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β-amyloid impairs axonal BDNF retrograde trafficking

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Abstract

The neurotrophin, brain-derived neurotrophic factor (BDNF), is essential for synaptic function, plasticity and neuronal survival. At the axon terminal, when BDNF binds to its receptor, tropomyosin-related kinase B (TrkB), the signal is propagated along the axon to the cell body, via retrograde transport, regulating gene expression and neuronal function. Alzheimer disease (AD) is characterized by early impairments in synaptic function that may result in part from neurotrophin signaling deficits. Growing evidence suggests that soluble beta-amyloid (Aβ) assemblies cause synaptic dysfunction by disrupting both neurotransmitter and neurotrophin signaling. Utilizing a novel microfluidic culture chamber, we demonstrate a BDNF retrograde signaling deficit in AD transgenic mouse neurons (Tg2576) that can be reversed by γ-secretase inhibitors. Using BDNF-GFP, we show that BDNF-mediated TrkB retrograde trafficking is impaired in Tg2576 axons. Furthermore, Aβ oligomers alone impair BDNF retrograde transport. Thus, Aβ reduces BDNF signaling by impairing axonal transport and this may underlie the synaptic dysfunction observed in AD.

Keywords

β-amyloid; Alzheimer; BDNF; neurotrophin; axonal transport; microfluidic chamber

1. Introduction

Alzheimer disease is defined pathologically by the accumulation of extracellular Aβ plaques and intracellular neurofibrillary tangles, which accompany synaptic and neuronal loss in the AD brain. While Aβ plaque accumulation is a clear risk factor associated with AD, cognitive decline precedes plaque pathology. Studies now suggest that soluble and/or oligomeric Aβ can cause synaptic deficits and correlate more closely with cognitive dysfunction than Aβ plaque load (Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000).

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Coincidentally, Aβ induces synaptic deficits similar to those observed in $BDNF^{-/-}$ and $TrkB^{-/-}$ mice. For example, soluble Aβ oligomers dramatically impair hippocampal long-term potentiation (LTP) and AD transgenic mice exhibit synaptic loss, impaired hippocampal LTP, and impaired hippocampal-dependent learning (Hsiao et al., 1996; Hsia et al., 1999; Mucke et al., 2000; Walsh et al., 2002; Cleary et al., 2005; Lesne et al., 2006). Thus, Aβ may impair brain function by disrupting BDNF signaling, which mediates neuronal plasticity involved in learning and memory (Tong et al., 2004; Garzon and Fahnestock, 2007). Supporting this notion, BDNF levels are reduced in pre-clinical stages of AD (Peng et al., 2005). BDNF enhances excitatory synaptic transmission, long-term synaptic plasticity, and hippocampal LTP, an analogue of learning (Kang and Schuman, 1995; Levine et al., 1995; Figurov et al., 1996; Akaneya et al., 1997; Gottschalk et al., 1998; Messaoudi et al., 1998). Reduced BDNF signaling leads to hippocampal and cortical synaptic plasticity deficits. Mice lacking either BDNF or TrkB exhibit impaired arborization, deficits in synaptic sprouting, decreased synapse number, impaired hippocampal LTP, impaired hippocampal-dependent learning, and cortical degeneration (Korte et al., 1995; Patterson et al., 1996; Causing et al., 1997; Martinez et al., 1998; Minichiello et al., 1999; Pozzo-Miller et al., 1999; Mizuno et al., 2000; Xu et al., 2000; Genoud et al., 2004).

When BDNF binds to TrkB, the phosphoinositide-3-kinase (PI3K), Ras/MAPK, and PLCγ/PKC pathways are activated. Signaling involves both local activation and long-distance retrograde transport of the BDNF/TrkB signal (Du and Poo, 2004). For axonal retrograde transport, TrkB is internalized into signaling endosomes that require the dynein motor protein to activate downstream signaling cascades (Grimes et al., 1996; Watson et al., 1999; Watson et al., 2001; Delcroix et al., 2003; Heerssen et al., 2004). The PI3K pathway plays a critical role in retrograde trafficking (Nielsen et al., 1999) and because Aβ interferes with BDNF-mediated PI3K activation (Tong et al., 2004), but not TrkB phosphorylation, we hypothesized that Aβ affects axonal retrograde transport and the downstream propagation of TrkB signaling.

Here, we directly tested this hypothesis. We have developed a microfluidic chamber that allows central nervous system (CNS) axons to be fluidically isolated to a compartment in which molecules, such as BDNF, can be selectively applied (Taylor et al., 2005). Using this chamber, we identified impaired TrkB retrograde processing in the axons of AD transgenic mouse (Tg2576) neurons. Furthermore, we found that Aβ oligomers cause these transport deficits. Therefore, in AD, transport deficits might underlie neuronal dysfunction and synaptic loss.

2. Materials and Methods

2.1. Assembly of microfluidic culture chambers

The chamber was fabricated in PDMS using rapid prototyping and soft lithography similar to previously published procedures (Taylor et al., 2003). Glass coverslips (24×40mm2, No. 1, Corning Inc., New York, NY), sonicated in 95% EtOH (30 min), and dried in a sterile hood, were immersed in sterile aqueous solution (0.5 mg/ml poly-L-lysine (Sigma, St. Louis, MO)) in PBS (24 hr, 5% CO_2, 37°C incubator), rinsed, and dried in a sterile hood overnight. The chambers were noncovalently assembled by conformal contact. The chambers consist of two parallel microfluidic channels, both connected by inlet and outlet wells. These two channels or compartments are separated by a solid barrier region with >150 microgrooves embedded in the bottom of the connecting barrier. A slight volume difference between the two channels (40 μl) was used to generate a fluidic resistance within the microgrooves, facilitating the isolation of BDNF to axons.

2.2. Cell culture of primary neurons

All procedures were performed under an IUCAC approved protocol. Primary cortical neuron cultures were derived from embryonic rat (E18) or mice (E16) as previously described (Loo et al., 1993; Cribbs et al., 1996). Briefly, dissected tissue was dissociated with trypsin, triturated, and plated in microfluidic chambers fitted with poly-L-lysine coated glass coverslips in serum-free Neurobasal supplemented with B27 (Invitrogen, Carlsbad, CA). Cells were plated at a density of 5×10^6 cells/ml and maintained in cultures until used.

Tg2576 neurons were derived from crossing Tg2576 mice containing the APP (amyloid precursor protein) double mutation K670N, M671L driven by the hamster prion promoter to B6SJL F1 mice (Jackson Labs, Bar Harbor, ME). The presence or absence of the human APP gene was demonstrated in individual pups by DNA genotyping. After DNA extraction, PCR analysis was performed using primers APP1502: 5′ - CTG ACC ACT CGA CCA GGT TCT GGG T - 3′ and APP1503: 5′ - GTG GAT AAC CCC TCC CCC AGC CTA GAC CA - 3′ as previously described (Hsiao et al., 1996). Non-transgenic littermates of the Tg2576 mice (APP) were used as the source of wild-type (WT) neurons. Neuronal purity was assessed by immunostaining with a mouse monoclonal β-III-tubulin (1:1000, Chemicon, Temecula, CA) and rabbit polyclonal GFAP (Glial Fibrillary Acidic Protein) (1:4000, DAKO, Denmark). Glial contamination was 4% (n=6). To block Aβ production, neurons were treated with γ-secretase inhibitor IX (1 μM, Calbiochem) overnight.

2.3. Purification of BDNF-GFP

Endotoxin-free BDNF-GFP plasmid (generous gift from Dr. Masami Kojima, was introduced by nucleofection (Amaxa, Gaithersburg, MD) into HEK cells followed by selection in DMEM containing 10% FBS and G418 (1mg/ml, plasmid contains a neomycin cassette). BDNF-GFP was isolated from stably transfected pre-pro-BDNF-GFP HEK293 cells as follows: After cells reached confluency, secreted pro-BDNF-GFP from the media was removed and concentrated with Amicon YM-30 centrifugal filters (5000g, 2 hr) (30,000 MW cutoff, Millipore, Billerica, MA). Pro-BDNF was converted to mature BDNF-GFP by treatment with plasmin (Sigma, St. Louis, MO) as previously described (Pang et al., 2004). Mature BDNF-GFP was further purified by size exclusion chromatography (Amicon YM-50) where the flow-thru contained the protein of interest. BDNF-GFP is indistinguishable from BDNF both biochemically and biologically (Hartmann et al., 2001; Kohara et al., 2001), and we confirmed that our purified BDNF-GFP was biologically active. To assess biological activity, BDNF-GFP (20 ng/ml) was added to rat primary neurons and the BDNF-mediated activation of CREB (phosphorylation) was examined and compared to commercially available BDNF (50 ng/ml, Peprotech, Rocky Hill, NJ). The BDNF-GFP concentration was determined by BDNF ELISA (Promega, Madison, WI).

2.4. Synthesis of Aβ oligomers

Oligomers were made as previously described (DeFelice et al, 2007). Briefly, Aβ aliquots that were lyophilized as a HFIP film (Chemicon, Temecula, CA) were stored at −80°C until used. Aβ was dissolved in neat, sterile DMSO (5 mM) and diluted in phosphate buffered saline (PBS), pH 7.4 to 100 uM and aged overnight (4°C), centrifuged (14, 000 × *g*, 10 min, 4°C) and the supernatants transferred to fresh eppendorf tubes and stored at 4°C until use. Confirmation of Aβ oligomers was carried out by Western analysis. Samples were diluted with Tricine sample buffer, separated on 10% Tris-Tricine gels, transferred to nitrocellulose, and Aβ oligomers were visualized following incubation with 6E10 and appropriate anti-mouse HRP conjugated goat secondary with SuperSignal West Pico (Pierce, Rockford, IL). To assess the toxicity of Aβ oligomers, treated cultures (24h) were stained with TOTO-3 and the percentage of apoptotic nuclei was quantitated.

2.5. Immunocytochemistry

Fluorescent immunocytochemistry was conducted as described previously (Blurton-Jones et al., 2004). In brief, cells were rinse with PBS and then paraformaldehyde fixed (4%) following either BDNF or BDNF-GFP treatment (2h), permeabilized in 0.25% Triton X-100 in PBS, (pH 7.4) and blocked with 5% goat serum. Cells were incubated in appropriate primary antibody overnight at 4°C. Following 3 rinses in PBS, cells were incubated in appropriate fluorescently-conjugated secondary antibodies (Alexa Fluorophores, Invitrogen, Carlsbad, CA). Imaging was carried out on a BioRad Radiance 2100 confocal system using lambda strobing mode to avoid non-specific cross-excitation or cross-detection of fluorophores. Antibodies used include rabbit polyclonal anti-TrkB (1:400, Upstate,), monoclonal anti-Rab7 (1:100, Sigma, St. Louis, MO), rabbit polyclonal anti-GFP (1:1000, Abcam, Cambridge, MA), TOTO-3 (1:500, Invitrogen, Carlsbad, CA), tau-1 (1:1000, Chemicon, Temecula, CA).

2.6. Time lapse microscopy

Wild-type or Tg2576 neurons (7DIV) were imaged using an inverted BioRad Radiance 2100 confocal microscope and a 60x oil emersion objective. Regions of interest (ROI) from 5 axon segments from each chamber were randomly selected for time-lapse imaging. Images were acquired every 5 sec for a total of 60 images (5 min). To determine rates of BDNF-GFP translocation in axons, kymographs were generated from the image stack of each time-lapse experiment. The velocity of each BDNF-GFP containing endosome was determined in each kymograph, and a statistical comparison of wild type versus Tg2576 was performed using a Student's paired *t* test.

2.7. Quantification of Rab7/TrkB co-localization

Axons were double-stained for Rab7 and TrkB following BDNF treatment (at 10 or 60 min). The extent (pixel counts) of co-localization (yellow) was quantified using Image J (NIH). The values for the co-localization were normalized to axon area. Axon area was determined by manually tracing axons in each ROI and quantifying the area (pixel counts) using Image J.

2.8. Quantification of BDNF-GFP

BDNF-GFP was added to the axonal compartment. After 2h, somal compartments were analyzed for BDNF-GFP transport. For Western analysis, media and projecting axons were first removed by aspiration. Then, neurons from each somal chamber were lysed in lysis buffer (BD Biosciences, San Jose, CA) supplemented with protease inhibitors; BDNF-GFP was immunoprecipitated with rabbit anti-GFP antibodies (Invitrogen, Carlsbad, CA) and then analyzed by Western blot with a mouse anti-GFP antibody (Invitrogen, Carlsbad, CA). Bands corresponding to BDNF-GFP were quantitated using Image J software (NIH). For immunocytochemical analysis, GFP was visualized with rabbit anti-GFP (Abcam, Cambridge, CA) followed by anti-rabbit Alexa-488 secondary antibodies. Cells were washed and then immuno-labeled with Toto-3 (Invitrogen, Carlsbad, CA) to identify nuclei. For each chamber, 3 ROIs were taken using the same settings, for each ROI, 5 random areas were chosen and GFP labeling was quantitated using Image J software (NIH). The mean pixel intensity for each area was then determined.

3. Results

3.1. Axonal TrkB processing is impaired in Tg2576 neurons

A microfluidic chamber has been developed that allows for the study of axonal biology including transport within CNS neurons (Taylor et al., 2005) (Figure 1A). The chamber

isolates axons by taking advantage of their ability to grow faster and longer than dendrites, does not require exogenously added neurotrophins, and also allows for the selective manipulation of axonal microenvironments. These chambers were employed to examine whether Tg2576 neurons exhibit axonal deficits. To study axonal function, we examined axonal TrkB processing following BDNF treatment in 7DIV neurons. After the addition of BDNF to only the axonal compartment, we found that TrkB levels in Tg2576 neurons (0.533±0.04) was significantly higher than wild-type (WT) neurons after 2h (0.248±0.04, $p<0.0001$) (Fig. 1C and 1D). TrkB levels (green) were normalized to the axonal marker, Tau-1 (red), to correct for the variation in axon numbers that project through each microgroove. In 7DIV neurons, Tau-1 levels are not altered between WT and Tg2576 neurons (Figure 1B) and are unaffected by the addition of BDNF (Supplemental Figure S1A and S1B). Prior to BDNF addition, TrkB levels within axons of Tg2576 neurons were similar to WT neurons (0.840±0.05 vs. 0.812±0.05, respectively, Supplementary Figure S1C). Impaired axonal TrkB processing was still observed when axonal TrkB levels were normalized to another axonal marker, BIII-tubulin that is also unaffected by BDNF (Supplemental Figure S2A and S2B). Furthermore, increased TrkB levels in Tg2576 neurons was not due to increased TrkB synthesis as cycloheximide-pre-treatment (100 μM, 1h prior to BDNF treatment) did not decrease TrkB levels in Tg2576 neurons. TrkB levels with cycloheximide pre-treatment were 106±4.5% of BDNF only controls (n=5).

Because Aβ interferes with BDNF-mediated PI3K activation (Tong et al., 2004), and because the PI3K pathway plays a critical role in retrograde trafficking (Nielsen et al., 1999), we hypothesized that in Tg2576 neurons that produce Aβ, axonal retrograde transport and the downstream propagation of TrkB signaling may be affected that would explain the increased TrkB levels in Tg2576 axons following BDNF treatment. Furthermore, impaired TrkB sorting and/or degradation that is present in Tg2576 neurons (Almeida et al., 2006) would also disrupt axonal retrograde flow. Thus, we examined whether TrkB axonal retrograde transport was impaired in Tg2576 neurons.

3.2. BDNF-mediated TrkB retrograde transport is impaired in Tg2576 neurons

Initially, to measure TrkB retrograde transport, we determined the extent to which the co-localization of Rab7 and TrkB changed within proximal and distal axons of both WT and Tg2576 neurons over time following BDNF treatment (Figure 2A). In mouse primary neurons, puncta containing co-localized Rab7 (green), a late endosomal protein involved in retrograde transport (Rink et al., 2005; Deinhardt et al., 2006), and TrkB (red) were quantitated within proximal and distal axons following BDNF treatment (Figure 2B). An increase in this ratio over time in proximal axons is indicative of TrkB transport (Deinhardt et al., 2006). The change in co-localization between 10 and 60 min was determined. In proximal axons, we found a significant increase in Rab7/TrkB co-localization over time (88.0% ± 25.1%, $p<0.05$, n=4), which was comparable to previous studies (Deinhardt et al., 2006)(Figure 2B). However, in Tg2576 neurons, Rab7/TrkB co-localization showed a miniscule increase over this same time period that was not significant (1.3%± 26.7% (n=5)). Conversely, in distal axons where TrkB is initially internalized to be transported, Rab7/TrkB co-localization significantly decreased by 36.7%±10.1% ($p<0.05$, n=3) in WT neurons. In contrast, Tg2576 distal axons exhibited a 19.6%.±10.2% (n=3) increase in Rab7/TrkB co-localization, although this change was not significant (Figure 2C). This result was suggestive of impaired retrograde transport of Rab7/TrkB-containing endosomes in Tg2576 neurons.

TrkB retrograde transport was further characterized using recombinant BDNF-GFP (BDNF fusion with green fluorescent protein at the C-terminus) that was isolated from the media of HEK-293 cells stably-expressing pro-BDNF-GFP. Both pro- and mature-BDNF-GFP are present in the media (Figure 3A), but plasmin-treatment produced predominantly mature

BDNF-GFP as determined by Western analysis. The calculated molecular weights of the pro- and mature-BDNF-GFP fusion proteins are approximately 62 kDa and 41kDa, respectively. Furthermore, antibodies to both GFP and BDNF recognized the band that migrated at approximately 41kDa. Next, the biologically activity of mature BDNF-GFP was assessed (Figure 3B and 3C). When m-BDNF-GFP (20 ng/ml) was added to rat primary neurons, BDNF-mediated activation of CREB (phosphorylation) was comparable to commercially available BDNF (50 ng/ml, Peprotech, Rockhill, NJ). Commercially available BDNF induced a 2.14±0.25-fold induction in phosphorylated CREB, while m-BDNF-GFP caused a 1.71±0.17-fold induction in phosphorylated CREB. Additionally, following m-BDNF-GFP treatment (Supplementary Figure S3), BDNF-GFP (anti-GFP, green) and TrkB (anti-TrkB, red) co-localized within structures resembling endosomes. Trk-containing endosomes carry cellular signals that are retrogradely transported within axons along dynein motors (Ye et al., 2003; Heerssen et al., 2004; Miaczynska et al., 2004; Howe and Mobley, 2005).

Within the microfluidic chamber, TrkB retrograde transport was then assessed by measuring the rate of axonal BDNF-GFP retrograde transport using time-lapse confocal microscopy. Live imaging revealed that axonally-applied BDNF-GFP is transported retrogradely in vesicles (Supplemental Movie 1). Representative kymographs in Figure 4A demonstrate BDNF-GFP vesicle retrograde transport is slower in Tg2576 when compared to WT. The vesicle velocity in WT neurons was in the range of fast axonal transport, albeit in the lower range (1-2 μm/sec). In contrast, vesicle velocities observed in Tg2576 axons were markedly reduced compared to BDNF-GFP-containing vesicles from WT axons (44.5±5.36% of WT, $p<0.005$) (Figure 4B). Next, we assessed the amount of BDNF-GFP that was transported to the somal side following BDNF-GFP axonal treatment (Figure 4C). Western blot analysis of somal lysates revealed a reduced BDNF-GFP signal from Tg2576 neurons when compared to WT neurons (Figure 4C). Somal BDNF-GFP levels in Tg2576 neurons was 63.4% of WT ($n=4$, $p=0.003$). This result further supports the notion that BDNF-TrkB retrograde trafficking is impaired in Tg2576 neurons. Immunofluorescent-labeling also confirmed that the accumulation of somal BDNF-GFP in Tg2576 neurons was impaired versus WT neurons (Figure 4D). Lastly, axonally-applied antibodies to TrkB, which mimic BDNF (Qian et al., 2006), also accumulate in the somal compartment to a lesser extent in Tg2576 versus WT neurons (data not shown). Taken together, these results suggest that BDNF-TrkB axonal retrograde transport is impaired in Tg2576 neurons. Because Tg2576 neurons accumulate human Aβ, these data suggest that Aβ disrupts axonal transport. However, a recent study found that APP overexpression can also cause transport deficits (Salehi et al., 2006). Therefore, we sought to determine the mechanism by which BDNF transport was impaired in Tg2576 neurons.

3.3. A secreted APP cleavage product impairs TrkB processing

First, we sought to determine whether APP overexpression might explain the impaired axonal TrkB processing in Tg2576 neurons. Tg2576 neurons overexpress APP (Hsiao et al., 1996) and its overexpression can result in transport deficits (Gunawardena and Goldstein, 2001; Salehi et al., 2006). Therefore, we examined the effect of γ-secretase inhibitors on TrkB processing in Tg2576 neurons. If APP overexpression directly caused the deficits, then inhibiting γ-secretase activity should not affect TrkB processing. Furthermore, by inhibiting γ-secretase activity, this should lead to increased APP-C-terminal fragments (APP-CTF). Salehi and co-workers found that transport deficits correlated with APP-CTFs levels in an APP trisomy model (Salehi et al., 2006), therefore, the prediction would be that γ-secretase inhibitors would further enhance the deficits. However, not only was there not an increase in the observed deficits, but TrkB processing deficits were ameliorated in Tg2576 neurons (Figure 1C and 1D) suggesting that, in Tg2576 neurons, the observed deficits required a γ-

secretase generated by-product, either Aβ or AICD (APP Intracellular domain). Of note, γ-secretase treatment did not affect TrkB levels prior to BDNF treatment in either WT or Tg2576 neurons (data not shown).

To further clarify whether the transport deficits were the direct result of an APP proteolytically-derived fragment, we examined whether WT neurons exposed to exogenous APP fragments within the microfluidic chambers exhibited TrkB transport deficits. For this study, WT neurons and their projecting axons were co-cultured with Tg2576 neurons that were placed in adjacent reservoirs, so that they were not in contact with the projecting axons, but acted as a source of APP fragments, which could readily diffuse and act on the WT axons (Figure 5). We observed that TrkB processing from WT neurons was impaired when they were co-cultured with Tg2576 neurons. The TrkB levels were higher in WT neurons that were co-cultured with Tg2576 neurons (0.904±0.06) when compared to WT neurons co-cultured with WT neurons (0.713±0.02). These results imply that a secreted factor was necessary to impair TrkB processing. Importantly, this data strongly suggested that the short-lasting intracellular AICD fragment was not responsible for impairing TrkB processing.

3.4. Aβ impairs TrkB retrograde transport

To directly examine whether Aβ mediates BDNF/TrkB retrograde signaling deficits, BDNF-GFP transport was assessed in the axons of WT neurons exposed to Aβ oligomers (Figure 6A). We chose to examine oligomers since increasing evidence suggests that oligomers are the earliest toxic species that act to impair neuronal function and synaptic plasticity (Lambert et al., 1998; Walsh et al., 2002). To assess BDNF retrograde transport, somal BDNF-GFP levels were measured following BDNF-GFP addition to the axonal compartment. We found that in WT neurons exposed to Aβ oligomers prepared *in vitro* (De Felice et al., 2007), the retrograde transport of axonally-applied BDNF-GFP was impaired (Figure 6B and 6C). Neurons exposed to Aβ oligomers demonstrated an approximately three-fold reduction in the accumulation of somal BDNF-GFP. Additionally, we quantitated the accumulation of somal BDNF-GFP over time by immunocytochemistry (Figure 6D-6F). BDNF-GFP levels as assessed with anti-GFP antibodies (Invitrogen, Carlsbad, CA) were strongly elevated (45.9±1.77 A.U.)) within WT neurons from the somal compartment following BDNF treatment (Figure 6D and 6E). However, in neurons that had been exposed to Aβ oligomers, the level of BDNF-GFP accumulation was greatly reduced (Figure 6F). Aβ oligomer treatment was not cytotoxic as <5% of nuclei within the microfluidic chamber were apoptotic. Taken together, these results strongly support the hypothesis that Aβ impairs BDNF-retrograde transport.

4. Discussion

Utilizing a novel microfluidic cell culture chamber that is ideally suited to study axonal biology, we show that Tg2576 neurons exhibit deficits in TrkB axonal processing. Importantly, these chambers allow for the fluidic isolation of microenvironments such that axons alone can be exposed to environments that are different from the cell body, replicating an important anatomical feature that occurs in the brain. We provide evidence that the impaired axonal processing is likely due to reduced retrograde transport of TrkB within axons. We found that the retrograde transport rate of BDNF-GFP vesicles and the accumulation of somal BDNF-GFP are significantly reduced in Tg2576 neurons. Furthermore, we demonstrate that exogenous Aβ exposure can lead to impaired TrkB processing and has a direct effect on BDNF retrograde transport. First, the use of γ-secretase inhibitors, which prevents the generation of Aβ, reverses the impairment in TrkB processing. Second, exogenous Aβ recapitulated the deficits in TrkB processing within the axons of wild-type neurons. Lastly, synthetic Aβ oligomers impaired the retrograde transport of

BDNF-GFP. Taken together, this study demonstrates a novel mechanism elucidating how Aβ downregulates BDNF signaling in AD by affecting BDNF retrograde trafficking.

BDNF has an important role in neuronal survival and in long-term potentiation (LTP) (Yuan and Yankner, 2000). Therefore, the downregulation of BDNF levels would impact cognition. In fact, in AD and mildly cognitively impaired (MCI) patients, BDNF mRNA and protein levels are reduced, and the decrease correlates with the severity of cognitive impairment (Phillips et al., 1991; Connor et al., 1997; Hock et al., 2000; Holsinger et al., 2000; Peng et al., 2005). Here, we found that in addition to lowering BDNF levels, amyloid impairs the BDNF-mediated retrograde signal. Neurotrophin retrograde signaling, including BDNF, from axon terminals to the soma has emerged as an important regulator of synaptic plasticity and survival (Howe and Mobley, 2005; Magby et al., 2006). When activated neurotrophin-bound receptor tyrosine kinases are endocytosed, the signal is propagated along the axon and toward the soma via signaling endosomes (Howe and Mobley, 2005; Zweifel et al., 2005). Therefore, in AD, transport deficits may contribute to cognitive decline and cell loss. In addition to AD, accumulating evidence suggests that transport deficits may play a role in the development or progression of other neurodegenerative diseases (Hall et al., 2000; Uemura et al., 2004; Salehi et al., 2006; Stokin and Goldstein, 2006).

Aβ can activate the GSK-3β kinase and this may underlie deficits in trafficking (Busciglio et al., 1995; Takashima et al., 1996; Zheng et al., 2002; Hoshi et al., 2003; Kim et al., 2003; Liu et al., 2004). GSK-3β phosphorylates kinesin-containing membrane-bound organelles (MBOs), releasing them from microtubules and thereby impairing anterograde transport (Morfini et al., 2002). However, our findings show a deficit in BDNF/TrkB retrograde transport and therefore, suggest the involvement of dynein, the motor protein responsible for the retrograde transport of BDNF/TrkB (Heerssen et al., 2004). To our knowledge, we are not aware of a mechanism by which Aβ acts directly on dynein. However, axonal swellings that occur prior to plaque deposition in APP^{Swe} mice might contribute to impair retrograde signaling (Stokin et al., 2005). Of note, we have observed similar varicosities in Tg2576 neurons in culture (data not shown).

The decrease in proximal Rab7/TrkB levels was also indicative of a transport defect. However we cannot exclude the possibility that deficits in endosomal sorting might contribute to the failure of BDNF/TrkB to initially reach the proper endosomal compartment required for functional transport. In these same neurons, the endosomal sorting of another cell surface receptor, EGFR, was defective (Alwan et al., 2003; Almeida et al., 2006).

Presenilin 1 (PS1) deficiency affects TrkB maturation (Naruse et al., 1998), but we did not detect differences in baseline axonal TrkB levels following γ-secretase inhibitor treatment (data not shown). Therefore, the inhibitors likely affected the fate of TrkB levels following BDNF treatment. However, we cannot rule out the possibility that by inhibiting γ-secretase activity we indirectly affected transport by impairing kinesin-mediated anterograde transport and therefore enhanced dynein-mediated retrograde transport. FAD-linked PS1 mutations have been shown to have deficits in anterograde transport (Pigino et al., 2003; Lazarov et al., 2007). Presenilin 1 (PS1), a component of the γ-secretase complex, may play a direct role in kinesin-based transport (Kamal et al., 2001; Cai et al., 2003; Sheng et al., 2003).

In Down's transgenic mice (Ts65Dn), neurotrophin retrograde trafficking is impaired (Salehi et al., 2006). Down's patients who suffer from a chromosomal 21 triplication (APP is located on chromosome 21) exhibit pathological features of AD (Wisniewski et al., 1985). Furthermore, duplication of the APP locus causes early-onset AD (Rovelet-Lecrux et al., 2006). While the accumulation of APP-CTFs correlated with axonal transport deficits in

Ts65Dn (Salehi et al., 2006), we found that γ-secretase inhibitors reversed the deficit suggesting that increased APP-CTF levels were not responsible for the transport deficits in Tg2576 neurons. Also, in contrast to Salehi and co-workers, we found that impaired retrograde trafficking in Tg2576 neurons was likely the result of Aβ. It is important to note that a critical difference between the TS65Dn model and Tg2576 mice utilized here is that the Ts65Dn mice express three copies of rodent APP. Thus, the Aβ that is generated in Ts65Dn mice is considerably less pathogenic than the human Aβ generated by Tg2576 neurons. Co-culture studies suggested that APP overexpression was not responsible for the TrkB deficits in Tg2576 neurons. Additionally, conditioned media from the 7PA2 CHO cell line (Cleary et al., 2005), which secrete Aβ, but not conditioned media from WT CHO cells also impairs TrkB processing (data not shown). Importantly, the deficits in BDNF retrograde transport were reproduced in wild-type neurons exposed to synthetic Aβ oligomers. In summary, there are numerous differences that might explain the disparity between our findings and those of Salehi and co-workers: we examined BDNF and not NGF transport; we examined transport in cortical neurons versus basal forebrain cholinergic neurons; and we used Tg2576 versus Ts65Dn mice.

Aβ can accelerate the accumulation of tau, a microtubule-associated protein, which deposits in AD brain as hyperphosphorylated aggregates or neurofibrillary tangles (Gotz et al., 2001; Lewis et al., 2001; Gamblin et al., 2003; Oddo et al., 2003a; Oddo et al., 2003b; Oddo et al., 2004; Rissman et al., 2004). Because the velocity of BDNF-GFP-containing vesicles is reduced in Tg2576 neurons, it suggests that Aβ/Aβ oligomers may target the molecular motors required for transport including tau. Interestingly, tau overexpression leads to axonal transport deficits (Ebneth et al., 1998; Stamer et al., 2002). Thus, the accumulation of tau and the development of AD tau pathology may contribute to diminished axonal flow leading to neurodegeneration (Terry, 1998). Supporting this hypothesis, axonal degeneration occurs in human tau overexpressing transgenic mouse brain (Spittaels et al., 1999). However, in 7DIV neurons, we did not detect any changes in the Tau-1 epitope, but cannot rule out the possibility that tau may still play a role as we did not assess all possible Aβ-induced tau modifications.

Aβ oligomers, which have been found to accumulate along microtubules within neuronal processes (Takahashi et al., 2004), can also induce tau hyperphosphorylation, a key step in the development of neurofibrillary tangles (De Felice et al., 2007). The binding of tau to microtubules is regulated by phosphorylation, and in general, is decreased by phosphorylation (Drechsel et al., 1992; Biernat et al., 1993; Bramblett et al., 1993; Alonso et al., 1994; Davis and Johnson, 1999; Evans et al., 2000) and tau isolated from AD brain does not bind to microtubules (Grundke-Iqbal et al., 1986; Bramblett et al., 1992; Yoshida and Ihara, 1993). Interestingly, tau phosphorylation alleviates deficits in kinesin-based cargo transport (Mandelkow et al., 2004). Therefore, phosphorylation may restore certain microtubule functions i.e. kinesin-based transport via disassociating tau from microtubules and therefore may initially serve a neuroprotective role (Rapoport et al., 2002). However, enhancing anterograde transport may come at the expense of retrograde transport. Hence, tau phosphorylation may regulate microtubule dynamics and influence microtubule-based axonal transport in both directions. In AD, this intricate balance may be disrupted leading to tau hyperphosphorylation, tangle formation, and neurodegeneration (Alonso et al., 2001) Of note, Aβ may also affect the relative abundance of the different tau isoforms within axons which would further influence axonal transport (Ebneth et al., 1998; Trinczek et al., 1999; Vershinin et al., 2007). It is interesting to speculate that the lack of cognitive deficits in tau-deficient AD transgenic mice (Roberson et al., 2007) may be due to an absence of tau-mediated disruption of axonal transport. Also, it may be soluble forms of tau that underlie the cognitive decline and not neurofibrillary tangles (Santacruz et al., 2005)

Our results contribute to an emerging literature on the significance of failed neurotrophin transport in Alzheimer disease. In addition to failed BDNF axonal trafficking in AD models, BDNF trafficking is affected in other neurodegenerative disease models including Parkinson's and Huntington's (Gauthier et al., 2004; Devon et al., 2006), which suggests that there may be a common mechanism of disrupted neurotrophin transport among neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer disease
Aβ	β-amyloid
APP	amyloid precursor protein
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
LTP	long-term potentiation
PI3K	phosphoinositide-3-kinase
PS1	Presenilin-1
Rab7	Ras-related GTP-binding protein 7
Tg2576	AD transgenic mouse line
WT	wild-type

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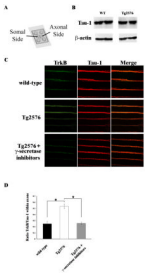

Figure 1.

TrkB axonal processing is impaired in Tg2576 neurons**. (A)** Depicted is a schematic of the microfluidic chamber used to facilitate the study of axonal TrkB processing in CNS neurons. The neurons are plated within the somal compartment and axons grow through the microgrooves and into the axonal compartment. A volume difference between the somal and axonal compartment (40 μl) was used to generate a fluidic resistance within the microgrooves, isolating BDNF treatment to just axons. **(B)** Levels of Tau-1 are not altered in Tg2576 neurons when compared to wild-type (WT) neurons. β-actin served as a loading control. **(C)** Representative images showing TrkB (green) and Tau-1 (red) levels in axons after axonal BDNF treatment (2h). Tau-1, an axonal marker was used to normalize the TrkB labeling found in each microgroove since the number of axons that projected through each varied. (**D**) Quantitation of TrkB within axons following BDNF axonal treatment in wild-type neurons, in Tg2576 neurons and in Tg2576 neurons pre-treated with γ-secretase inhibitors. The data are presented as mean± SEM. * denotes $p<0.0001$.

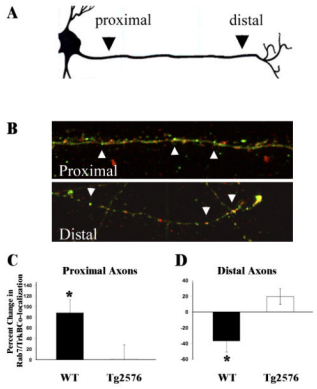

Figure 2.

Retrograde transport of TrkB/Rab7-positive endosomes is impaired in Tg2576 neurons. **(A)** The co-localization between TrkB and Rab7 was determined in proximal and distal axons at both 10 and 60 min. **(B)** In axons from mouse primary neurons, co-localization (yellow) between Rab7 (green) and TrkB (red) can be found within puncta in both proximal and distal regions. Representative images from both axonal regions are depicted. The percent change in Rab7/TrkB co-localization between 60 min and 10 min in proximal **(C)** and in distal **(D)** axons was determined and was used as an indirect measure of BDNF transport. The data are presented as mean± SEM. The percent change in the co-localization within both proximal and distal axons of WT neurons was significant (*)($p<0.05$, n=4 and n=5, respectively). In the case of Tg2576 neurons, the percent increase of co-localization in proximal axons was reduced when compared to WT (88.0% increase vs. 1.3%) and was not significant (**). As TrkB undergoes retrograde transport, an expected decrease in TrkB/Rab7 co-localization is observed in the distal axons of WT neurons (−36.7%), while we observed an increase in the distal axons of Tg2576 (19.6%), but was also not significant.

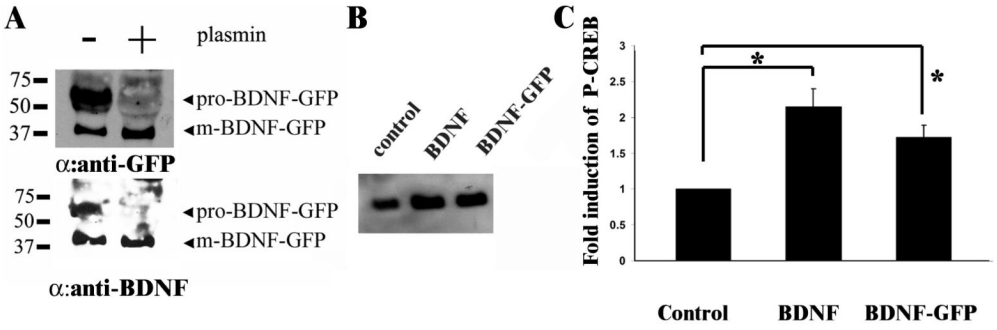

Figure 3.

Purified BDNF-GFP is biologically active. **(A)** pro-BDNF-GFP was sequentially purified as described in Methods and converted to mature-BDNF-GFP with plasmin treatment (1 h, 37°C). Plasmin treatment converted pro-BDNF-GFP to mature BDNF-GFP as determined by antibodies to both GFP (top) and BDNF (bottom), respectively. **(B)** Mature-BDNF-GFP was added to rat primary neurons to assess biological activity. After treatment (20 min), cells were lysed and lysates were analyzed by Western blot analysis and probed with a phospho-CREB-specific antibody. A representative blot is shown. **(C)** Quantification of p-CREB following BDNF treatment using ImageJ Software (NIH). BDNF (50 ng/ml) showed over a two-fold increase in p-CREB. BDNF-GFP also showed a robust induction of p-CREB. $p<0.01$, $n=3$. The data are presented as mean± SEM.

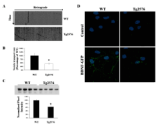

Figure 4.

The retrograde transport of BDNF-GFP is reduced in Tg2576 neurons. **(A)** Representative kymograph of BDNF-GFP vesicles within axons of WT (top) and Tg2576 (bottom) neurons. The time arrow represents 5 min. and the retrograde arrow indicates 65.2 μm. **(B)** Individual BDNF-GFP vesicle velocities were assessed using ImageJ (NIH). The average velocity of BDNF-GFP vesicles was significantly reduced in Tg2576 neurons compared to WT (*p<0.005, n=37 for WT, n=24 for Tg2576). **(C)** Lysates from somal compartments were immunoprecipated with rabbit anti-GFP antibody (Invitrogen) followed by Western blot analysis with mouse anti-GFP antibodies and revealed a BDNF-GFP band that was decreased in Tg2576 somal lysates when compared to WT. Quantification of BDNF-GFP on the somal side demonstrated a significant reduction (*p=0.003, n=4) in net BDNF-GFP transport in Tg2576 vs WT neurons. **(D)** Immunocytochemical analysis of Tg2576 and WT somal compartments. In the absence of BDNF (Control, Top panels), BDNF-GFP-labeling was not detected in somal chambers, but after 2hr, BDNF-GFP levels were increased in the somal compartment of WT, but to a lesser extent in Tg2576 (Lower panels).

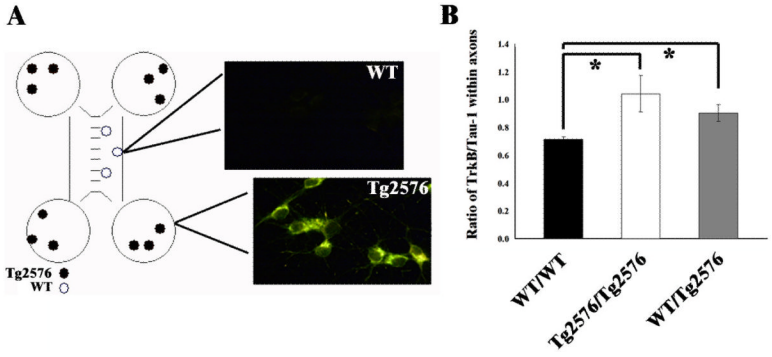

Figure 5.

An APP cleavage product impairs TrkB processing. **(A)** The microfluidic chamber was used to co-culture one-type of neuronal population with another. For example, wild-type (WT) can first be plated in the chambers followed by the addition of Tg2576 neurons in the reservoirs where they can grow and survive but do not come in contact with the neurons in the chamber. However, the neurons within the reservoirs can secrete soluble factors (i.e. Aβ) that can readily diffuse and act on the neurons within the chambers. In this example, neurons located within the chambers were fixed and stained with 6E10 to validate that no Tg2576 neurons from the reservoirs diffused into and contaminated the chambers. (**B**) Axonal TrkB processing was determined from co-cultures and quantitated (WT/WT-wild-type neurons in chambers and wild-type neurons in the reservoirs; Tg2576/ Tg2576- Tg2576 neurons in the chambers and Tg2576 neurons in the reservoirs; WT/ Tg2576-wild-type neurons in the chambers and Tg2576 neurons in the reservoirs). The data are presented as mean± SEM (*$p<0.01$, $n=4$).

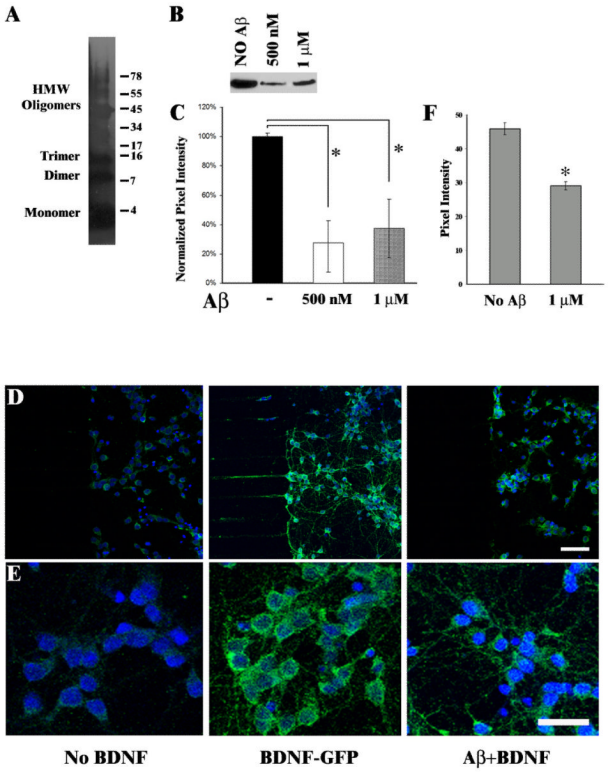

Figure 6.

Aβ oligomers impair BDNF retrograde transport. (**A**) Representative oligomer preparation. Prepared oligomers were separated by SDS-PAGE and analyzed by Western using 6E10 to confirm that the protocol yielded oliogomers. The molecular weight of bands that correspond to monomers, dimers, and trimers, and higher molecular weight (HMW) oligomers are identified. (**B**) Representative Western blot analysis of lysates isolated from somal compartments examining BDNF-GFP levels with an anti-GFP antibody. Aβ oligomers impair the accumulation of somal BDNF-GFP. (**C**) Quantitation of somal BDNF-GFP revealed that Aβ oligomers significantly impaired the accumulation of BDNF-GFP. The data are presented as mean± SEM. *$p<0.03$, n=3. (**D**) Immunocytochemical analysis of BDNF-GFP within the somal compartment following BDNF-GFP (2hr) near the barrier where axons enter the microgrooves. Scale bar = 60 microns. (**E**) Higher magnification showing individual neurons. Scale bar=30 microns. (**F**) Immunocytochemical quantification of BDNF-GFP in the somal compartment. The data are presented as mean± SEM. *$p<0.0001$, n=55 for no Aβ and n=45 for the 1 μM Aβ treatment.