in rats has been just as beneficial in preventing cholinergic neuron loss upon fimbria-fornix lesion injury [66]. Currently ongoing and future clinical trials in human subjects using both BDNF and NGF viral delivery should inform about their safety and efficacy, and their potential benefits.

Neurotrophin-peptide mimetics/ small molecules with neurotrophic properties

It is worth mentioning that many of the challenges facing either direct infusion of neurotrophic factor or cell-, viral vector-mediated gene delivery methods may be overcome with small molecules that target the receptor for the neurotrophic factor instead of introducing the neurotrophic factor itself. This would allow for specific activation of only one type of receptor, such as TrkA or TrkB and not p75, or vice versa, potentially alleviating the side effects. To this end, the discovery and use of peptide mimetics, short peptides that have improved bioavailability and lower degree of proteolysis [67], and small molecules ligands for the Trk receptors [68] have attracted intensive interest. A potent peptide mimetic of BDNF has been shown to activate TrkB, promoting neuronal survival in embryonic chick dorsal root ganglion sensory neurons [69]. Also, small molecule BDNF mimetics have high potency and specificity towards TrkB and can promote neuronal survival as well, while also inducing differentiation and synaptic function in cultured hippocampal neurons [70]. When administered to mouse models of AD, Huntington, and PD, the small molecule was able to rescue cellular death to the same extent of full protein BDNF [70]. Further studies using small molecule mimetics of BDNF confirmed their broad application in both restoring TrkB function and improving respiratory function in mouse models of Rett Syndrome [71] and in facilitating functional recovery after stroke while promoting an increase in the number of neurons adjacent to stroke site [72]. Currently, a number of clinical trials are being carried out using neurotrophic factor mimetics [68]. Results from these trials, especially concerning side effects and efficacy, will broaden and enhance neurotrophic factor -based therapy for treating neurodegenerative disorders.

Combinational therapy using neurotrophins and other small molecules

Neurodegenerative disorders are very complex diseases. Although neurotrophic factor-based strategies have offered great potential, the biggest unknown is whether such approach by itself is adequate in halting and reversing the course of progression of these diseases.

As years have passed and many clinical trials later, the idea that "a single magic bullet approach" or one drug can act as the sole solution for treating neurodegenerative disorders has proven not very successful. This is highlighted well in the case of Alzheimer's disease. For example, acetyl cholinesterase inhibitors (Aricept) and N-methyl-D-Aspartate (NMDA) receptor antagonists (Mementine) have been approved for the treatment of AD, but both treat symptoms and show only moderate efficacy. Unfortunately, both fail to slow the rate of cognitive decline in AD patients [73]. Furthermore, another painful lesson came from the recent failure of a Phase III trial using gamma secretase inhibitors to treat AD, a "disease-modifying" compound which has been a sought after drug target for some time [74]. It is clear that various treatments with small molecule drugs such as these, have yielded only modest results at best. Novel small molecules for AD, including disease-modifying gamma secretase modulators, are currently under extensive evaluation for their potential for AD treatment [75]. Given past failure in monotherapy in this arena, it may become necessary to use a combination of approaches, i.e. combinational therapies, to attack the different disease

causing mechanisms simultanously. We can envision that by a combinational use of a small molecule with neurotrophins may work synergistically to restore neuronal function and to minimize possible side effects as discussed above.

Going forward, the idea of combinational therapies for treating various neurodegenerative disorders is worth serious consideration, given the fact that so many attempts with monotherapies have not yielded any success. For many non-neurodegenerative disorders co-therapies targeting multiple disease pathways and symptoms are actively being used and evaluated. For rheumatoid arthritis, disease modifying antirhematic drugs (DMARDS) are used in combination with fast acting glucocorticoids to alleviate the symptoms of inflammation quickly [76]. The combination of DMARDS and glucocorticoids was shown to cause a reduction in both the tolerability and side effects of DMARD infusion alone [76-79]. Moreover combinational therapy using various DMARDS and glucocorticoids together resulted in short and long term improvements when compared to DMARDS alone [76]. For cardiovascular disease involving cholesterol, treatment with statins is the standard. However, statins have shown over time to eventually lead in some cases to regression of the disease [80]. Co-therapy using statins and niacin have shown to lead to significant decreases in disease causing low-density lipids, while raising beneficial high-density lipids [80,81] which may lead to tighter lipid control and therapeutic benefit for those with statin-treated cardiovascular disease. Even for diabetes, insulin, which has been the main glucose lowering treatment, has been evaluated in combination with recombinant human insulin-like growth factor I (IGF1), a pathway that if restored can lead to much higher glucose lowering than insulin alone. Subjects receiving both IGF1 and insulin together decreased their insulin need, while those treated with insulin alone had an increase in insulin usage [82]. Examples such as these highlight various properties of combinational therapies in treating a disorder and confirm why co-therapy in neurodegenerative disease may prove to be most successful. First, these examples show that one can quickly relieve symptoms of a disease with one or more drugs while concomitantly treating the disease itself with another. This is important since treating a disease will most likely have a longer time course inherent to its action than solely relieving symptoms. Secondly, combinational therapy using two or more drugs can compensate for one drug's inactivity over time and therefore potentially inhibit regression of the disease. The final property illustrated here is that combinational therapy using two or more proteins/small molecules can work synergistically together so that one or both are needed in lower dose or less frequently, a convenience that someone suffering from an illness would definitely benefit from.

Conclusions

NGF and its other family members provide potent trophic support to neurons. Their robust effects in rescuing degenerating neurons cannot be matched in this regard by any small molecules or compounds identified thus far. Neurotrophin-based therapies may well prove to be an effective means to combat epidemic neurodegenerative diseases. Yet, many daunting challenges remain to be resolved. Furthermore, it remains to be seen if such strategies that aim at a single target will be sufficient to cure these diseases. In the end, the inherent complexity of neurodegenerative diseases may require combinational therapies that target multiple pathways for effective treatment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AW drafted the manuscript, CW critically revised the manuscript. Both authors read and approved the final manuscript.

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CHAPTER 2

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Enhanced β-secretase processing alters APP axonal transport and leads to axonal defects

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Alzheimer's disease (AD) is a neurodegenerative disease pathologically characterized by amyloid plaques and neurofibrillary tangles in the brain. Before these hallmark features appear, signs of axonal transport defects develop, though the initiating events are not clear. Enhanced amyloidogenic processing of amyloid precursor protein (APP) plays an integral role in AD pathogenesis, and previous work suggests that both the Aβ region and the C-terminal fragments (CTFs) of APP can cause transport defects. However, it remains unknown if APP processing affects the axonal transport of APP itself, and whether increased APP processing is sufficient to promote axonal dystrophy. We tested the hypothesis that β-secretase cleavage site mutations of APP alter APP axonal transport directly. We found that the enhanced β-secretase cleavage reduces the anterograde axonal transport of APP, while inhibited β-cleavage stimulates APP anterograde axonal transport. Transport behavior of APP after treatment with β- or γ-secretase inhibitors suggests that the amount of β-secretase cleaved CTFs (βCTFs) of APP underlies these transport differences. Consistent with these findings, βCTFs have reduced anterograde axonal transport compared with full-length, wild-type APP. Finally, a gene-targeted mouse with familial AD (FAD) Swedish mutations to APP, which enhance the β-cleavage of APP, develops axonal dystrophy in the absence of mutant protein overexpression, amyloid plaque deposition and synaptic degradation. These results suggest that the enhanced β-secretase processing of APP can directly impair the anterograde axonal transport of APP and are sufficient to lead to axonal defects *in vivo*.

INTRODUCTION

Amyloid precursor protein (APP) plays an essential role in the pathogenesis of Alzheimer's disease (AD), a prevalent neurodegenerative disease in which patients suffer cognitive decline and memory loss. Pathological hallmarks of AD brains include intracellular neurofibrillary tangles and extracellular amyloid plaques. A primary component of amyloid plaques is Aβ, a peptide derived from the sequential proteolytic processing of APP by β- and γ-secretases. APP is a transmembrane protein that undergoes axonal transport (1–5), but it is not clear how proteolytic processing of APP affects its axonal transport properties. It is known that full-length APP and its C-terminal fragments (CTFs) accumulate at terminal fields (6) following anterograde axonal transport and may undergo further processing into Aβ that is released at the synapse (7). Full-length APP and cleavage fragments are reported to localize into distinct neuritic vesicles (8,9); however, there is no precise comparative characterization of their individual transport behaviors.

Elucidating the influence that proteolytic processing has on APP axonal transport may provide important insight into understanding the phenotypes characteristic of axonal transport defects that develop both early and late in AD. Before the hallmark pathologies of amyloid plaques and neurofibrillary tangles develop, axonal swellings can be found in sporadic AD brains (10). These types of axonal swellings, consisting of accumulated organelles and vesicles, have previously been described in animal models with mutations in genes encoding motor protein components, as well as in animal models that overexpress wild-type (WT) or familial AD (FAD) mutant APP (2,10–16).

Transgenic mice overexpressing FAD Swedish APP mutations (Tg-swAPP^{Prp}) develop the phenotypes typical of

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axonal transport defects similar to those seen in sporadic AD (10). Swedish mutations of APP enhance the β-secretase cleavage of APP and are sufficient to cause FAD (17). Intriguingly, β-secretase cleaved CTFs (βCTFs) generated from the β-secretase cleavage of APP are also found to be increased in sporadic AD brains (18), as are protein levels and the enzymatic activity of the APP β-secretase (BACE) (19,20). A previous study has shown that elevated βCTF levels induce endosomal defects (21), which is also an early feature of sporadic AD (22). These findings highlight the importance of investigating the β-secretase cleavage of APP in disease mechanisms, and as a potential regulator of APP axonal transport. Other perturbations to APP, such as overexpression and deletion, have also been found to disrupt axonal transport, with overexpression phenotypes dependent on the APP C terminus (2,14,23–25). In addition to the finding that axonal transport defects can be caused by the overexpression of the APP C terminus (2), Aβ is reported to cause axonal transport defects when added exogenously (26–32). Since the β-secretase cleavage of APP generates βCTFs that are subsequently cleaved by γ-secretases to generate Aβ, it is possible that the enhanced β- or γ-secretase cleavage of APP could affect the axonal transport of APP specifically. However, the relative contributions to axonal transport defects of APP processing to βCTF or Aβ, or of APP overexpression, are unknown and have not been directly studied.

Here, we tested the hypotheses that the enhanced β-secretase cleavage of APP directly alters APP axonal transport in cultured mouse hippocampal neurons and is sufficient to lead to axonal defects *in vivo* without overexpression. We performed a detailed characterization of the live axonal transport of full-length APP, full-length APP mutated at its β-secretase cleavage site and C-terminal APP cleavage products, in order to compare their axonal transport behaviors. Direct examination of WT and mutant APP real-time axonal transport demonstrated that enhanced β-secretase cleavage reduces APP anterograde axonal transport, while inhibited β-secretase cleavage stimulates it. Our analyses specifically implicated βCTF production, rather than αCTF or Aβ production, for these differences in axonal transport behaviors. βCTFs also showed decreased anterograde axonal transport compared with full-length APP. To clarify if the overexpression of mutant APP is necessary to induce axonal defects, we examined a gene-targeted mouse. Analysis revealed that FAD Swedish mutations of APP, which enhance β-secretase cleavage, are sufficient to induce plaque-independent axonal dystrophy in the form of axonal dilation, even when mutant APP is expressed at endogenous levels. Axonal defects occurred in the absence of any detectable synaptic degradation. These results suggest that the enhanced β-secretase processing of APP can reduce the anterograde axonal transport of APP itself and can contribute to the development of axonal defects *in vivo*.

RESULTS

APP-YFP β-secretase cleavage site mutations alter its cleavage

To test if alterations in the β-secretase cleavage of APP can affect APP axonal transport properties, we generated and characterized two mutant forms of human APP tagged with yellow fluorescent protein (APP-YFP). FAD Swedish APP-YFP possesses two mutations at the β-secretase cleavage site (K595N and M596L) to enhance β-secretase cleavage (17,33). M596V APP-YFP has a single mutation at the same site to inhibit β-secretase cleavage (34) (Fig. 1B). To confirm that Swedish and M596V mutant APP-YFP undergo enhanced or suppressed β-secretase cleavage, respectively, we performed western blots from cells transfected with WT, FAD Swedish (**SWE**) or M596V (**MV**) APP-YFP. The C-terminal β-secretase cleavage product of APP-YFP, βCTF-YFP, was increased in cells transfected with SWE APP-YFP and decreased in cells transfected with MV APP-YFP compared with WT APP-YFP (Fig. 1C). Levels of full-length, endogenous mouse APP remained relatively unchanged. Thus, as expected, mutations of the β-secretase cleavage site of APP-YFP alter its cleavage by β-secretase.

FAD Swedish mutations reduce the anterograde axonal transport of APP-YFP

Although the overexpression of FAD Swedish APP causes axonal transport phenotypes in *Drosophila* and mouse (2,10), the effects of these mutations specifically on APP vesicle movement dynamics in axons remain untested. In order to understand the role that proteolytic processing plays in APP axonal transport, we first compared the axonal transport dynamics of SWE APP-YFP to those of WT APP-YFP.

Primary cultures of mature hippocampal neurons with well-established axonal projections from WT mice were transfected at 10 days *in vitro* with either WT or SWE APP-YFP. Experiments were carefully designed so data could be collected in parallel, and only data from multiple repetitions of these experiments performed on several different days were compared with each other. In addition, several culture preparations were transfected, and several different plasmid preparations of each construct were used. Care was taken to collect data only from axons of neurons lacking any gross morphological signs of cellular toxicity. A large amount of fluorescent protein was present within transfected cell bodies (Fig. 2A), but individual puncta of moving APP-YFP-containing vesicles were visible within axons distal to the cell body (Fig. 2B, arrows). Neurons transfected with WT or SWE APP-YFP showed similar numbers (Fig. 2C) and intensities (Fig. 2D) of fluorescent particles in axons, suggesting that there is no major difference in the amount of tagged protein that enters axons under these conditions.

Movement of APP-YFP vesicles across time was plotted on kymographs (Fig. 2E) and analyzed. FAD Swedish mutations induced obvious changes to the axonal transport of APP-YFP vesicles, including a significant decrease in the percentage of SWE APP-YFP particles moving in the anterograde direction when compared with WT APP-YFP (Fig. 2F; $P = 0.017$). More precise characteristics of axonal transport dynamics include segmental velocity and run length, which are defined as the speed and distance a particle travels in one direction without being interrupted by a pause in movement or a reversal in direction traveling. Segmental velocities and run lengths of anterograde and retrograde SWE APP-YFP were comparable with WT (Fig. 2G and H). Thus, FAD Swedish mutations, which enhance the β-secretase cleavage of APP, alter axonal transport of APP itself by reducing its anterograde transport.

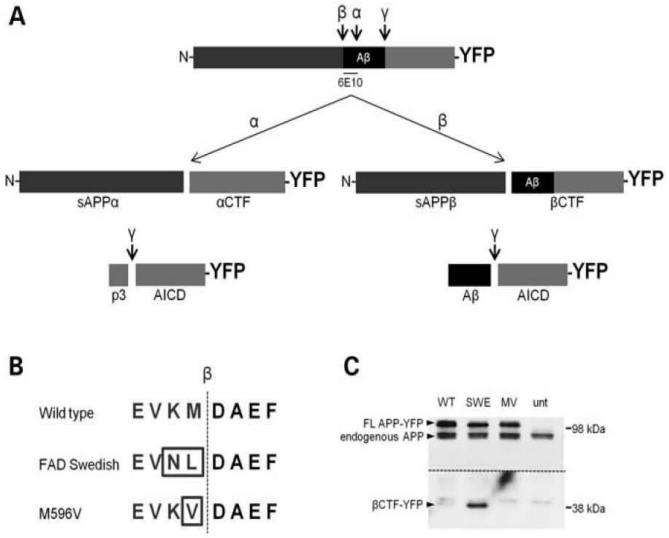

Figure 1. APP-YFP β-secretase cleavage site mutations alter its cleavage. (**A**) A schematic representation of the APP protein tagged with YFP, with the secretase cleavage sites indicated. Aβ region is indicated in black, and the 6E10 antibody epitope is identified. Cleavage products formed upon α-, β- and γ-secretase cleavage are indicated. (**B**) Amino acid sequence flanking the β-secretase cleavage site (indicated by dotted line) for WT, SWE and MV APP-YFP. (**C**) Western blot of SH-SY5Y cells transfected with WT and mutant (SWE and MV) APP-YFP and untransfected (unt) cells as a control. The 6E10 antibody epitope lies in the Aβ region, so it will recognize full-length APP-YFP and βCTF-YFP, but not the αCTF-YFP. Top arrowheads identify full-length APP-YFP and full-length endogenous APP bands, and bottom arrowhead identifies βCTF-YFP bands. Both YFP bands are absent in the untransfected control. Above the dotted line is the membrane at a low exposure. Below the dotted line is the same membrane at a higher exposure to reveal the βCTF-YFP bands. This higher exposure also reveals a set of non-specific bands present in all samples that lies immediately above the βCTF-YFP bands.

Inhibition of β-secretase cleavage by the MV mutation enhances the anterograde axonal transport of APP

Because FAD Swedish mutations at the β-secretase cleavage site of APP enhance its cleavage by β-secretase and reduce APP anterograde axonal transport, we tested the ability of β-secretase cleavage inhibition to stimulate anterograde APP axonal transport. Thus, we examined the axonal transport of APP-YFP with an M596V mutation at the β-secretase cleavage site, which inhibits the β-secretase cleavage of APP (34) (Fig. 1C). Mouse hippocampal neurons transfected with MV APP-YFP had similar numbers and intensities (Fig. 3A and B) of fluorescent particles in axons as neurons transfected with WT APP-YFP, suggesting that there is no major difference in the amount of tagged protein that enters axons.

The MV mutation stimulated the anterograde axonal transport of APP-YFP (Fig. 3C), including a significant increase in the percentage of particles moving in the anterograde direction ($P = 0.007$) and a decrease in the retrograde direction ($P = 0.045$) compared with WT APP-YFP (Fig. 3D). Anterograde, but not retrograde, segmental velocity ($P = 0.026$) and run length ($P = 0.003$) of MV APP-YFP significantly increased compared with WT (Fig. 3E and F). Strikingly, SWE and MV mutations have opposite effects on the β-secretase cleavage of APP and opposite effects on APP axonal transport, suggesting that the β-secretase cleavage of APP can contribute to the regulation of APP axonal transport properties.

Pharmacological inhibition of β-secretase stimulates APP anterograde axonal transport, but γ-secretase inhibition reduces it

Given that mutations to the β-secretase cleavage site of APP alter APP axonal transport, we further tested the ability of β-secretase cleavage to regulate APP axonal transport by using a non-genetic approach. Specifically, since FAD Swedish mutations enhance the β-secretase cleavage of APP, a key prediction is that the pharmacological inhibition of β-secretase should suppress the reduced anterograde axonal transport phenotype of FAD Swedish APP-YFP. We found that 40 μM β-secretase inhibitor significantly increased the percentage of FAD Swedish APP-YFP particles moving in the anterograde direction ($P = 0.006$) and decreased the percentage of retrograde particles ($P = 0.019$) compared with FAD Swedish APP-YFP with vehicle alone (Fig. 4A). Neurons treated with the drug did not show any gross signs of cellular toxicity (data not shown), and a lower 10 μM dose had a reduced effect on transport (Fig. 4A). These results in which impaired anterograde axonal transport of SWE APP-YFP can be reversed with β-secretase inhibition suggest that the FAD Swedish APP transport phenotype is likely due to enhanced β-secretase cleavage caused by the mutations.

Although a β-secretase inhibitor was able to reverse the effects of FAD Swedish mutations on APP axonal transport, it will inhibit the cleavage of both transfected APP-YFP and endogenous APP. We set out to distinguish which is

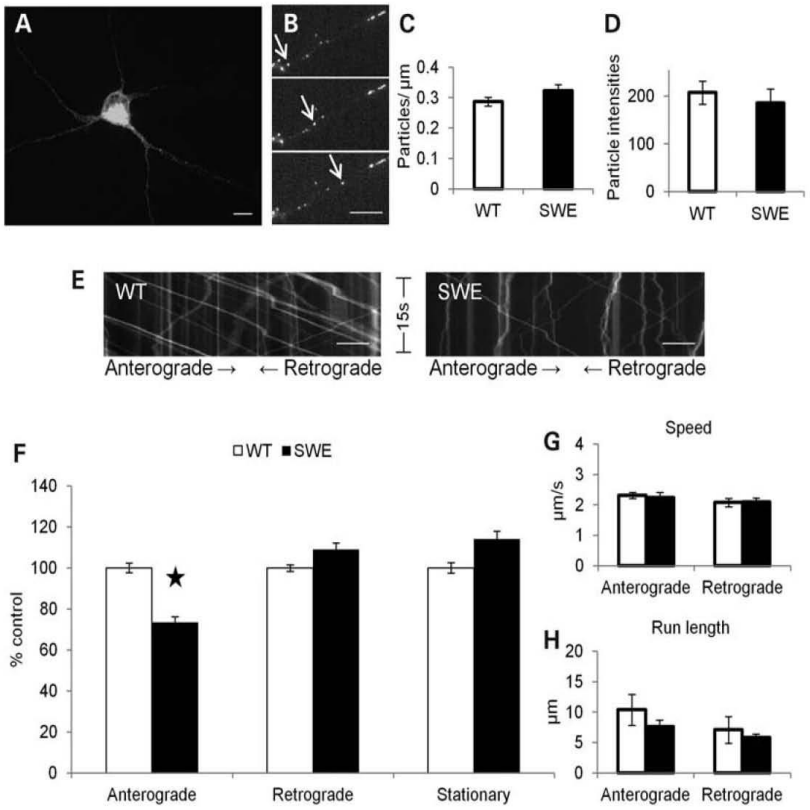

Figure 2. FAD Swedish mutations reduce APP anterograde axonal transport. (**A**) Confocal image of a mouse hippocampal neuron transfected with SWE APP-YFP. Scale bar = 10 µm (**B**) Fluorescent SWE APP-YFP particles in an axon. Arrows follow a single particle traveling in the anterograde direction across chronological frames (top to bottom) from a movie used in data analysis. Scale bar = 10 µm. (**C**–**H**) WT APP-YFP (white) $n = 40$ axons, 1009 particles and SWE APP-YFP (black) $n = 40$ axons, 1129 particles. (C) The mean number of fluorescent particles per length of transfected mouse hippocampal axon. (D) The mean fluorescent intensities of axonal particles. (E) Representative kymographs for WT and FAD Swedish APP-YFP axonal transport from 15s, 10 Hz movies. Right or left descending particles represent anterograde or retrograde moving vesicles, respectively. Vertical lines represent stationary particles. (F) Percentage of anterograde ($P = 0.017$), retrograde and stationary SWE APP-YFP particles compared with WT. Average segmental velocities (G) and segmental run lengths (H) for anterograde and retrograde APP-YFP particles obtained from kymograph analyses. (Student's *t*-tests, $^{*}P < 0.05$.

responsible for altering SWE APP-YFP axonal transport. If the inhibitor alters SWE APP-YFP axonal transport by acting directly on SWE APP-YFP, then the inhibitor treatment of MV APP-YFP would be predicted to have no effect on MV APP-YFP axonal transport, since it already has inhibited β-secretase cleavage as a result of the M596V mutation. We found that MV APP-YFP treated with 40 µM β-secretase inhibitor showed normal axonal transport that was comparable with MV APP-YFP with vehicle alone (Fig. 4B). 40 µM β-secretase inhibitor dramatically increased FAD Swedish APP anterograde axonal transport, but had no effect on MV APP axonal transport, indicating a direct role for βCTF production on APP axonal transport instead of an indirect or toxic effect of the inhibitor. In addition, these results suggest that the stimulation of APP anterograde axonal transport caused by the MV mutation is likely due to its inhibited β-cleavage of APP-YFP.

Typically, the β-secretase cleavage of APP is followed by γ-secretase cleavage to generate Aβ, the primary component of amyloid plaques. Some studies report that Aβ can induce general axonal transport defects in cell culture (26–32,35,36), but evidence from transgenic mice and *Drosophila* suggests that Aβ is not the only fragment of APP that causes axonal transport defects that result from perturbations to APP *in vivo* (2,24,37). To investigate the role that Aβ plays in altered APP transport upon increased βCTF generation, a γ-secretase inhibitor was used with SWE APP-YFP to reduce Aβ production and raise βCTF-YFP levels. Pharmacological inhibition with 5 µM, but not a lower dose of 100 nM, γ-secretase inhibitor further reduced SWE APP-YFP anterograde axonal transport, by decreasing the percentage of anterograde ($P = 0.002$) and increasing the percentage of retrograde ($P = 0.001$) axonal transport compared with SWE APP-YFP with vehicle alone (Fig. 4C). Neurons treated with this drug did not show any signs of cellular

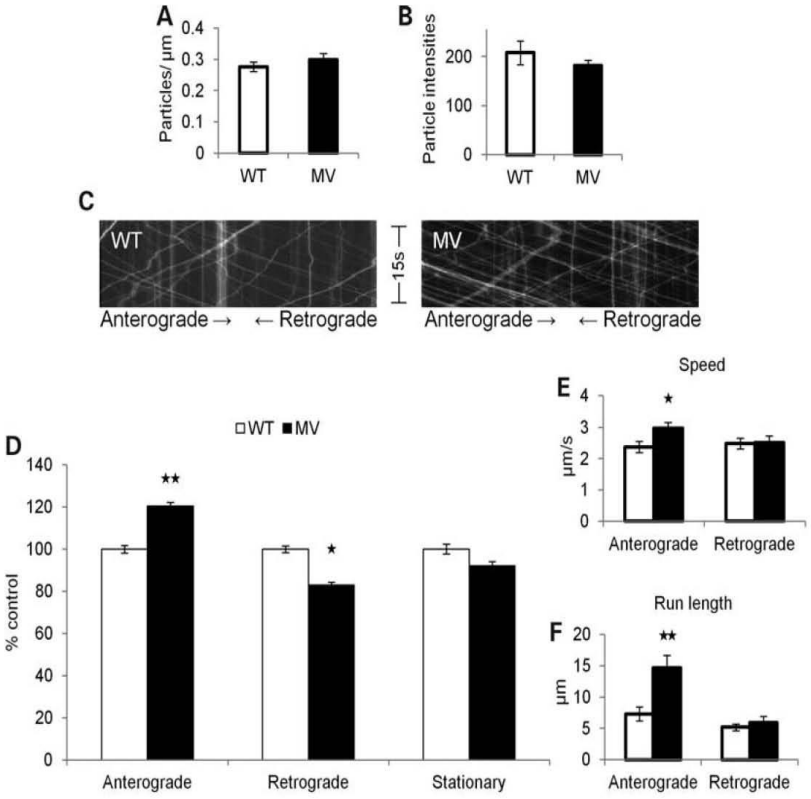

Figure 3. MV mutation stimulates APP anterograde axonal transport. (**A**–**F**) WT APP-YFP (white) $n = 44$ axons, 1172 particles, and MV APP-YFP (black) $n = 52$ axons, 1577 particles. (A) The mean number of fluorescent particles per length of transfected mouse hippocampal axon. (B) The mean fluorescent intensities of axonal particles. (C) Representative kymographs for WT and MV APP-YFP axonal transport. (D) Percentage of anterograde ($P = 0.007$), retrograde ($P = 0.045$) and stationary MV APP-YFP particles compared with WT. Average segmental velocities (E) and segmental run lengths (F) for anterograde ($P = 0.026$ and $P = 0.003$, respectively) and retrograde APP-YFP particles obtained from kymograph analyses (Student's *t*-tests, $^{*}P < 0.05$, $^{**}P < 0.01$).

toxicity (data not shown). The ability of the γ-secretase inhibitor to alter APP axonal transport in an opposing manner to the β-secretase inhibitor argues for specificity of these inhibitors and against indirect drug toxicity effects. Thus, reducing Aβ production and increasing βCTF-YFP levels exacerbated the decreased anterograde axonal transport phenotype of SWE APP-YFP. These data suggest that enhanced β-secretase cleavage by FAD Swedish mutations is sufficient to impair anterograde APP axonal transport by a mechanism that is likely βCTF-dependent and Aβ-independent.

APP βCTFs exhibit impaired anterograde axonal transport

Since our data suggest that the enhanced β-secretase cleavage of APP impairs APP anterograde axonal transport, while inhibited β-secretase cleavage stimulates it, it implies that these phenotypes simply result from alterations in βCTF levels. However, our experiments described so far inherently do not distinguish full-length APP-YFP from CTF-YFP axonal transport. To test the hypothesis that βCTFs have reduced anterograde axonal transport compared with full-length APP, we examined the axonal transport of CTFs tagged with enhanced green fluorescent protein (EGFP).

We transfected neurons with βCTF-EGFP and compared its axonal transport with that of WT APP-EGFP. Cells transfected with βCTF-EGFP do not express any full-length APP-EGFP (Fig. 5A). Mouse hippocampal neurons transfected with either full-length WT APP-EGFP or βCTF-EGFP showed similar numbers of fluorescent particles in axons, suggesting that there is no major difference in the amount of tagged protein that enters axons (Fig. 5B). However, βCTF-EGFP exhibited severely reduced anterograde axonal transport compared with WT APP-EGFP (Fig. 5C), including a significantly decreased percentage of anterograde ($P < 0.001$) and an increased percentage of retrograde ($P = 0.003$) axonal transport, along with an increase in the percentage of stationary particles ($P = 0.024$; Fig. 5D). Both anterograde segmental velocity ($P = 0.001$) and run length ($P < 0.001$) significantly decreased compared with WT APP-EGFP, while retrograde segmental velocity increased ($P = 0.025$; Fig. 5E and F). Thus, βCTF-EGFP exhibits reduced anterograde axonal transport compared with WT APP-EGFP, with a phenotype much more drastic than the SWE APP-YFP phenotype. These data

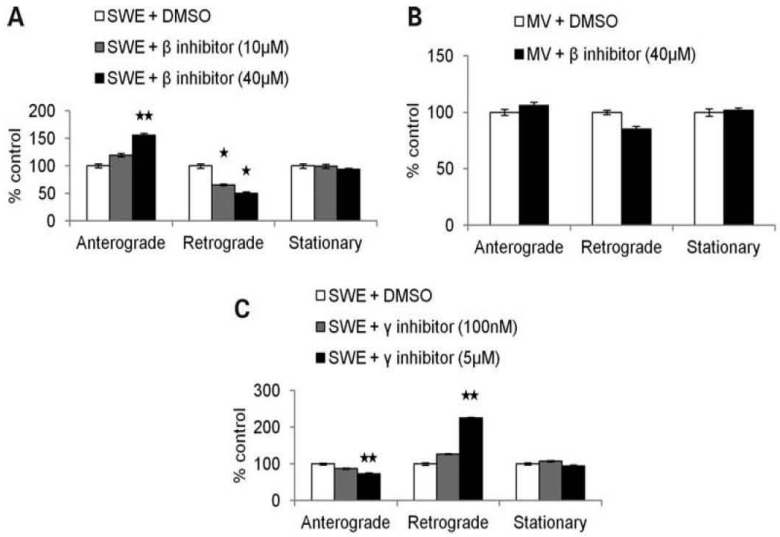

Figure 4. Pharmacological inhibition of β- and γ-secretase activity alters APP axonal transport. (**A**) Percentage of anterograde, retrograde and stationary SWE APP-YFP particles treated with 10 μM β-secretase inhibitor (gray, $n = 25$ axons, 598 particles) or 40 μM β-secretase inhibitor (black, $n = 22$ axons, 445 particles) compared with DMSO (white, $n = 25$ axons and 697 particles for 10 μM data set, $n = 18$ axons and 234 particles for 40 μM data set). Anterograde axonal transport significantly increased with 40 μM β-secretase inhibitor ($P = 0.006$), while retrograde transport decreased with both 10 and 40 μM inhibitor ($P = 0.018$ and $P = 0.019$, respectively). (**B**) Percentage of anterograde, retrograde and stationary MV APP-YFP particles treated with 40 μM β-secretase inhibitor (black, $n = 33$ axons, 309 particles) compared with DMSO (white, $n = 29$ axons, 373 particles). (**C**) Percentage of anterograde ($P = 0.002$), retrograde ($P = 0.001$) and stationary SWE APP-YFP particles treated with 100 nM ($n = 49$ axons, 1499 particles) or 5 μM γ-secretase inhibitor (black, $n = 22$ axons, 674 particles) compared with DMSO (white, $n = 35$ axons and 995 particles for 100 nM data set, $n = 12$ axons and 322 particles for 5 μM data set) (Student's t tests, $^{*}P < 0.05$, $^{**}P < 0.01$).

support the hypothesis that the altered axonal transport of FAD Swedish APP is due to an increased proportion of βCTFs and demonstrate directly divergent live axonal transport behavior of full-length APP and βCTFs.

Inhibiting the β-secretase cleavage of APP by the M596V mutation enhances its anterograde axonal transport, but this could possibly be due to an increased proportion of either full-length APP or αCTFs. To address this issue, we transfected neurons with αCTF-EGFP to compare its axonal transport with WT APP-EGFP. Western blots of transfected cells showed the presence of the αCTF-EGFP product when probed with a C-terminal APP antibody (Fig. 5G). However, neurons transfected with αCTF-EGFP only exhibited diffuse fluorescence in neurites. Therefore, the axonal transport of punctate particles was not observed (Fig. 5H), as with APP-EGFP and βCTF-EGFP.

Axonal defects in an FAD Swedish APP gene-targeted mouse

Taken together, our *in vitro* results indicate a direct impact of APP processing on axonal transport properties of APP vesicles, such that FAD Swedish mutations that enhance the β-secretase cleavage of APP inhibit its anterograde axonal transport. Flies and mice overexpressing FAD Swedish APP also develop phenotypes typical of axonal transport defects (2,10). Enhanced β-secretase processing is thought to occur in both sporadic and FAD (17–20,33), and AD brains characteristically display axonal dystrophy (38–42). APP is not overexpressed, however, in sporadic or FAD, as it is in these FAD Swedish APP animal models previously studied. It remains unanswered whether FAD Swedish mutations enhancing the β-secretase cleavage of APP are sufficient to cause axonal defects, or if the overexpression of the mutant protein is required as well.

To address this issue, we tested the hypothesis that FAD Swedish mutations could lead to axonal defects in a mouse model that does not overexpress APP. We examined axonal phenotypes of neurons in a gene-targeted mouse that has FAD Swedish mutations and a humanized Aβ region targeted to the mouse APP gene (Fig. 5A). Previous studies report increased β-secretase cleavage and Aβ generation without amyloid plaque deposition (43,44), emphasizing the use of this mouse as a model of the earliest changes that might occur in the disease process.

We assessed axonal morphologies in the cholinergic basal forebrain, a location known to show axonal transport defects in AD brains and Tg-swAPP^{Prp} mice (10). Immunohistochemistry for choline acetyltransferase (ChAT), the enzyme that catalyzes the final step in acetylcholine biosynthesis, was performed to identify cholinergic axonal morphology in the septal nucleus. A significant increase in dilated axons over 1.5 μm in diameter was observed in the septohippocampal region of the septal nucleus of 12-month-old gene-targeted mice compared with WT, age-matched littermates (Fig. 6B), including both the total length and number of dilated axons (Fig. 6C and D, $\alpha < 0.05$). This phenotype was specific to the septohippocampal subregion of the septal nucleus and was not observed in the

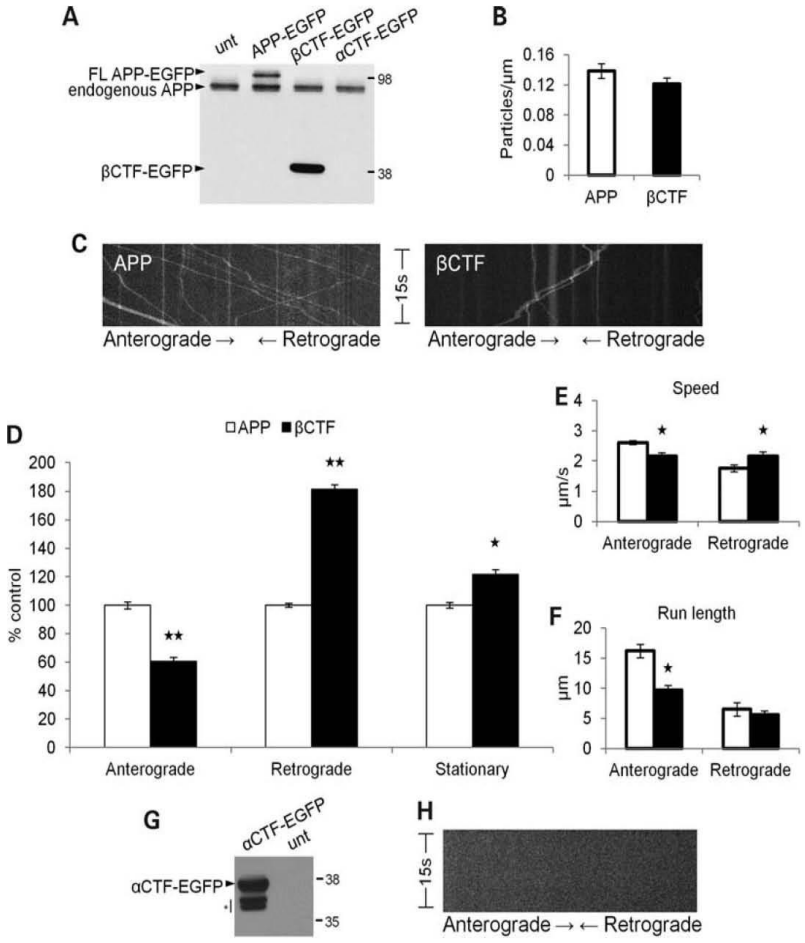

Figure 5. βCTFs have reduced anterograde axonal transport. (**A**) Western blot of SH-SY5Y cells transfected with WT APP-EGFP, βCTF-EGFP, αCTF-EGFP and untransfected (unt) cells as a control. The 6E10 antibody epitope lies in the Aβ region, so it will recognize full-length APP-EGFP and βCTF-EGFP, but not αCTF-EGFP. Top arrowhead identifies the full-length APP-EGFP band present only in APP-EGFP transfected cells, and bottom arrowhead identifies the βCTF-EGFP band that is absent in αCTF-EGFP transfected cells. (**B**–**F**) WT APP-EGFP (white) $n = 52$ axons, 658 particles, βCTF-EGFP (black) $n = 38$ axons, 448 particles. (B) The mean number of fluorescent particles per length of transfected mouse hippocampal axon. (C) Representative kymographs for WT APP-EGFP and βCTF-EGFP axonal transport. (D) Percentage of anterograde ($P < 0.001$), retrograde ($P = 0.003$) and stationary ($P = 0.024$) particles for βCTF-EGFP compared with WT APP-EGFP. Segmental velocities (E) and run lengths (F) for anterograde ($P = 0.001$ and $P < 0.001$, respectively) and retrograde ($P = 0.025$) particles obtained from kymograph analyses. (G) Western blot of cells transfected with αCTF-EGFP and the untransfected control, probed with a C-terminal APP antibody. Below αCTF-EGFP product lies two bands of unidentified/degradation products (indicated by asterisk), which were previously reported with the βCTF-EGFP plasmid from which this αCTF-EGFP plasmid was constructed (85). (H) Representative kymograph for αCTF-EGFP showing no axonal transport (Student's *t*-tests, $^{*}P < 0.05$, $^{**}P < 0.01$).

medial or intermediate septal subregions (Fig. 6C and D). Thus, FAD Swedish mutations to APP that we have shown alter axonal transport of APP *in vitro* can also lead to axonal defects *in vivo*, even when mutant APP is not overexpressed.

We demonstrated *in vitro* that the β-secretase processing of APP can alter its axonal transport, which could potentially also affect the delivery of necessary components to axon terminals. Because FAD Swedish APP gene-targeted mice develop axonal changes, we tested if synapses in their corresponding terminal field were also affected. Therefore, we tested for decreased innervation of septal nucleus terminal fields in the hippocampus. Immunohistochemistry was performed for both ChAT and the presynaptic marker synaptophysin, and density of labeling was measured in the hippocampus. FAD Swedish APP gene-targeted mice show comparable levels of synaptic density to WT mice through the age of 24 months, using both ChAT (Fig. 6E) and synaptophysin (Fig. 6F–I) as probes. Cholinergic axonal dilation in gene-targeted mice does not correlate with decreased presynaptic density in corresponding terminal fields. Thus, the axonal defect observed in

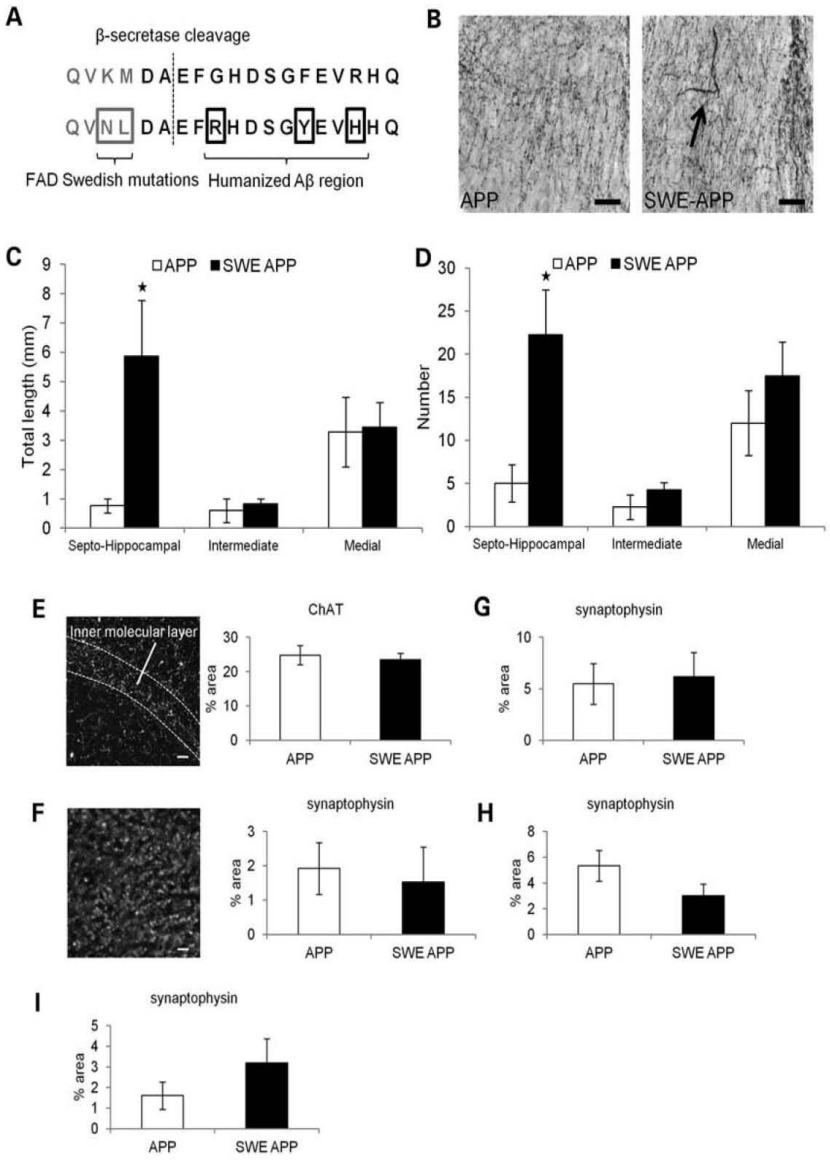

Figure 6. Axonal defects in FAD Swedish APP gene-targeted mice. (**A**) WT (top) and mutant (bottom) amino acid sequences near the β-secretase cleavage site for mouse APP. Mutated amino acids are boxed in the bottom row. (**B**) ChAT immunohistochemistry in the septohippocampal subregion of the septal nucleus in the basal forebrain. Dilated axon (arrow) shown in the FAD Swedish APP brain. Scale bar = 30 μm. (**C**) The average length and (**D**) the average number of dilated axons with diameter over 1.5 μm in septohippocampal, intermediate and medial subregions of the septal nucleus at 12 months [WT, $n = 4$ (white); APP, $n = 4$ (black)]. (**E**) (Left) Example image of ChAT staining in the dentate gyrus of the hippocampus. (Right) The average percent area labeled by ChAT in inner molecular layer of the dentate gyrus in 18 m mice [WT, $n = 4$ (white); APP, $n = 4$ (black)]. (**F**) (Left) Example image of synaptophysin staining in the dentate gyrus. (Right) The average percent area labeled by synaptophysin in the inner molecular layer of dentate gyrus in 6 m mice (WT, $n = 4$; APP, $n = 3$) and (**G**) 18 m mice (WT, $n = 3$; APP, $n = 4$). The average percent area labeled by synaptophysin in the hippocampal region (**H**) CA1 in 18 m mice (WT, $n = 3$; APP, $n = 4$) and (**I**) CA3 in 24 m mice (WT, $n = 3$; APP, $n = 3$) (Mann–Whitney rank-sum test, $^*\alpha < 0.05$).

Swedish APP gene-targeted mice occurs in the absence of other AD-associated pathology, such as synaptic loss and amyloid plaque deposition, and likely represents one of the earliest events in the pathological cascade of disease progression.

DISCUSSION

We investigated the effects of β-secretase cleavage on APP axonal transport and axonal morphology. FAD Swedish

mutations of APP that enhance β-secretase cleavage were sufficient to impair APP anterograde axonal transport *in vitro* and lead to axonal defects *in vivo*. An opposing MV mutation that inhibits the β-cleavage of APP had the opposite effect and stimulated APP anterograde transport, as did the pharmacological inhibition of β-secretase activity. Increasing βCTF and reducing Aβ levels with a γ-secretase inhibitor further reduced Swedish APP anterograde transport, and direct examination of βCTFs revealed dramatic impairment of axonal transport. Taken together, these observations suggest a βCTF-dependent mechanism to impair APP anterograde transport. Furthermore, the brains of mice with FAD Swedish mutations targeted to the mouse APP gene also developed axonal defects in the absence of synaptic degradation or amyloid plaque formation, arguing for axonal changes as an early event in disease progression.

The enhanced β-secretase cleavage of APP was previously suggested to lead to axonal transport defects (10,37), but it was not possible to distinguish effects of APP overexpression from effects of the Swedish mutations that the stimulate β-cleavage of APP. Although a previous study reported that FAD Swedish mutations do not impair APP axonal transport by the criterion of APP accumulation at the proximal side of a nerve ligation, the data demonstrated an increased ratio of retrograde to the anterograde axonal transport of FAD Swedish mutant APP compared with non-mutant APP (45), which is consistent with our findings. In fact, this previous biochemical experiment also provided only an indirect comparison between mice with overexpressed FAD Swedish human APP and mice overexpressing human PS1, an APP processing component, but with endogenous mouse APP (45). Our study made a direct comparison between non-mutant and FAD Swedish mutant human APP, both expressed at similar levels and with endogenously expressed, non-mutant proteolytic processing components. Live imaging of real-time axonal transport demonstrated that FAD Swedish mutations increasing β-secretase cleavage can modestly impair the anterograde axonal transport of APP itself. Identifying the more moderate consequences of the enhanced β-secretase cleavage of APP can, in principle, help elucidate potentially important phenotypes relevant to neurodegeneration. Disease takes decades to develop, especially in late-onset AD, in which there is enhanced APP processing in the absence of APP mutations.

Earlier studies have suggested the divergent axonal transport of CTFs and full-length APP based on their distribution into distinct axonal transport vesicles (8,9). We now provide for the first time a detailed comparative analysis of CTF live axonal transport behavior that supports the qualitative data previously available. We directly examined βCTF axonal transport to demonstrate a dramatic reduction in anterograde transport compared with full-length APP. Increased βCTF levels caused by FAD Swedish mutations of APP likely underlie the comparatively moderate transport phenotype resulting from the mutations. This interpretation is supported by the finding that increasing βCTF levels by using a γ-secretase inhibitor on FAD Swedish APP also severely reduced anterograde transport. Conversely, preventing the β-cleavage of FAD Swedish APP with a β-secretase inhibitor produced a large increase in APP anterograde axonal transport. A sizeable increase in anterograde transport was also seen with MV mutant APP, in which β-cleavage is precluded. Thus, not only does β-secretase cleavage appear to mediate APP axonal transport, but the magnitude of β-secretase cleavage directs the size of the effect on APP axonal transport. Our data indicate that full-length APP has an increased propensity toward anterograde axonal transport toward the synapse than βCTFs.

Several studies report that Aβ can induce defects in the axonal transport of mitochondria and dense core vesicles, and these Aβ-dependent effects appear to target general axonal transport machinery. The total number of transporting vesicles decreased, and both anterograde and retrograde transport and velocities were similarly decreased (26–32,35,36). Our data, on the other hand, provide evidence that mutations affecting APP processing at the β-secretase cleavage site altered the axonal transport of APP itself. There was no decrease in the total number of transporting vesicles, and anterograde and retrograde transport were altered in an opposing manner. Furthermore, the reduced anterograde axonal transport of FAD Swedish APP could be reversed with β-secretase inhibition and exacerbated with γ-secretase inhibition. Because the γ-secretase inhibition of FAD Swedish APP increases βCTF and reduces Aβ production, our data suggest that there is also a βCTF-dependent and Aβ-independent mechanism for APP axonal transport inhibition. In fact, the direct examination of βCTF axonal transport demonstrated that βCTFs have a higher propensity toward retrograde axonal transport compared with full-length APP. Our findings are consistent with previous research that found Aβ-independent defects in axonal transport (2,24,37), in addition to βCTF-dependent and Aβ-independent endosomal changes (21).

The β-secretase cleavage of APP is thought to occur in early endosomes (3,46,47), and endosomes become enlarged upon increased βCTF levels (21) and as an early phenotype in AD (22). In axon terminals of mice carrying an extra copy of the APP gene, early endosomes containing APP, CTFs and the neurotrophic factor nerve growth factor (NGF) were enlarged. Retrograde axonal transport of NGF decreased proportionally with increasing CTF, but not APP, levels, and NGF retrograde axonal transport also decreased in Tg-swAPP^{Prp} mice (24). Because *Drosophila* and mouse models that overexpress either mutant or WT APP exhibit axonal transport defects (2,10,24), it suggests that perturbations to APP processing and amount are both capable of affecting axonal transport. This may be crucial in understanding mechanisms that can contribute to sporadic or late-onset AD, in which there are no mutations to APP. Sporadic AD brains exhibit phenotypes suggestive of axonal transport defects (10) and undergo enhanced APP processing, even though there are no known genetic mutations.

Impaired axonal transport and endosomal defects have been shown to develop concurrently (24) and exhibit βCTF-dependence (21), suggesting a potential mechanistic link. Endosomes in both cell bodies and axon termini have been proposed to play a role in sorting and assembly of APP transport vesicles (48–51). Decreased activity of endosomal component rab3A has previously been reported to reduce the association of APP transport vesicles with motor protein kinesin-I and decrease APP transport into neurites (48). In

poly-L-lysine-coated coverslips and stored in a 37°C incubator with 5% CO_2. At least three separate cultures prepared on different days were used for each experiment. SH-SY5Y cultures were grown in F-12 nutrient and Hamm media supplemented with 10% FBS.

Transfection and drug treatment

After 10 days in culture, hippocampal neurons were transfected with APP-YFP for 2 h with 800 ng of WT or mutant DNA per well of a 24-well plate. Low transfection efficiency was obtained using Lipofectamine 2000 (Invitrogen). Experiments were designed to collect data 12–16 h after transfection from control and experimental groups in parallel. From a single 24-well plate of cultured hippocampal neurons, half the wells were transfected with WT APP-YFP, while the other half was transfected with mutant APP-YFP. Transfected hippocampal cultures in some experiments were treated with either β-secretase inhibitor II (Calbiochem) or γ-secretase inhibitor compound E (Calbiochem). For drug treatment experiments, the entire plate was transfected with either Swedish or MV APP-YFP. An hour before imaging the next day, half the wells were treated with drug dissolved in dimethyl sulfoxide (DMSO), and half the wells were treated with an equal volume of DMSO only. For APP-, βCTF- and αCTF-EGFP transfections, 500 ng of DNA was used per well, and data were collected 4–10 h after transfection. At least three separate culture preparations and transfections were performed for each experimental group, using multiple preparations of plasmid DNA. SH-SY5Y cells were used for western blot experiments because of their high transfection efficiency. Transfections the day after splitting followed a protocol similar to hippocampal transfection.

Axonal APP-YFP movie collection

Transfected axons were located in primary hippocampal cultures using an inverted epifluorescent microscope (TE-2000U, Nikon) and a 100× oil immersion objective (Nikon, 1.4 NA, 0.126 μm/pixel) connected to a CCD camera (Roper Scientific). During imaging, cultures were kept at 37°C using a heated stage and 5% CO_2 chamber. Twelve to 16 h after transfection, movies of axons expressing fluorescent protein were collected using Metamorph 7.0 software (Universal Imaging Corporation) run on a PC computer. Directionality was determined by tracking axons far away from cell bodies or axon termini, and individual transfected axons could be distinguished due to the small number of transfected neurons in the primary culture. Data were not collected if movement directionality could not be determined, or if cells appeared unhealthy. Particles moving from cell bodies to axon termini were considered anterograde, and those moving from termini to cell bodies were considered retrograde. Continuous, 15-second movies of 150 frames with 100 millisecond exposure for each frame were collected for APP-YFP or APP-EGFP axonal movement. Phase and fluorescent images of cell bodies were also collected to ensure that there were no signs of gross toxicity to the cells or substantial differences in the amount of transfected fluorescent protein present. All movies were collected within an hour of the culture being placed in the heat- and CO_2-controlled microscope incubator chamber.

APP-YFP movement analysis

Using Metamorph software, a kymograph was generated for each movie to plot time on the *y*-axis and distance on the *x*-axis for the movement of each fluorescent particle. Manual analyses were conducted on particle movement trajectories plotted on these kymographs. Percentages of anterograde, retrograde and stationary particles were extracted by following particle movement from initial starting point to ultimate ending point along the kymographs. Segmental velocity and run length are the speed and distance a particle travels in a given direction, during a segment of movement that is not interrupted by a pause in movement or a reversal in movement direction. These were calculated using kymographs by tracing particle movement across an uninterrupted segment of movement, then calculating distance traveled and time taken to travel that distance. Each data set analyzed consists of movies collected from at least three separate culture preparations and transfections performed on different days, using multiple plasmid preparations. Only data from multiple repetitions of these experiments performed in parallel on several different days were pooled for analyses.

Western blot analysis

Protein analysis of transfected cultures was performed by western blots. For each experiment, one 10 cm plate of SH-SY5Y cells was transfected with each construct. The next day, cells were rinsed of culture media and scraped from the plate using PBS with a protease inhibitor cocktail (Roche). Cells were collected on ice, and then pelleted by centrifuging at 4°C for 10 min at 1000*g*. After removing PBS, the cell pellets were resuspended and homogenized using NP-40 lysis buffer. After protein concentration was determined using the Bradford assay, an equal volume of 2× lithium dodecyl sulfate sample buffer (Invitrogen) was added with β-mercaptoethanol to a final concentration of 4%. Equal amounts of protein samples were loaded onto 4–12% Bis–Tris gels, using SeePlus 2 as a molecular weight marker (Invitrogen), and gels ran at 90 V until dye reached the end of the gel. Transfer onto nitrocellulose membranes was done at 0.3 A for 90 min using 25 mM Tris-base, 190 mM glycine and 20% methanol. Ponceau S stain (Sigma) indicated the quality of the transfer and was used as a guide when cutting membranes into separate sections for incubation with different antibodies. Membrane blocking was performed for 1 h at room temperature with 5% milk in Tris-buffered saline with 0.25% Tween (TBST), while primary antibody diluted in blocking solution was incubated overnight at 4°C. Human-specific APP antibody 6E10 (Covance) was used at 1:1000. This antibody recognizes the first 16 amino acids of the Aβ region and will consequently detect full-length APP and βCTFs, but not αCTFs. C-terminal APP antibody (Zymed) was used at 1:500 to detect αCTF. Tubulin was used at 1:10 000 as a loading control. Antibodies were used sequentially to detect levels of βCTF-YFP and tubulin in the same membrane.

Horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody was diluted in TBST at 1:10 000, and incubation was done for 2 h at room temperature. Membranes were developed with ECL Western blotting substrate (Pierce). Dissected mouse brains were collected and immediately frozen in liquid nitrogen. Sample preparation and western blot technique were performed in a similar fashion.

Mice and genetic crosses

Gene-targeted mice obtained for these studies possess FAD Swedish mutations and a humanized Aβ region targeted to the mouse APP gene and were originally on the 129/CD-1 genetic background (Cephalon, West Chester, PA, USA) (42). We backcrossed these mice onto the C57BL/J6 genetic background for five generations and bred to each other the resulting C57BL/J6 mice heterozygous for the gene-targeted APP mutations. Mice homozygous for the APP gene-targeted mutations and their age-matched, WT littermates were included for study.

Immunohistochemistry

At various ages, mice were transcardially perfused first with 0.1 M phosphate buffer (PB), pH 7.2, followed by 4% paraformaldehyde in PB (4% PFA). Dissected brains were post-fixed overnight with 4% PFA at 4°C. Coronal sections 50 μm thick were cut using a vibrating blade microtome (Leica 1000S), collected in PB and stored in 30% glycerol cryoprotectant until immunohistochemistry was performed the next day. For light microscopy, every 6th section was collected in PB into a serially sampled set representative of the cholinergic basal forebrain and rinsed in PB three times for 5 min each rinse. Brain slices there then quenched for 1 h at room temperature with 0.6% hydrogen peroxide and rinsed again in PB. Tissue was blocked and permeabilized for 1 h at room temperature with 10% serum and 0.2% Triton X-100 in PB, then incubated in primary antibody ChAT (Invitrogen) diluted 1:100 in the same solution for 72 h at 4°C. After washing brain sections with PB six times for 10 min each rinse, tissue was blocked again. Primary antibody signal was enhanced using a biotinylated secondary antibody (Jackson ImmunoResearch) at 1:200 for 1 h at room temperature, followed by rinsing with PB. Tissue was then incubated with biotin–avidin complexes from the ABC vectastain kit for 1 h at room temperature, rinsed and developed with Nova Red (Vector Laboratories). After rinsing with water and mounting onto microscope slides, brain slices were dehydrated in an ethanol series, cleared with xylene and mounted with Permount (Fisher). Slides were then coded for blind data collection and analysis using a stereological approach. Fluorescence microscopy was conducted in a fashion similar to light microscopy. Three serial sections through the hippocampus for each mouse were collected in PB, rinsed, blocked and permeabilized, then incubated in either primary antibody synaptophysin (Millipore) or ChAT (Invitrogen) at 1:100 overnight at 4°C. After washing brain sections with PB three times for 5 min each rinse, they were incubated with a fluorophore-conjugated secondary (AlexaFluor) at 1:200 for 1 h at room temperature. Tissue was mounted with fluorescence-preserving Vectashield media (Vector Laboratories).

Stereology

Brain stereology was performed with an Axioplan Zeiss light microscope associated with a Bioquant Nova Stereology software image analysis system (Bioquant R&M Biometrics, Inc.). In brief, dystrophic cholinergic axon length and number were determined using random, systematically-sampled serial sections of 50 μm brain slices. The first slice was selected as the most anterior slice containing the septal nucleus, with every other slice spaced apart by 250 μm. Random serial sections were analyzed using the Bioquant system to calculate lengths and numbers of dystrophic axons by tracing non-varicose, dilated axons with widths over 1.5 μm in the entire area of each septal nucleus subregion contained in each brain slice. A minimum of three mice per experimental group were analyzed at each age point.

Immunofluorescent analyses

Fluorescent images were collected with an inverted Nikon Bio-Rad Laboratories FV-1000 confocal imaging system and 100× oil immersion objective. Before data collection, confocal acquisition settings to be used for all images were determined to ensure for an intensity range below saturation. Images were collected at six different sites within the synaptic region of interest for each brain slice. Single images were collected for synaptophysin at each location. For ChAT imaging, z stacks were collected, consisting of 10 images separated by 1 μm, and made into maximum projections. Analysis consisted of using ImageJ software to apply a standard threshold selection to all synaptophysin or all ChAT images and calculate the percent area labeled. Mean values were obtained for each animal, and animals within the same genotype were pooled for statistical analyses and graphical plotting.

Statistics

For analyses of APP-YFP movement parameters, Student's *t*-tests were used to determine significant differences between mutant and WT, or drug-treated and vehicle-treated, groups. Percentages of movement in each direction, mean segmental velocities and mean segmental run lengths were calculated for each movie collected. These movie values were used for statistical comparisons between experimental groups. Single stars indicate statistical significance with a P-value of <0.05, and double stars indicate a P-value of <0.01. Graphs plot the percent of the control value for each construct or drug-treatment group, and error bars represent the standard error of the mean (SEM). For analyses of gene-targeted mice, each graph plots genotype means obtained from several animals, and error bars represent the SEM. Asterisks indicate statistical significance. For brain stereology and immunofluorescent image analyses, a Mann–Whitney (two sample rank sum) nonparametric statistical test was used at an α significance of 0.05.

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Conflict of Interest statement. None declared.

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Chapter 2, in full, is a reprint of the material as it appears in *Human Molecular Genetics*, Rodrigues, Elizabeth; Weissmiller, April; and Larry Goldstein, 2012. The dissertation author was a co-author on this paper.

CHAPTER 3

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to both cognitive decline and impairment of daily function. One of the pathological hallmarks of AD, β-amyloid (Aβ)-containing plaques, provides the rationale for the amyloid cascade hypothesis which proposes that Aβ accumulation leads to neuronal dysfunction and death(Hardy and Selkoe, 2002; Scheuner et al., 1996). Aβ40 and Aβ42 peptides, produced by sequential processing of the APP by β-secretase and γ-secretase, are the principal components of amyloid plaques in the brain (Fig3.1a). In addition, several lines of evidence suggest that soluble and oligomerized Aβ40 and Aβ42 play an important role in AD pathogenesis (Davis et al., 2011; Freir et al., 2011; Jin et al., 2011; Li et al., 2011; Shankar et al., 2008). However, in recent years data collected has shown that APP CTFs, independent of Aβ, are capable of eliciting neuronal dysfunction and therefore may play an important role in disease mechanisms of both AD (Jiang et al., 2010; Rodrigues et al., 2012; Stokin et al., 2008) and Down syndrome (Salehi et al., 2006).

Unfortunately, the ability to ask how APP CTF accumulation can directly cause neuronal dysfunction phenotypes has been limited to studies in which APP and or its CTFs are overexpressed (Gunawardena and Goldstein, 2001; Rodrigues et al., 2012; Torroja et al., 1999) or to studies of mouse models (Calkins et al., 2011; Pigino et al., 2003; Stokin et al., 2005) in which a direct, temporal link between altered APP

processing and phenotype cannot directly be assessed Recently, inhibition of the γ-site cleavage of APP using γ-secretase inhibitors (GSIs), which eliminate all Aβ species while increasing APP CTFs, has gained attention due to failure of these drugs to provide any therapeutic benefit in Phase III clinical trials (Doody et al., 2013). GSI-mediated toxicity has since been confirmed both in mouse models of AD and in wild-type mice, which the authors attribute to APP CTF accumulation (Mitani et al., 2012).

Therefore, we postulated that we would be able to use a GSI compound to study neuronal phenotypes in a temporally controlled manor (i.e., directly following drug effect) and obtain mechanistic insight into the result of APP CTF accumulation. We sought to investigate the effects of GSI treatment on neuronal trafficking and signaling in response to brain-derived neurotrophic factor (BDNF), a trophic factor that is critical for various aspects of the nervous system and synaptic maintenance during adulthood (Chao, 2003; Korte et al., 1995; Patterson et al., 1996; Sendtner et al., 1992). In our studies we used a potent GSI, BMS-299897 (Anderson et al., 2005) (henceforth called GSI throughout this study) (Fig. 3.1b). The GSI selected represents a class of molecules known as arylsulfonamides, which constitute the core structure of a large family of GSIs including the reportedly Notch sparing GSI currently in clinical trials called avagacestat (Coric et al., 2012; Martone et al., 2009). Our results provide evidence of abnormal neuronal trafficking of multiple cargos following GSI-treatment, of which retrograde BDNF function is directly related to APP processing. Interestingly, internalized BDNF vesicles contain increased amounts of APP and/or APP CTFs, along with increased amounts of the anterograde protein subunit, kinesin

light chain 1 (KLC1). Through application of GSI, we show a novel, yet pathological role for APP CTF accumulation as it impacts retrograde axonal trafficking. Furthermore, we provide potential mechanistic insight into progression of neuronal dysfunction due to elevated APP CTF levels.

Results

Effects of GSI on APP processing and Aβ production

To determine the effect of GSI treatment in neurons, we first confirmed that the drug inhibited the processing of endogenous APP. A dose-response experiment using GSI showed that the GSI inhibited Aβ peptide formation (Fig. 3.1b). To further confirm that the GSI affected substrate processing we assessed the carboxyl-terminal fragment (CTF) production of both APP and N--cadherin, another gamma-secretase substrate. Consistent with published reports(Anderson et al., 2005; Kounnas et al., 2010), treatment of neurons with GSI increased C-terminal fragments of both APP and N-cadherin (Fig. 3.1c,d). In addition γ-secretase inhibition would also be expected to cause Notch CTF accumulation and impede the translocation of the Notch intracellular domain (NICD) to the nucleus, thus inhibiting downstream transcriptional events. We tested this by quantifying the mRNA levels of Hes1 and Hey1, two transcription factors activated by NICD, and found that GSI treatment significantly decreased Hey1 and Hes1 mRNA levels (Fig. 3.1e). These results are evidence that the GSI inhibited processing of several key substrates of gamma secretase.

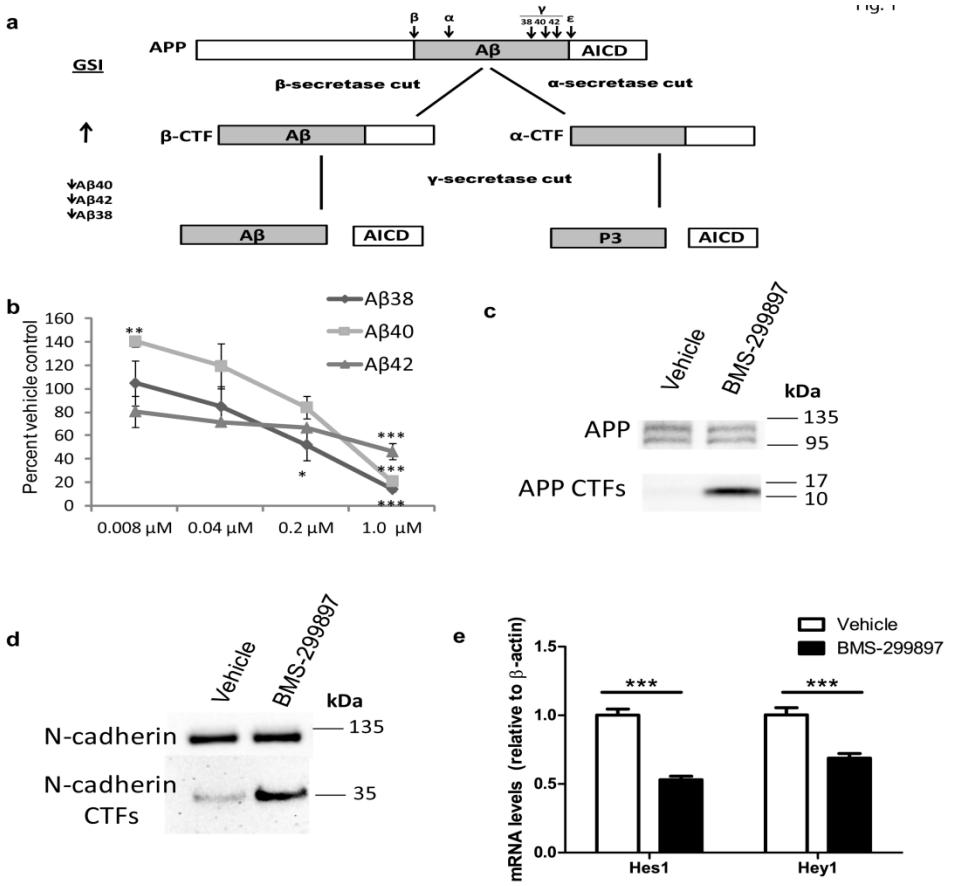

Figure 3.1 Effect of GSI treatment on APP processing and C-terminal fragment production using BMS-299897 (a) Diagram of APP processing and how GSI treatment affects both Aβ peptide formation and C-terminal fragment production. First, beta-secretase or alpha secretase cuts APP, leading to C-terminal fragments which are then sequentially processed by gamma-secretase at multiple sites to create several Aβ peptides and the APP intracellular domain (AICD). (b) Secreted Aβ peptides from cortical neurons treated 24 hours with GSI, as determined by ELISA assay. In a dose-dependent manner, GSI treatment decreased levels of Aβ42, Aβ 40, and Aβ 38, consistent with inhibited γ-secretase cleavage. (n = 3, *P < 0.05, **P < 0.01, **P < 0.001 using Student's *t*-test). (c,d) Western blots showing the processing of two substrates of gamma-secretase, APP and n-cadherin, using 1μM GSI in cortical neuronal lysates. GSI treatment caused accumulation of bands corresponding to the size of APP and N-cadherin C-terminal fragments, providing further evidence of inhibition of γ-secretase by GSI. GSI treatment did not alter the levels of the full-length proteins. This concentration of GSI was used in all further experiments. (e) Hes1 and Hey1 mRNA levels reveal that after 24h drug treatment, GSI-treated cortical neurons have decreased levels of Hes1 and Hey1 mRNA compared to vehicle. mRNA levels are relative to mRNA levels of β-actin (n=3, mean±S.E, ***P<0.001 using Student's *t*-test).

GSI treatment caused deficits in transport of QD-BDNF in hippocampal neurons

To assess the impact of GSI on BDNF trafficking we analyzed the transport of quantum-dot conjugated BDNF (QD-BDNF) in hippocampal neurons grown in compartmentalized chambers, as described previously (Poon et al., 2011; Xie et al., 2012). Real-time monitoring of QD-BDNF movement within the microgrooves and analysis of the corresponding kymographs (Fig 3.2a) revealed striking differences. Vehicle-treated axons showed the expected processive movement, largely in the retrograde direction. However, both the average velocity and total distance travelled by QD-BDNF molecules were decreased by 50% following GSI-treatment as compared to vehicle-treated neurons (Fig 3.2b,c) . To define the patterns of movement, we sorted and quantified the percentage of individual QD-BDNF molecules moving retrogradely, anterogradely, or remaining stationary. Surprisingly, GSI treatment reversed directionality of a large percentage of QD-BDNF signals such that a higher percentage of QD-BDNFs now moved anterogradely rather than retrogradely (Fig. 3.2d). A detailed analysis of separated QD-BDNF movements indicated that the reduction in average velocity of QD-BDNF by GSI treatment was largely due to defective retrograde, but not anterograde, transport velocities (Fig 3.2e).

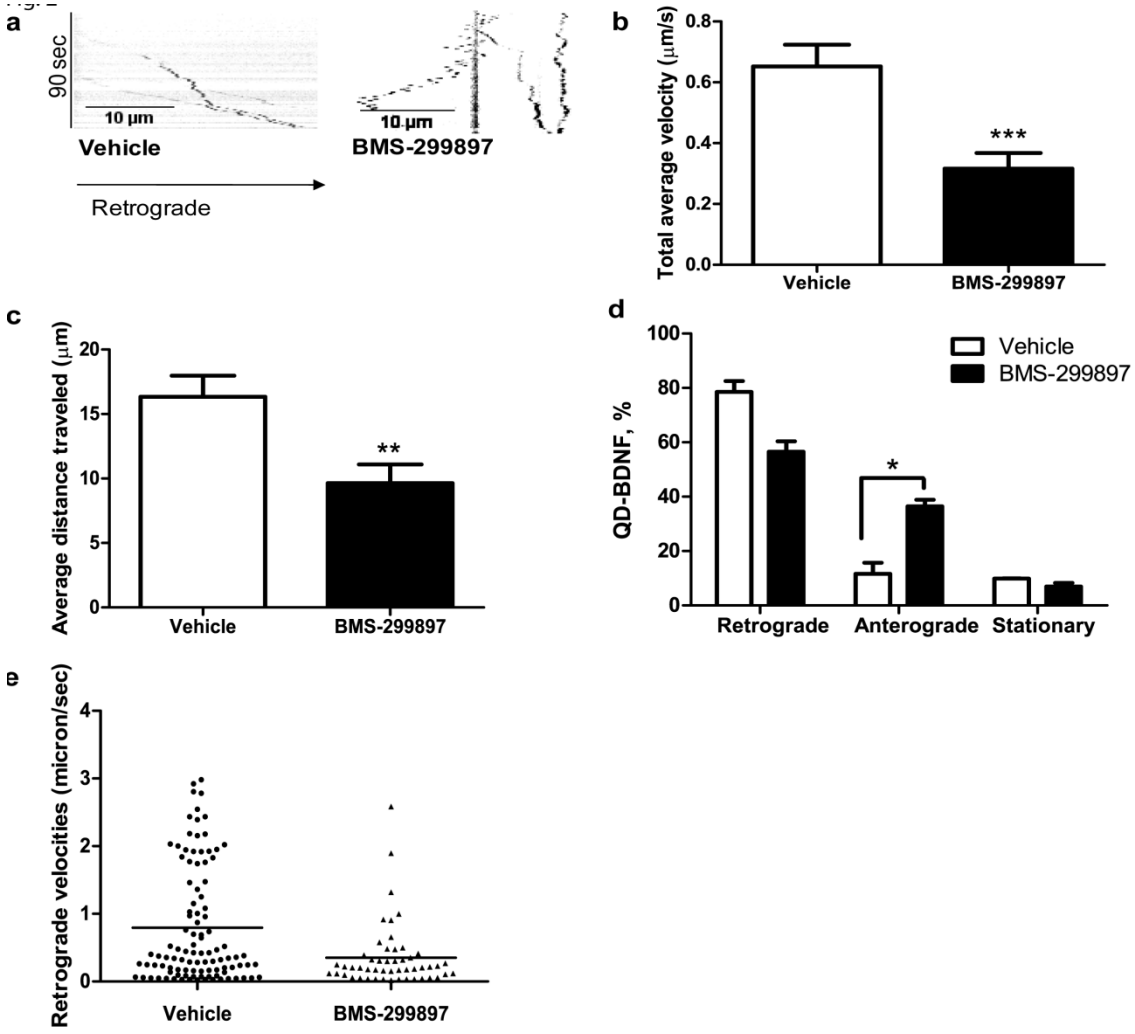

Figure 3.2 Retrograde trafficking of quantum-dot conjugated BDNF in compartmentalized hippocampal neuronal cultures and somatodendritic BDNF-induced signaling (a) Kymographs of QD-BDNF movement within axons inside the chamber microgrooves. Retrograde direction is depicted by arrow and time course is vertical axis (b) Total average velocity of QD-BDNF molecules for each treatment. GSI treatment significantly decreased the total average velocity compared to vehicle. (c) The average distance travelled also showed a significant impairment in distance traveled for GSI treatment compared to vehicle. (d) Breakdown of individual directionalities of all QD-BDNF showing that there is a decrease in the percentage of QD-BDNF moving retrograde following GSI treatment with a corresponding increase in the percent of QDs moving anterograde. Percentages of stationary QD-BDNF between each treatment did not differ. (e) Actual retrograde velocities of individual QD-BDNF molecules moving in the retrograde direction. QD-BDNF from GSI-treated neurons predominantly move at slower velocities (<0.2µm/sec). Time-lapse recordings were collected from two independent experiments for each drug treatment with 15-20 separate movies being collected blindly for each chamber. The data represented here are collective data for 70-125 QD-BDNF molecules. (mean±S.E, *P = 0.03, **P = 0.001, ***P < 0.001 using Student's t-test)

BDNF-mediated signaling was reduced in GSI-treated cultures

BDNF signaling from dendrite-to-nucleus and axon-to-nucleus occurs independently in hippocampal and cortical neurons(Cohen et al., 2011; Kohara et al., 2001) due to the presence of TrkB receptors in different regions of the neuron (Gomes et al., 2006). This creates the possibility that changes in movement of BDNF can translate to differential changes in signaling depending on location of BDNF addition. To compare somatodendritic versus axonal signaling under the influence of GSI, we examined BDNF-induced signaling in mass cultures and in axons alone. Cortical neurons were used because of the larger amount of axonal material that can be obtained from the chamber cultures. We assessed the activation state of two proteins downstream from the TrkB receptor: Akt (part of the phosphoinositide 3-kinase (PI3K/Akt) signaling cascade) and ERK (part of the mitogen-activated protein kinase (MAPK) signaling cascade). In mass cultures, early and sustained activation of TrkB and Akt was comparable following treatment with GSI or vehicle (Fig 3.3a-c). However, a different pattern was seen for activation of ERK in GSI-treated neurons in that activation was significantly decreased compared to control treated cultures at 5 min (Fig. 3.3a,d). This data suggest a significant impact of GSI downstream of the receptor for BDNF, which is specific to ERK activation.

We next investigated ERK activation elicited by BDNF in axons using a 3-chamber microfluidic device in which adequate amounts of axonal protein could be

extracted (Fig. 3.3e). Probing these cultures with specific antibodies demonstrated marked enrichment of the axonal protein Tau in the distal axon compartment as expected while MAP2, a dendritic protein, was enriched in the cell body/dendrite compartment. Using this system, compartmentalized cortical cultures were drug-treated in all compartments and the response in the axons after 5 min of BDNF addition in the axon compartment was examined since this time point had shown the largest difference between drug treatments in mass culture. To account for the number of axons that were collected for each compartment, the response examined was normalized to the amount of tubulin. In line with the mass culture data, a significant impairment in activation of ERK was observed in axons from GSI-treated cultures as compared to vehicle -treated neurons (Fig. 3.3f).

To better define if impaired axonal pERK signaling induced by BDNF in axons prevented downstream signaling at the nucleus, we proceeded to examine the activation state of CREB (cAMP response element-binding protein) in nuclei of the cell bodies, following axonal addition of BDNF for 30 min. Activated CREB (pCREB) has been shown to be present in the nucleus in response to axonal application of BDNF (Zhou et al., 2012). Importantly, it is a critical transcriptional activator of many genes involved in dendritic development and morphology thought to be downstream of the MAPK pathway (Li et al., 2009). Images of pCREB within cell bodies no further than 300μm away from the microgrooves revealed significant differences between GSI-treated compartments and that of vehicle. While vehicle-treated cultures showed about 55% of nuclei with pCREB expression, in GSI-treated

cultures this was decreased to 35% (Fig. 3.3g). This result reveal that GSI-mediated deficits in axonal BDNF trafficking and signaling result in decreased BDNF-induced transcriptional activation at the nucleus.

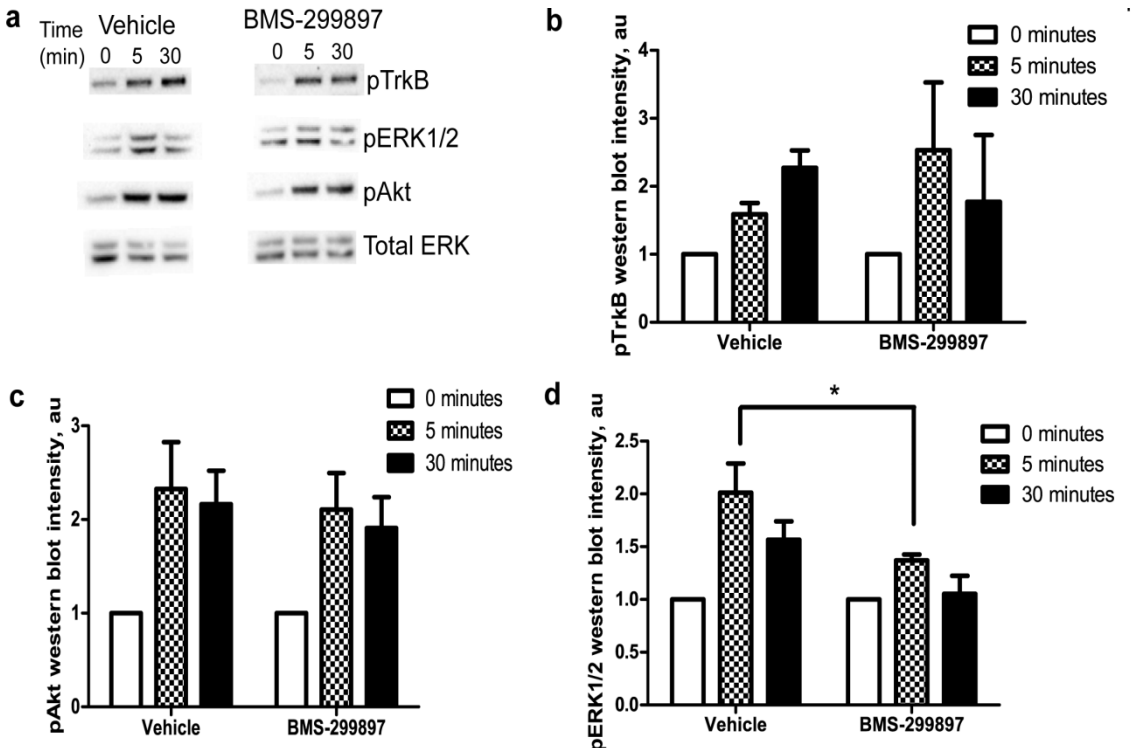

Figure 3.3 Activation of BDNF-induced signaling from mass cultures stimulated with BDNF globally and axons stimulated with BDNF in axons only (a) Western blots from mass cultures showing pTrkB, pAkt, pERK1/2 at 0, 5, or 30 min after addition of 50ng/ml BDNF. Quantification of pTrkB (b) and pAkt (c) shows that GSI does not differ from vehicle in its effect on response to BDNF at 5 and 30 minutes (mean±S.E., pTrkB n = 3, pAkt n = 5). (d) Quantification of pERK1/2 revealed a lesser activation in GSI-treated neurons in response to BDNF at 5 min (mean±S.E., n = 8, *P = 0.04using Student's *t*-test).

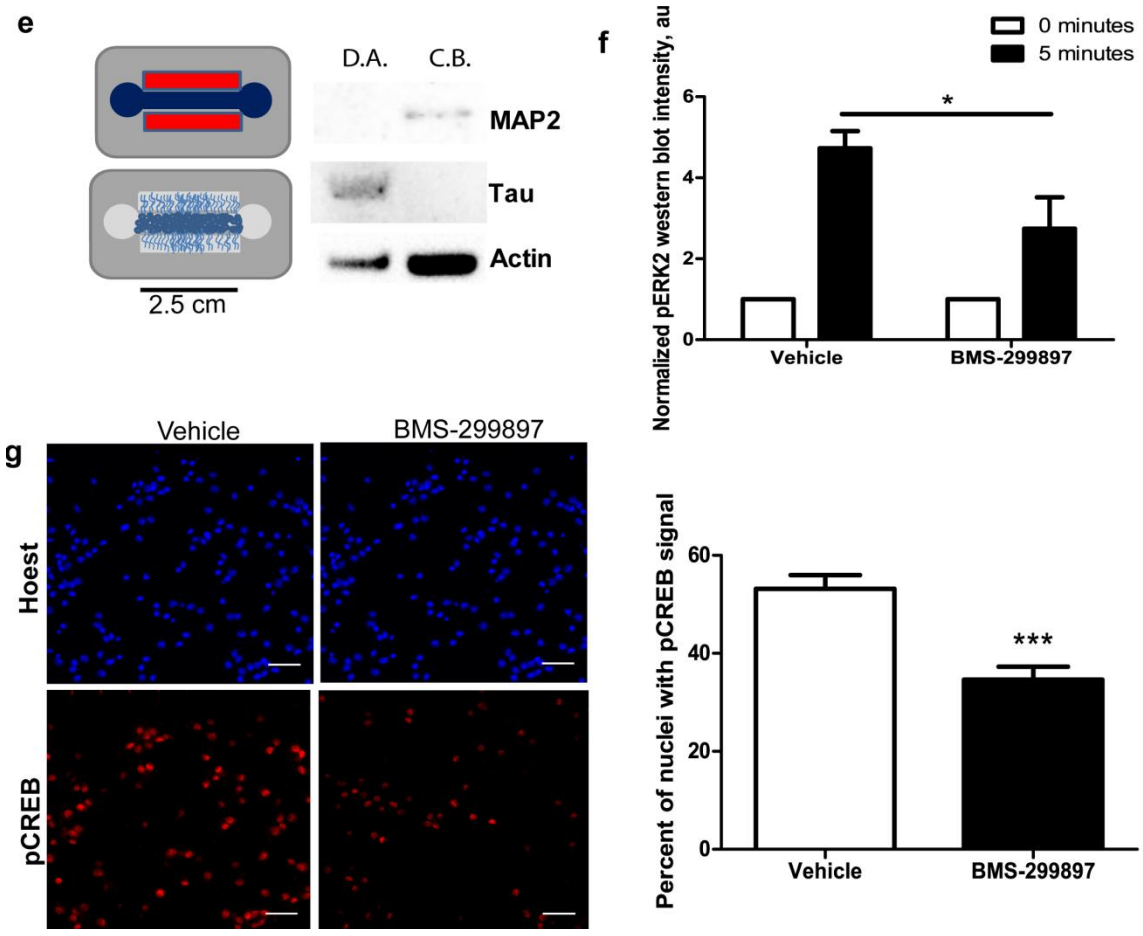

Figure 3.3 (cont'd) (e) Diagram of chambers used for axonal signaling experiments. Distal axons (DA), enriched for Tau, can be separately collected from cell body (CB) compartment, enriched for the dendritic protein, MAP2. Western blot for distal chambers is all sample lysate compared against 1/4th of cell body lysates collected. (f) Quantification of pERK2 normalized to tubulin showed significant impairment in activation at 5 minutes in GSI-treated axons compared to vehicle-treated (mean±S.E., n = 5, *P=0.02). Drugs were applied to all compartments and BDNF was added only at the axon. All signaling data analyzed are from 3 or more separate signaling experiments. (g) Images of activated CREB in nuclei of compartmentalized hippocampal neurons after BDNF addition to the axon compartment for 30 min. GSI-treatment reduced BDNF-mediated pCREB expression in nuclei of cell bodies (mean±S.E., n = 11, ***P<0.001). Images were immediately adjacent to the cell body side of the chamber and represent the cell bodies within 300 microns from microgroove separation. Drugs were applied to all compartments and 50ng/ml BDNF was added only in the axon compartment. scale bar = 50μm.

BDNF-containing vesicles colocalized more with APP/APP CTF and contain increased amounts of APP/APP CTF and kinesin light chain 1 following GSI treatment

Our findings pointed to a change in BDNF trafficking and signaling induced by GSI treatment but the underlying mechanism remained unclear. We therefore proceeded to examine axonal BDNF-containing vesicles asking if and how the γ-secretase substrate APP is implicated. APP is a likely candidate for axonal disruption given that it is directly altered by γ-secretase inhibition and it has a well documented role in trafficking and signaling (Inomata et al., 2003; Kamal et al., 2000; Perez et al., 1999; Schettini et al., 2010; Szodorai et al., 2009). As APP was initially discovered to undergo fast axonal anterograde transport(Koo et al., 1990) and both APP and APP CTFs have been shown to be located in axon terminals (Buxbaum et al., 1998), we narrowed our examination to axons using compartmentalized chambers which were drug treated in both the axon and cell body compartments but stimulated with biotinylated BDNF for 10 minutes only at the axon. Density analysis revealed that the number of internalized, trafficking BDNF vesicles was not significantly different in GSI-treated axons compared to vehicle-treated axons (mean~0.8 QD-BDNF/μm for vehicle, 0.6 QD-BDNF/μm for GSI, Fig. 3.4a first panel), suggesting that internalization of BDNF was not altered following GSI treatment.

Next we employed recently developed colocalization software (Szpankowski et al., 2012) which allowed us to quantitatively examine large numbers of BDNF-containing vesicles. Using this software, changes in specific populations of vesicles,

which may not be detected readily by eye, can be defined. With BDNF as a reference point, we assessed colocalization of APP with BDNF and with specific antibodies to the kinesin light chain (KLC1), a subunit of the motor protein kinesin. Kinesin was of particular interest as our transport data for QD-BDNF revealed a marked switch in directionality towards the anterograde direction suggesting that an anterograde motor protein may be involved. Furthermore, APP has been shown to transport in a kinesin-dependent manner (Gunawardena and Goldstein, 2001; Inomata et al., 2003; Kamal et al., 2000; Szodorai et al., 2009; Szpankowski et al., 2012) and therefore any alterations in APP processing might impact kinesin localization. The specificity of the KLC1 antibody used has recently been confirmed using transgenic mouse lines for KLC1 (Szpankowski et al., 2012). To confirm the C-terminal APP antibody we choose was specific to APP, we performed quantitative immunofluorescence analyses in hippocampal neurons generated from APP null (APP-/-) and APP heterozygous (APP+/-) mice containing one additional copy of APP. In the APP-/- we saw little, if any, punctate staining, while in the APP+/-, staining was punctate and bright, findings in agreement with published reports (Guo et al., 2012) (Fig. 3.4b).

We then proceeded with the BDNF vesicle analysis. The analysis of BDNF-containing vesicles show significantly more colocalization with APP and/or APP CTFs (APP/APP CTFs) following GSI-treatment compared to vehicle treatment (Fig. 3.4c). In addition, the intensity of APP/APP CTF on BDNF vesicles following GSI treatment was ~2-fold higher than vehicle (Fig. 3.4d), indicating increased amounts of APP on the vesicle. While we did not witness any significant changes in

colocalization of KLC1 with BDNF (Fig. 3.4c), the intensity of KLC1 on BDNF vesicles was increased by 3-fold following GSI treatment (Fig. 3.4e), pointing to increased amounts of kinesin on BDNF vesicles following GSI-treatment. Taken together, these data provide evidence that BDNF-containing vesicles following GSI treatment colocalize more with the gamma-secretase substrate APP, and contain higher amounts of APP/APP CTF and the anterograde motor subunit KLC1. This suggests a potential mechanism to explain altered BDNF trafficking and signaling under gamma secretase inhibition.

GSI treatment does not affect colocalization of dynein heavy chain with BDNF, nor does it significantly alter dynein heavy chain levels

In order for bidirectional transport to take place both kinesin and dynein, at times bound to the same transport cargo, must coordinate their directionality based on variety of factors, one being the amount of motor on a particular cargo (Derr et al., 2012; Hendricks et al., 2010; Soppina et al., 2009). Given that we observed increased KLC1 amounts on the BDNF vesicle we further examined if dynein levels were altered on internalized BDNF vesicles following GSI treatment by using the antibody to dynein heavy chain, a part of the dynein complex, which has been used recently with high specificity (Szpankowski et al., 2012). After 10 min of BDNF application to the axon compartment, internalized BDNF vesicles showed no difference in colocalization of BDNF with DHC following GSI treatment (Fig. 3.4g). Furthermore, the intensity of DHC levels was not different between GSI and vehicle treatment,

suggesting that GSI treatment leads to a motor-specific phenotype which does not include dynein (Fig. 3.4f). .

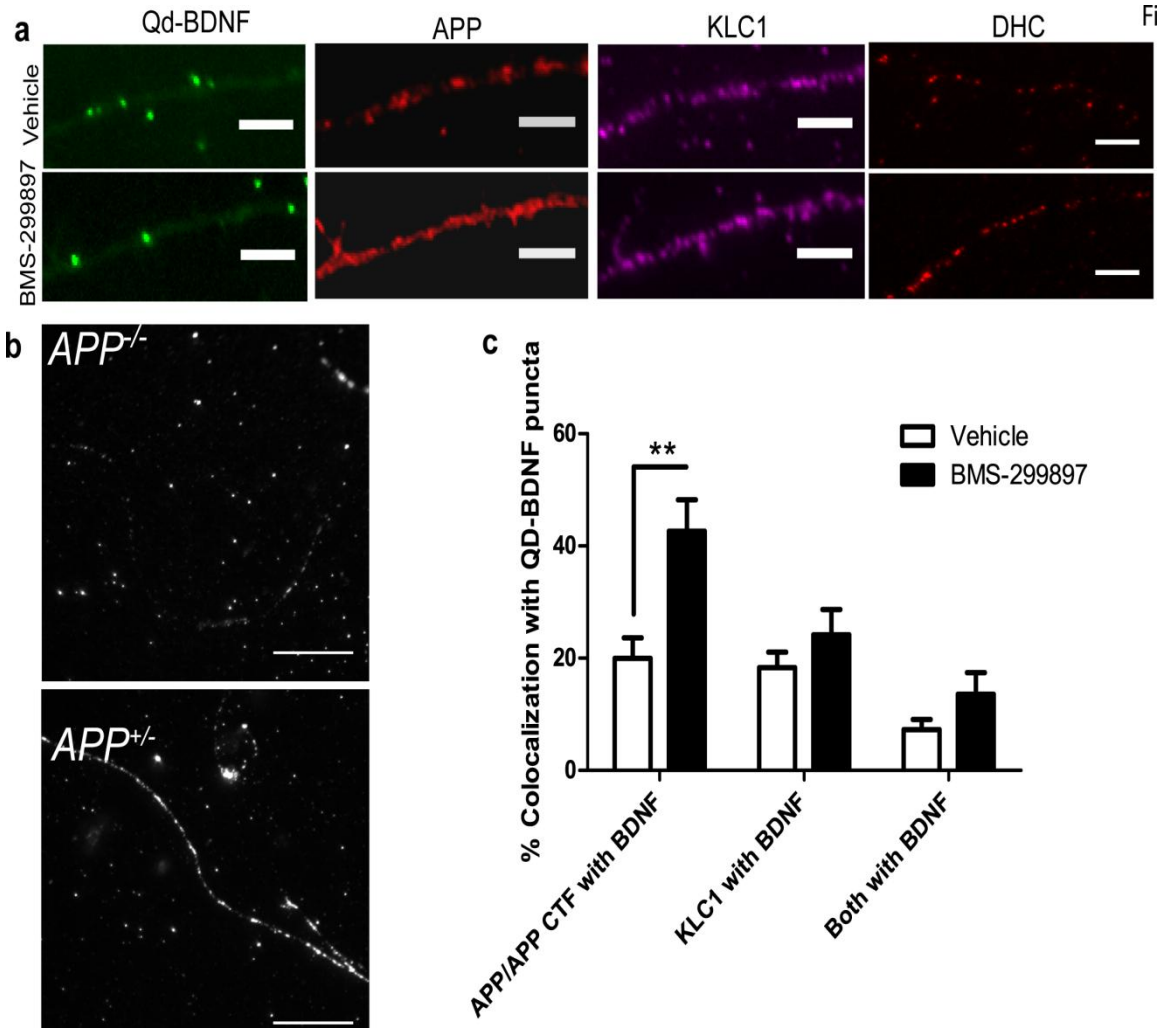

Figure 3.4 BDNF-containing vesicle studies in the hippocampal axons of compartmentalized chambers after addition of biotinylated BDNF (a) Sample immunostaining images which were used to run program and to which Gaussians were fitted to determine individual puncta. Image shows BDNF (pseudo-colored green), APP/APP CTF (red), KLC1 (pseudo-colored magenta), and DHC (red). BDNF, APP, and KLC1 are same axon, DHC is staining from its separate experiment (b) Sample images from hippocampal neurons generated from $APP^{-/-}$ and $APP^{+/-}$ embryonic mice and stained with C-terminal Epitomics APP antibody. Little staining is present in $APP^{-/-}$ neurons, while increasing APP by one copy using $APP^{+/-}$ results in increased punctal fluorescence (c) Colocalization analysis of BDNF with antibodies for APP and KLC1. Internalized BDNF vesicles following GSI-treatment show increased colocalization with APP.

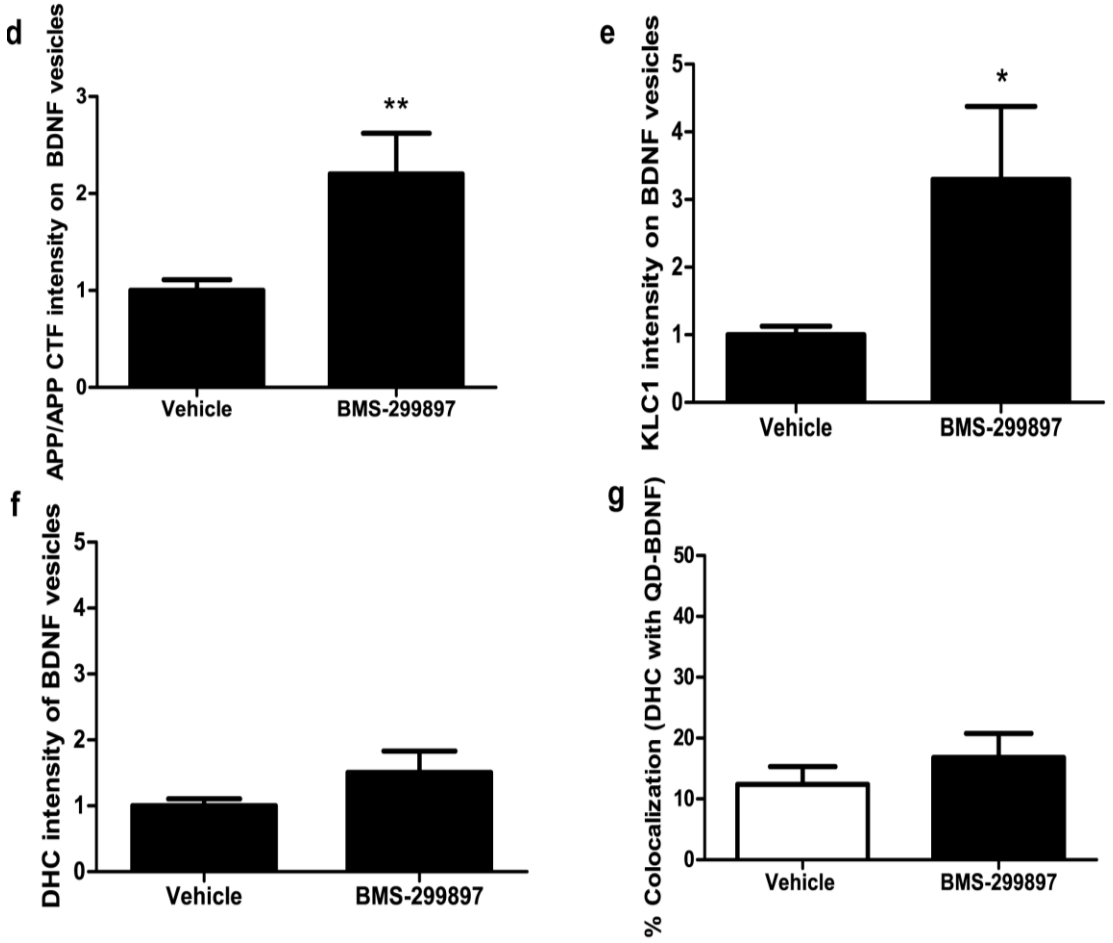

Figure 3.4 (cont'd) (d) APP/APP CTF intensities on BDNF vesicles are increased following GSI-treatment indicating increased amounts of APP/APP CTF on BDNF vesicles (e) KLC1 intensity on BDNF vesicles are increased following GSI treatment (mean±S.E., n=35-40 axons and 230-300 BDNF puncta , **P=0.002, 0.004, *P=0.02 using Student's t-test). (f) DHC intensity on BDNF vesicles is similar on BDNF vesicles following GSI treatment (g) Colocalization analysis of BDNF with an antibody to DHC. Internalized BDNF vesicles following GSI treatment show no difference in colocalization with DHC. (mean±S.E., n=19 axons and 120-150 BDNF puncta) scale bar = 5μm.

GSI-treatment causes increases in the amount of APP, more specifically APP CTF, in axons

Next we examined GSI-treated axons without BDNF stimulation to determine what effect drug treatment had on APP within the axons. In these experiments, GSI or vehicle was applied to both the axon and cell body side, but BDNF was not added. In the axon compartment, GSI treatment increased individual puncta intensities to approximately three times that of vehicle-treated, suggesting that GSI results in more APP and/or APP CTFs on individual APP vesicles (Fig. 3.5a). Frequency distribution analysis of the APP intensity amplitudes revealed a broadening of the distribution of punctal intensities, a finding also consistent with increased amounts of APP and/or APP CTFs in individual vesicles (Fig. 3.5b).

Given that inhibition of γ-secretase causes accumulation of APP CTFs and that the antibody used was to the C-terminal region of APP, we sought to determine whether the APP in the distal axons was the full-length protein or its CTFs. However, testing of specific alpha-CTF and beta-CTF antibodies by staining neurons derived from APP transgenic mice revealed that neither antibody was specific enough for this study (data not shown). Therefore, western blots of axonal and cell body compartments collected following GSI treatment were assessed for their full length APP and APP CTF levels. Following GSI treatment, there was a robust APP CTF accumulation in both the axons and cell body compartments with modest effects to full length APP. This resulted in an increased ratio of APP CTFs to full length APP in axons, favoring the presence of APP CTF in axons (Fig 3.5b). In summary, these data

suggest that the APP phenotypes witnessed for both BDNF and APP vesicles following GSI-treatment could be due to increased presence of APP CTFs rather than full-length APP.

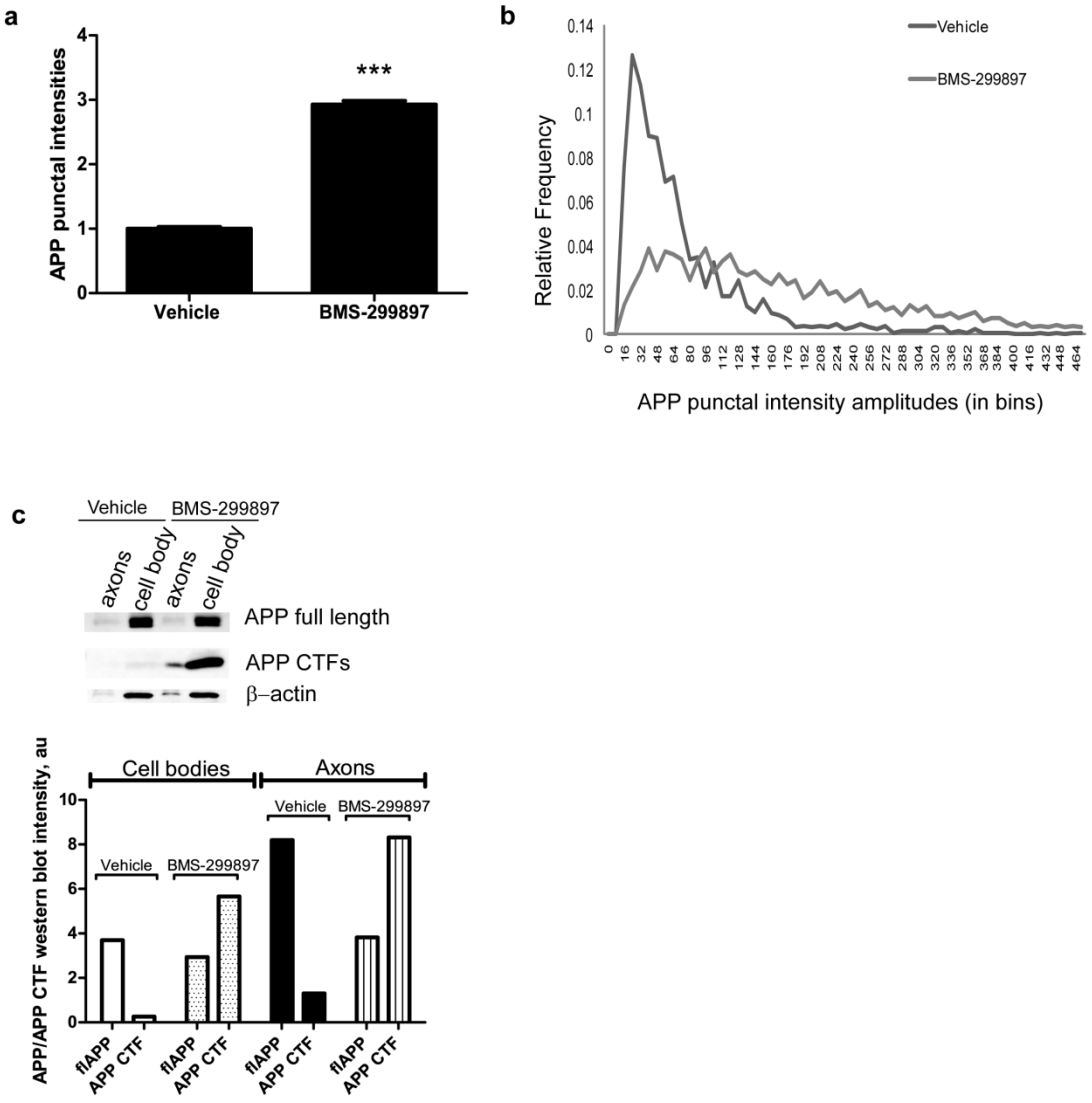

Figure 3.5 APP-positive vesicle characteristics in distal hippocampal axon compartments treated with either GSI or vehicle (a) Intensity of all APP puncta are increased following GSI treatment (mean±S.E, n = 1500 APP puncta, ***P<0.001 using Student's t-test). (b) Frequency histogram showing all amplitudes of intensities for APP-positive puncta for each drug treatment. Vehicle treated neurons show smaller APP intensities compared to GSI-treated axons which show a broad distribution of APP intensities. (c) Western blot depicting the localization and amount of full-length APP and APP CTF fragments in either cell bodies or axons of hippocampal neurons cultured in compartmentalized biochemistry chambers. Graphs show the APP CTF over full-length APP ratio (normalized to β-actin) and reveal that GSI-treatment favors APP CTF in the axon compared to APP CTFs in vehicle-treated axons. All imaging data represented are cumulative from two independent experiments.

Reducing APP levels rescued axonal transport deficits in GSI-treated hippocampal neurons

Given our results for changes in vesicular proteins we proceeded to determine if the GSI-mediated deficits we had witnessed so far could be explained by failed processing of APP as we initially hypothesized, rather than that of other GSI substrates. We tested this idea by reducing APP protein levels using siRNA prior to drug treatment and then assessing BDNF trafficking. Both APP and APP CTFs were decreased after siRNA-induced knockdown when compared to control siRNA transfected neurons (Fig. 3.6a). QD-BDNF transport experiments revealed that lowering APP levels improved the velocity and directionality of BDNF movement in GSI-treated neurons (Fig. 3.6b). Specifically, the average velocity of QD-BDNF went from moving about 50% of the vehicle to a velocity essentially equal to vehicle (Fig. 3.6c). In addition, lowering APP levels increased the percentage of BDNF molecules moving retrogradely, while lowering the percentage of anterograde movement (Fig. 3.6d). Importantly, lowering APP levels also improved the percentage of nuclei expressing pCREB in 30 min post-BDNF application to the axon (Fig. 3.6e). These results indicate that of the many known substrates for gamma-secretase, APP serves as an important mediator of GSI-facilitated BDNF trafficking and signaling deficits. Furthermore, they provide data for how altered APP processing can directly impair trafficking of a retrograde signal.

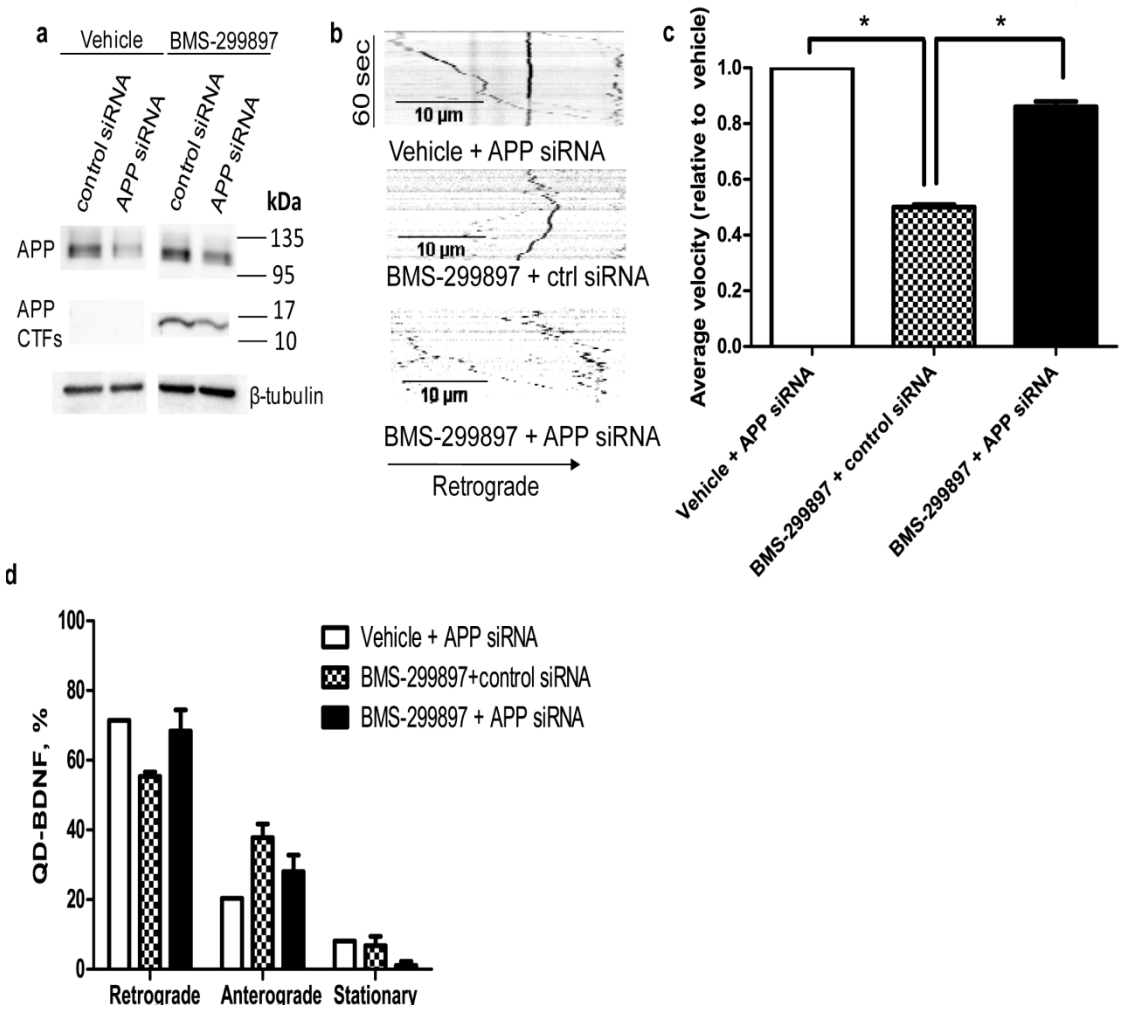

Figure 3.6 Knockdown of APP prior to GSI treatment rescues velocity of QD-BDNF and directionality. (a) Western blot of typical knockdown experiment. Knockdown of APP with siRNA against APP or control was performed 72 hrs prior to drug treatment. Earlier time points showed only small knockdown of the protein even though mRNA levels were decreased close to 80% in 24h. On average, APP and APP CTF were knocked down to 70% of normal levels per experiment. (b) Kymographs of QD-BDNF movement within axons inside the microgrooves with siRNA type indicated. Retrograde direction is depicted by arrow and time course is on vertical axis. Analysis of QD-BDNF for total average velocities (c) and sorting of QD-BDNFs for directionality (d) revealed that knocking down APP prior to GSI treatment partially rescues the velocities of QD-BDNF and the deficits in directionality previously seen. Time-lapse recordings were collected from one experiment for vehicle with siRNA against APP and two independent experiments for BMS-299897 pre-transfected with either control siRNA or siRNA against APP, with 15-20 separate movies collected for each chamber. The data represented here are collective data for 50-130 QD-BDNF molecules. (mean±S.E, *P = 0.02 using Student's *t*-test).

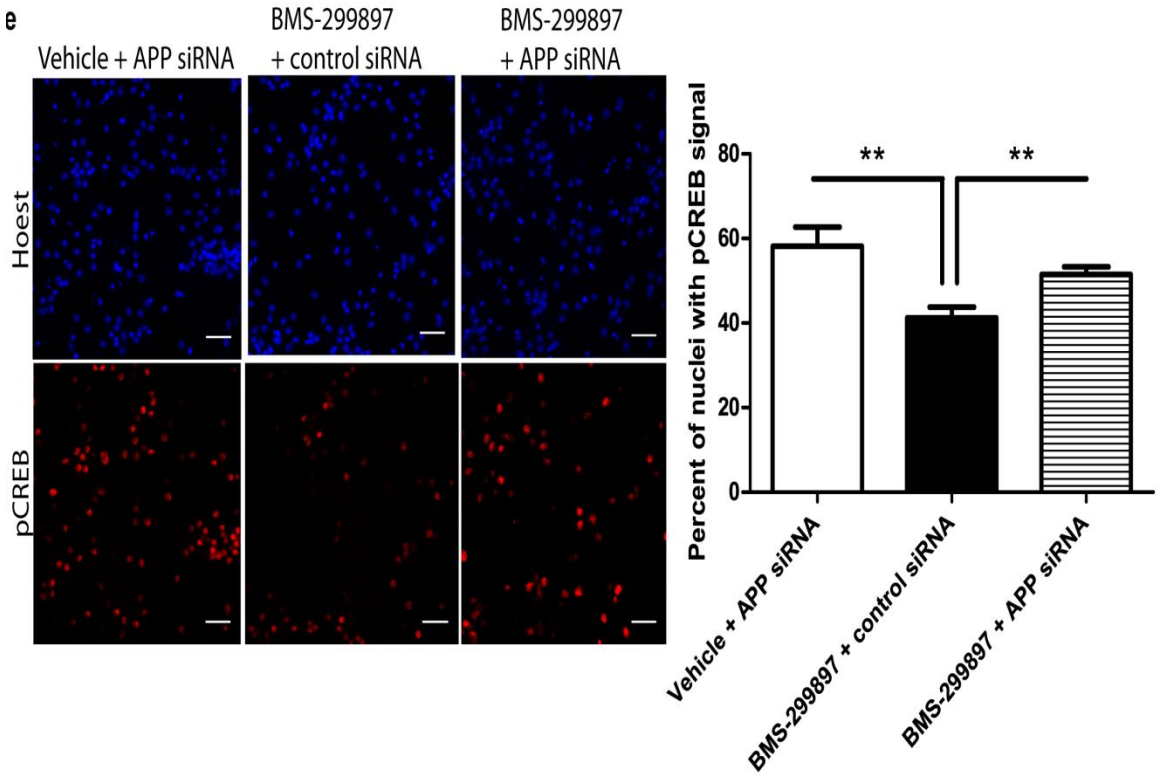

Figure 3.6 (cont'd) (e) Images acquired following similar siRNA/drug treatment and addition to BDNF at the axon in compartmentalized chambers.Quantitation represents the percent of nuclei which express pCREB (mean±S.E., n = 10 images, **P=0.004, 0.003 using Student's *t*-test). scale bar = 50μm.

GSI treatment induced transport deficits for mitochondria and synaptic vesicles and elicited organelle accumulation

To define whether or not the GSI-induced trafficking problems were specific to retrogradely transported signals we examined the transport of mitochondria and synaptophysin vesicles which are both anterogradely transported and, at least in the case of mitochondria, are trafficked in dendrites as well as axons. To assess transport, mass cultures of hippocampal neurons were drug-treated from DIV4 to DIV7 to allow

for the development of potential transport phenotypes (Fig. 3.7a). Analysis of mitochondrial density revealed no significant differences in the number of mitochondria per micron between GSI treatment as compared to vehicle (Fig. 3.7b). However, there was a significant increase in the size of mitochondrial puncta following GSI treatment with GSI-treatment leading to an increase from ~4μm^2 to 6μm^2 ($P<0.001$). For synaptic vesicles, as marked by synaptophysin, the opposite finding was made in that the density was significantly lower in GSI-treated neurons ($P=0.03$), while size remained similar to vehicle (Fig. 3.7c). To better understand what ultrastructural changes were caused by GSI treatment, we imaged drug-treated neurons using transmission electron microscopy following same experimental paradigm (Fig. 3.7a). Both mitochondria and vesicles were sparse in neurites of vehicle-treated neurons (Fig. 3.7d). High magnification pictures of mitochondria revealed that, as expected, they were localized along microtubules (Fig. 3.7e). In contrast, GSI-treated neurons displayed striking abnormalities. First, there was abnormal accumulation of mitochondria (Fig. 3.7f,i). High magnification revealed that these were not enlarged nor fused mitochondria but many separate mitochondria lined up with each other (Fig. 3.7g), suggesting that the increased size of mitochondrial puncta in fluorescent imaging was due to accumulation of mitochondria. We also observed accumulation of vesicles, some of which occurred in swellings that protruded from the neurite (Fig. 3.7h,i). Taken together, these data indicate that the GSI markedly affected neuronal structure and lead to phenotypes indicative of inefficient trafficking.

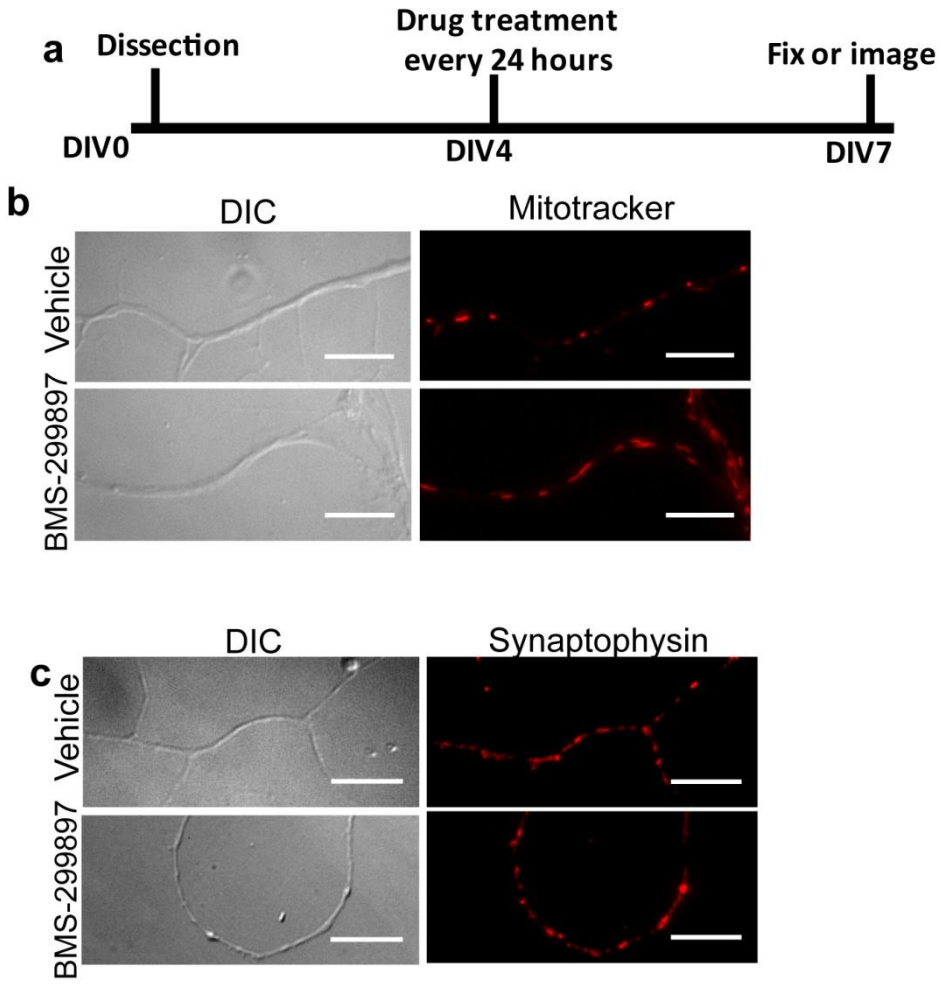

Figure 3.7 Treatment with GSI caused changes in apparent transport of multiple organelles. (a) Experimental paradigm used to examine the trafficking of mitochondria and synaptic vesicle precursors. Drug treatment was initiated on DIV4 and was continued until DIV7. (b) Representative pictures and quantitative analysis of drug-treated hippocampal neurons using Mitotracker. Analysis of images revealed that the density of mitochondria along the neurite were not different between drug treatments (n=15-20 neurites), however there was a large increase in the size of mitochondria with GSI treatment (n=95-150 puncta, mean±S.E, ***P<0.001 using Student's *t*-test). (c) Representative pictures and quantitative analysis of same experiment examining synaptophysin-positive puncta. Analysis revealed that the density of synaptophysin-positive puncta was significantly decreased (n=29-40 neurites, mean±S.E, *P<0.05 using Student's *t*-test) with GSI treatment compared to vehicle, but that the size was similar for both drug treatments (n=26-27 puncta), scale bar = 20μm.

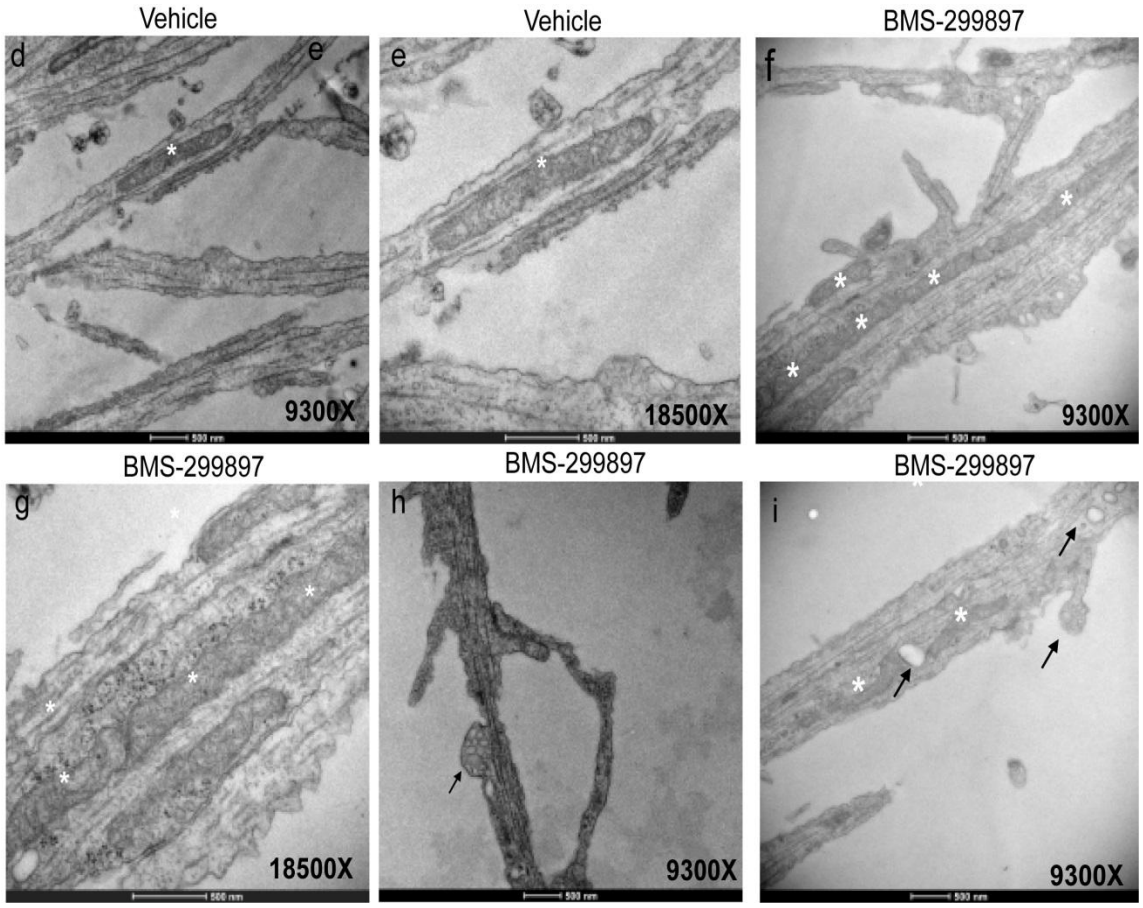

Figure 3.7 (cont'd) Same experimental paradigm was used to assess the ultrastructural phenotypes of hippocampal neurites for both GSI and vehicle using electron microscopy. Vehicle (d, e) treatment showed no obvious phenotype with respect to mitochondria, vesicles, and other ultrastructural characteristics, while GSI treated cultures showed accumulations of mitochondria along the neurite (f, g, i) and synaptic vesicle accumulations (h, i). Asterisk denotes mitochondria, arrows point to vesicles. EM data were verified by two independent researchers.

Discussion

Altered amyloid precursor protein (APP) processing, specifically increased APP CTF levels, are implicated in axonal dysfunction of multiple neurological disorders. Here we analyzed a GSI of well-defined structure for its effect on BDNF trafficking and signaling in hippocampal and cortical neurons. We found that treatment with the GSI, BMS-299897, caused a significant impairment in trafficking of QD-BDNF movement, revealing dramatic phenotypes that correlate to increases in APP CTFs.

Interestingly, we found that GSI-treated neurons showed powerful effects on axonal and somatodendritic signaling in response to BDNF. In mass cultures, in which somatodendritic and axonal regions are treated simultaneously, only ERK activation appeared decreased, a finding we also witnessed when we looked specifically at axonal signaling of BDNF. This is in line with previous studies which have shown ERK activation to be dependent on retroendocytic mechanisms (Delcroix et al., 2003; Howe et al., 2001; Zhang et al., 2000). Activation of Akt, on the other hand, has been shown to be independent of endocytosis(Zhang et al., 2000), even though there have been components of the PI3K pathway identified on vesicles induced by signaling of NGF in PC12 cells(Howe et al., 2001). This suggests that surface signaling mechanisms following GSI treatment remain intact. Suppressed ERK activation seen by GSI treatment highlights the importance of endosomal

trafficking for proper signaling response. At least two possibilities exist to explain failed activation of ERK signaling. First, it may be that failure in trafficking of the BDNF signaling endosome leads to premature degradation of ERK along the transport route. Second, it is possible that active complexes necessary for downstream signaling of TrkB/BDNF are not present on the internalized BDNF signaling endosome following GSI treatment. One important physiological consequence of this failed transport for the neuron was decreased activation of the transcriptional activator CREB in the nucleus of neurons treated with GSI following BDNF addition to the axon. Given these data, continued failed trafficking and signaling over time would be expected to result in poor synaptic function and reduced maintenance of synapses.

To address the mechanism behind GSI-induced trafficking defects of BDNF, we performed extensive, quantitative immunofluorescence analysis of BDNF, APP, and KLC1 containing axonal puncta. Strikingly, we found that following GSI treatment BDNF-containing vesicles exhibited increased colocalization with APP/APP CTFs and contained increased relative amounts of both APP/APP CTFs and KLC1. In a similar experiment, we found that internalized BDNF vesicles between vehicle and GSI did not show any changes in motor colocalization or loading of DHC. Therefore, one possible mechanism to explain decreased retrograde velocities and the switch to more frequent anterograde movement is that increased presence of kinesin on BDNF vesicles alters the ability of the vesicle to move efficiently in the retrograde direction by changing the ratio of opposing motors. It is possible that GSI, potentially through APP CTF accumulation, regulates the amounts of kinesin on retrograde BDNF-

containing vesicles in axons as has been suggested for APP transport vesicles (Gunawardena and Goldstein, 2001; Kamal et al., 2000; Szodorai et al., 2009; Szpankowski et al., 2012). We are not currently aware of the type of APP, full length or CTF, that is on the BDNF vesicle but western blots of axons versus cell bodies indicated a strong increase in the ratio of APP CTF in the axon compartment of GSI-treated neurons suggesting that APP CTFs do play a role. In line with this our knockdown experiments showed that decreasing levels of full-length APP and APP CTF can rescue trafficking deficits, indicating that altered APP processing can dramatically affect retrograde trafficking, an observation that has been noted in other studies(Poon et al., 2011; Salehi et al., 2006). Further examination of both mitochondria and other anterograde transport vesicles revealed changes to their morphology that were suggestive of stalled transport following GSI treatment, indicating that the GSI-induced deficits were not specific to retrograde cargoes and may potentially involve the anterograde motor subunits, adaptors, or regulatory proteins. In fact, one particular mitochondrial study revealed that while mitochondrial transport was dependent only on the heavy chain subunit of kinesin. If KLC was expressed in that same paradigm, the heavy chain was no longer recruited to the mitochondria (Glater et al., 2006), mimicking what we saw with stalled mitochondria. Furthermore, multiple studies have provided evidence that altered APP processing, including overexpression of APP and/or APP CTF, can cause altered APP movement(Rodrigues et al., 2012), organelle accumulation(Gunawardena and Goldstein, 2001; Stokin et al., 2005; Torroja et al., 1999) and mitochondrial

dysfunction and trafficking(Calkins et al., 2011; Calkins and Reddy, 2011; Rui et al., 2006), some of which may be the result of APP's proposed ability to recruit kinesin motor complexes. At this time we cannot conclude to what degree KLC1, or any other motor protein subunits, play a role in the mitochondrial and other vesicle trafficking deficits seen here. However, the idea that APP and its altered processing under GSI treatment can directly lead to motor loading abnormalities for these compartments is intriguing and would be consistent with several studies in the field.

Building on our findings, we propose a model for how altered APP processing can impact BDNF signaling: in a healthy neuron, normal and efficient retrograde trafficking of BDNF is allowed in the retrograde direction under dynein control, even though some amount of kinesin is present. Under GSI treatment, APP proteolysis is reduced leading to increased APP and/or APP CTFs along with increased KLC1 on internalized BDNF vesicles .This increase in kinesin tips the balance of predominant BDNF retrograde transport to slow retrograde transport and an increased propensity to move in the anterograde direction (Fig. 3.8). Over time, non-APP anterograde compartments such as mitochondria are also affected and cause neuronal phenotypes suggestive of inefficient transport. A continued impairment in bidirectional transport for a neuron would eventually result in synaptic demise as the normal, daily neuronal process of moving proteins and organelles to and from the cell body becomes compromised.

In conclusion, we have demonstrated that GSI treatment has a detrimental effect on key facets of neuronal structure and function including BDNF trafficking and

signaling due to effect on APP processing. These data exhibit potential mechanistic insight into the biological processes and subsequent impairment driving all forms of Alzheimer's disease that include increases in APP CTFs or in other disorders in which APP gene dose is increased. While generally the pathophysiology witnessed in Alzheimer's disease is slow and progressive, the use of GSI may help to elucidate the actual contribution that non-Aβ products of APP play in the disease.

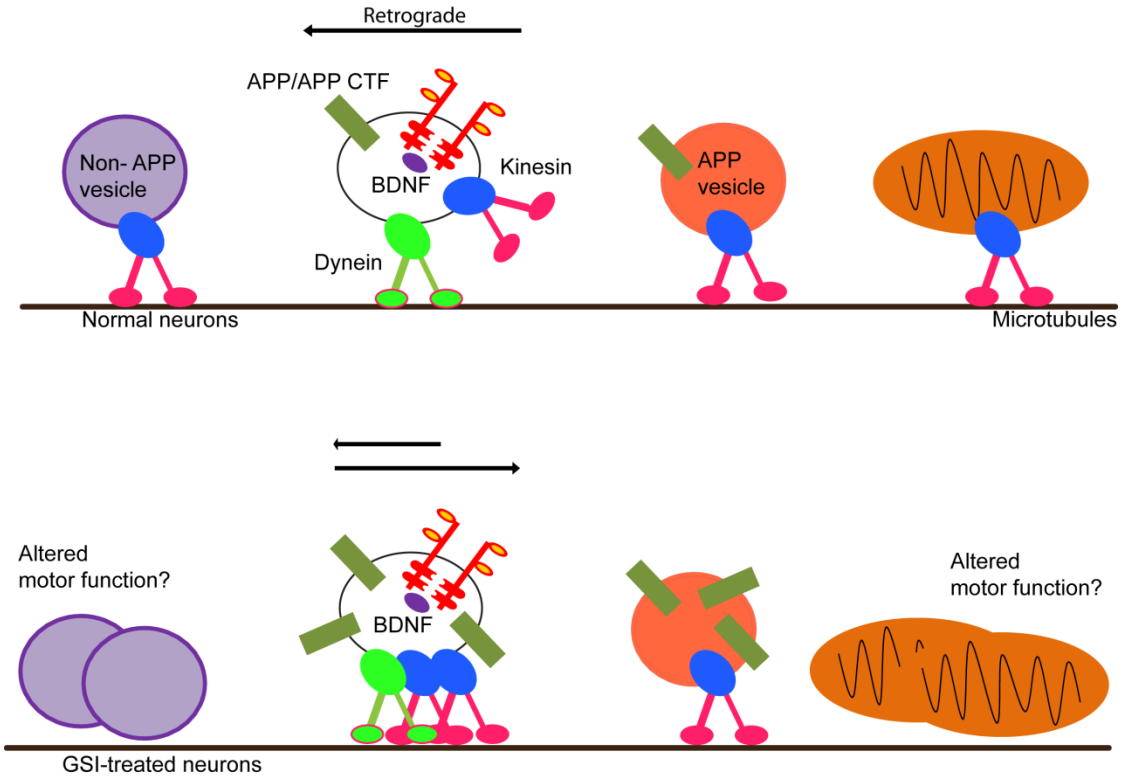

Figure 3.8 Simplified model for APP-dependent trafficking deficits under gamma-secretase inhibition. In a healthy neuron, normal and efficient retrograde trafficking of BDNF is allowed in the retrograde direction under dynein control, even though some amount of kinesin is present. Under GSI treatment, APP proteolysis is reduced leading to increased APP and/or APP CTFs and KLC1 on internalized BDNF vesicles without a concomitant increase in dynein. This increase in kinesin tips the balance of BDNF retrograde transport to slow retrograde transport and an increased propensity to move in the anterograde direction, resulting in overall poor trafficking and signaling of BDNF to the nucleus. This effect on trafficking may not be specific to retrograde BDNF because GSI treatment leads to phenotypes for mitochondria and other vesicles that is indicate of inefficient transport.

Materials and methods

Primary cell culture: Rat E17-18 primary embryonic mixed cortical and hippocampal neurons were dissected and plated into 12 well plates coated with poly-D-lysine at 500K cells/well. For imaging experiments, hippocampal neurons were cultured into microfluidic chambers with 450μm microgrooves (Xona microfluidics) that had been prewashed using 1% Alconox and presterilized by UV. For biochemistry experiments, cortical neurons were cultured in home-made larger microfluidic chambers with 450μm microgrooves using patterned SU8-coated silicon wafers as master molds. All neurons were dissected into Hanks Balanced Salt Solution (HBSS) containing 10mM HEPES and 1% Penicillin/Streptomycin and then plated in plating media containing neurobasal, 2% B27, 1% Glutamax and 5% FBS. The next day, two-thirds of the plating media was replaced with a maintenance media containing neurobasal, 2% B27 and 1% Glutamax. Two-thirds of the media was replaced every 2-3 days until experiments. For mass culture signaling studies, cultures were starved for two hours in neurobasal prior to treatments, stimulated with 50ng/ml BDNF and followed by lysis in PBS containing 1% NP-40, 0.1% SDS, 0.1% deoxylcholate, 1mM PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride), 200uM sodium orthovanadate and protease inhibitor cocktail (Roche). Axonal signaling was performed similarly except that BDNF was added only to distal axon chambers. Axons were collected by running a small volume of lysis buffer into the distal compartments several times.

Drug treatment and siRNA transfections: At 7 day in vitro (DIV7), neuronal cultures were treated for 24 hrs with 1μM BMS-299897 or the vehicle DMSO (final concentration: 0.01%). Transfections were performed on 100k neurons at DIV4 with siRNA against APP or control siRNA (Sigma) using NTER Nanoparticle transfection system (Sigma). Knockdown experiments were optimized by examining both mRNA and protein expression of APP. Knockdown efficiency was confirmed by western blot for each experiment.

Live Imaging of Quantum dots-labeled BDNF (QD-BDNF): monobiotinylated BDNF was prepared in a method similar to that described previously (Sung et al., 2011). QD-BDNF, which signals and transports like normal BDNF (manuscript in prep), was prepared as described previously (Cui et al., 2007). We chose QD655 which has an emission that can be visualized in the Texas Red channel. Following starvation for two hours in neurobasal media, QD-BDNF (0.1nM) was added to the distal chamber and allowed to incubate for two hours. After 2 hrs, the chamber was placed on a Leica DMI6000B inverted microscope with an 100x oil objective lens. The microscope is equipped with an environmental chamber (37oC , 5% CO2). All images were captured with a CCD camera (Rolera-MGi Fast 1397 from Qimaging). Images were collected at 1 frame/second for a total of two minutes per movie. Ten to twenty movies were collected per chamber. For each treatment, two separate chambers cultured on two different days were used to collect movies. For each condition, 75-130 QD-BDNF signals from two independent experiments were analyzed. Kymograph was created from each movie and was analyzed using the Metamorph

Software. Direction of movement was determined from angle measurement acquired for each line on kymographs using cutoffs we specified: retrograde (-1- -89.4), anterograde (<-90.6), and stationary (90□0.5). Actual movies and kymographs included in results section were processed from the original Metamorph file in Image J to bring out the contrast of QD-BDNF movement and add scale bar with time stamp. All live-imaging experiments was performed blindly to prevent any bias.

Antibodies and Reagents: Rabbit monoclonal pERK1/2, pAkt, and rabbit polyclonal total ERK antibodies were from Cell Signaling Technologies. Rabbit polyclonal phospho-TrkB antibodies were kindly provided by Dr Moses Chao (NYU), the rabbit monoclonal phospho-TrkB antibodies were from Epitomics. Other antibodies used include: pCREB(ser133) rabbit polyclonal (Cell Signaling), β-actin and β-tubulin mouse monoclonal (Sigma), C-terminal APP rabbit monoclonal (Epitomics), rabbit polyclonal C-terminal APP(Kounnas et al., 2010), goat polyclonal KLC1 (Santa Cruz Biotechnology). Alexa-based secondary antibodies were used to reveal the primary antibodies. For reprobing of biotinylated BDNF, QD605 (Life Technologies) was used.

Western blotting: Samples were run on a 4-12% Novex precast gel (Life Technologies). For APP CTF detection, 4-20% precast gels were used. Immunoblotting was performed following standard practice (Zhang et al., 2013). All blots were developed using the BioRad ECL substrate and visualized on a BioRad ChemiDoc XRS+ with the BioRad ImageLab software. All quantification of band

densities was done using blots within the linear range of exposure and analyzed using the BIoRad ImageLab software.

Immunostaining: Neurons were cultured on coverslips and were fixed in 4% paraformaldehyde (PFA) in PBS for 15 minutes, were permeabilized in 0.2% Triton X-100 in PBS. Coverslips were blocked using 3% BSA, 5% goat serum in PBS for 1 hr at room temperature. Primary antibodies were added in block solution for 1 hr at room temperature or overnight at 4□C. Secondary antibodies were added in 3% BSA for 1 hr at room temperature. Coverslips were washed and mounted in anti-fade and sealed using nail polish. Visualization of neurons was done on a Leica DMI6000B inverted microscope with an 100x oil objective lens. Image J was used to add scale bar to each image. For quantitative analysis of chamber cultures, chambers were fixed live for 30 min at 37□C using 4% PFA and 4% glucose and then permeabilized with 0.1% Triton X-100 in PBS. Chambers were blocked using 10% donkey serum, 3% BSA, and 0.1% Triton X-100. Primary antibodies were added overnight at 4□C probed with Alexa-conjugated donkey secondaries for 1 h at room temp. All chamber staining, image acquisition and quantitation were performed using a Deltavision RT deconvolution imaging system (Applied Precision Instruments), with coded samples whose identity was not known by the analyzer. Results were analyzed using a designed colocalization software as previously described (Szpankowski et al., 2012). Briefly, the program used immunostaining images and to fit multiple Gaussians to determine individual puncta based on spread of intensity. A 300nm radius was drawn around the center of each Gaussian fit and x- and y- coordinates for each channel were

determined by program. Any other puncta that fell into that radius was considered colocalized with it. Regions of interest (ROIs) were drawn around axons of interest and puncta were filtered based on size to filter out any unspecific binding of antibodies or artifacts. For mean intensities, the top and bottom five percent of intensity amplitudes were discarded in the intensity averages. For pCREB analysis, images at 20x were taken starting at one side of the chamber and ending at the opposite side, covering all the cells directly adjacent to the microgrooves. Image J was used for analysis in which images were thresholded equally and counted automatically with defined size cutoffs.

Aβ Meso scale assay: Aβ38, 40, and 42 were detected as previously described (Kounnas et al., 2010). Briefly, for dose-response assay, 500K embryonic mixed cortical neurons were treated in triplicate in 400µl of media containing various concentrations of drugs or vehicle. Media was collected 24 hrs later into three separate aliquots. Aβ peptides in media were detected using Aβ triplex meso scale assay. The amount of Aβ peptides in solution was quantitated as percentage of vehicle control.

Mitochondria and synaptic vesicle analysis: Deep Red FM Mitotracker (Molecular Probes) was used to visualize mitochondria, following the protocol provided. A rabbit polyclonal synaptophysin antibody (GenTex) was used on fixed coverslips. All images were acquired and analyzed blindly.

Electron microscopy: Drug-treated DIV7 neurons were fixed and sectioned at the Cellular and Molecular Medicine Electron Microscopy Facility at UCSD following a standard EM flat embedding protocol for cells on coverslips. Images were acquired on a FEI Tecnai Spirit G2 BioTWIN Transmission Electron Microscope.

mRNA quantification: Quantification of mRNA levels of Hes1 and Hey1, was achieved using cDNA synthesized from total RNA obtained using RNAeasy Micro Kit (Qiagen), following the manufacturer's protocol. Triplicate quantitative real-time PCR reactions were performed for each sample, using transcript-specific primers (Qiagen). Minus reverse transcriptase (-RT), minus template, and minus primer controls were included to ensure lack of signal in the assay background. Melting curve analyses were performed to test for the specificity and quality of the qPCR amplifications. Relative expression was calculated using the comparative threshold (ΔΔCT) method and normalized to the expression of GAPDH.

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CONCLUSION

Research collected over the past several decades has revealed multiple functions for neurotrophic factor trafficking and signaling. In the central nervous system, BDNF has been shown to be required not only for development, but the maintenance of the adult brain and its synaptic activity. Alterations in BDNF levels and function have been implicated in a host of various neurological disorders such as depression, obesity, AD, Down Syndrome, and Huntington's disease. These findings further strengthen the role that BDNF plays in overall neuronal function.

In this dissertation, I have presented evidence that altered processing of APP can directly modulate the ability of BDNF to elicit proper trafficking and signaling behavior. Previous work from our lab has shown that APP plays a critical role in the transport of nerve growth factor (NGF) in basal forebrain cholinergic neurons of a mouse model of Down Syndrome, providing initial evidence which implicates APP in the movement of a retrogradely transported cargo. This was consistent with other studies that have also indicated that overexpression of APP and/or various processed fragments of APP, can elicit trafficking phenotypes on a variety of cargoes.

Given that the majority of these studies have been limited to either mouse models of the disease, in which no temporal control of APP expression can be obtained, or in cultured neuronal systems, in which APP and/or its fragments are overexpressed, we believed it was beneficial to try endogenous manipulation of the APP processing system using small molecule drugs. By using gamma-secretase inhibitors as a means to pharmacologically increase APP C-terminal fragments (APP

CTFs) while significantly decreasing smaller Aβ species, we discovered several key findings directly specifically at APP CTF accumulation. Briefly, we found that APP CTF accumulation through inhibition of gamma-secretase was capable of reducing BDNF trafficking and signaling, both in axons and mass cultures. Furthermore, we provided evidence that BDNF vesicles themselves contain increased amounts of APP and/or APP CTF while also recruiting increased amounts of the anterograde motor protein subunit KLC1. The model we present raises the possibility that changes in APP processing can directly influence the motility of retrograde axonal signals through influencing the amount of motor protein that is recruited to the same vesicle.

This data is consistent with the "tug-of-war" model of bidirectional transport, which states that changes in the ability of anterograde and retrograde motors to initiate movement can dramatically influence net processivity. A simplistic interpretation of our data would suggest that increased levels of kinesin, potentially through increased APP and/or APP CTF, would be sufficient to disrupt normal, retrograde transport and cause either stalling or switch in directionality. Transport, however, is much more complex than simply motor loading, and so the data presented in this dissertation set the groundwork for future studies in this field which ask the questions: 1) How do changes in motor loading influence the activity of a motor? 2) Do changes in motor loading influence microtubule binding affinities? and, 3) Do changes in motor recruitment lead to abnormal recruitment and retention of other adaptors and cargo receptors and how do those work to overall influence net flux direction?

Answering these questions, particularly in axons where trafficking of cargoes is extremely regulated, will require advancements in both technology and an understanding of all proteins and cargoes involved. Even more work will be required to apply the answers to these fundamental questions to the pathophysiology of diseases in which APP CTF load is increased like that in familial Alzheimer's disease and in Down syndrome.

Particularly for BDNF, the main neurotrophic factor in the central nervous system, little work has been done to understand the properties and trafficking movements of this retrograde cargo and therefore the findings here are some of the first insights into the composition of a BDNF retrograde trafficking vesicle and how that composition can be manipulated during the pathology of a disease. Given that the BDNF trafficking vesicle is ordinarily composed of signaling proteins and scaffolding proteins related to signaling, experiments which delineate exactly what changes in protein composition on a vesicle that has an abundance of APP will be important. It is possible that the recruitment of APP also facilitates the recruitment of APP-specific scaffolding and receptor proteins. This may work to essentially compete away proper placement or complexing of BDNF:TrkB proteins, leaving signaling abnormal either initially during formation or during long-term transport. Any kinase or signaling activity of TrkB and its downstream signaling proteins, if present on the BDNF trafficking vesicles following APP CTF accumulation, could actually work to abnormally regulate APP's recruited components. APP has been shown to be regulated by phosphorylation in the internalization domain, very near the C-terminal

end. Furthermore, TrkA has been shown to be able to bind and phosphorylate APP. If this is true for TrkB as well, then the presence of TrkB and APP on a vesicle would lead to very abnormal signaling patterns and would have the potential to modulate activity of several adaptor and scaffolding proteins specific to either TrkB or APP.

A separate issue which could also be at play is that poor trafficking of BDNF vesicles, along with poor trafficking of other organelles could result in premature degradation of the TrkB signal as the BDNF vesicle encounters phosphatases or other endosomes later in the endocytic pathway earlier than expected due to a global trafficking deficit. If this were to be true it would mean that not only can accumulation of APP CTFs cause direct problems for a retrograde BDNF vesicle by modulating protein recruitment to the vesicle, but that it can also cause global trafficking dysfunction as well.

Overall, the results from this study prevent novel ideas in the field of retrograde neurotrophin signaling in terms of its trafficking patterns and the proteins involved. The results also have impact for the field of AD in which altered APP processing can lead to neuronal dysfunction, apart from Aβ accumulation.