UNIVERSITY OF CALIFORNIA,
IRVINE

The role of neuropathology on brain-derived neurotrophic factor endosomal transport: Underlying biological mechanisms of Neurological Disorders

DISSERTATION

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for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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DEDICATION

To

Mom and Dad…who supported

To

my Abuelos…who encouraged

To

Grandma…who inspired

and

To my wife

Caitlin…who saw it in me

for all your sacrifice, love and support,

this is dedicated to you.

May we never forget to remember their love when they can no longer remember ours

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Abstracts Published

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Carlos AJ, Aguilar BL, Cotman CW. Mechanisms of Neurotrophic Resistance in Alzheimer's Disease by the "IL-1 Effect and Potential Restorative Effects of Neural Stem Cell Based Therapy. Second Annual Meeting for UC Irvine School of Medicine Clinical, Basic, and Translational Science Festival, Irvine CA 2013

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ABSTRACT OF THE DISSERTATION

The role of neuropathology on brain-derived neurotrophic factor endosomal transport: Underlying biological mechanisms of Neurological Disorders

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Accumulation of Aβ and chronic inflammation are hallmark neuropathological findings that define Alzheimer's Disease. Both Aβ and pro-inflammatory cytokines have been linked to poor cognitive function, likely owing to impaired neuronal signaling and attenuation of neurotrophic factor signaling. BDNF endosomal transport is a key signaling system facilitating many aspects of healthy brain function and synaptic plasticity. Furthermore, proper intracellular transport via the endosomal trafficking system is crucial to maintain steady-state BDNF signaling. This dissertation focuses on how Aβ and IL-1β act both synergistically and distinctly to induce a state of abnormal neuronal endosome transport of BDNF. Utilizing a specialized microfluidic isolation chamber for *in vitro* primary neuronal cultures, we demonstrate that Aβ oligomers compromise BDNF retrograde transport by impairing endosomal transport rate, resulting in impaired downstream signaling driven by BDNF. In a similar fashion, we show that IL-1β also attenuates BDNF endosomal trafficking flux and the dispersion of BDNF signaling endosomes throughout neurite networks in cultures. Distinct from Aβ however, the mechanism for IL-1β-induced deficits to BDNF endosomal signaling did

not arise from impaired rate of transport of the BDNF-TrkB receptor complex. Rather, IL-1β may be associated with a Ub-dependent presynaptic sorting deficit. Our data suggests that ubiquitin C-terminal hydrolase L1 (UCH-L1), a deubiquitinating enzyme that functions to regulate cellular ubiquitin, mediates these trafficking deficits, since the irregularity in BDNF trafficking can be reversed by increasing cellular UCH-L1 levels. UCH-L1 is important for regulating neurotrophin receptor sorting and supporting retrograde transport. Thus, this work supports the idea that in AD or other neurological conditions where chronic inflammation is present, down-regulated UCH-L1 may drive BDNF trafficking deficits, compromising synaptic plasticity and neuronal survival. Collectively, the data indicate that both Aβ and IL-1β distinctly contribute to BDNF trafficking deficits, and that Aβ-induced deficits can be rescued *in vitro* by UCH-L1 overexpression.

Chapter 1 Introduction

Affecting nearly 5.3 million people, Alzheimer's disease (AD) is the leading cause of dementia in the elderly, the sixth leading cause of death in the United States, and a major fiscal burden totaling up to $220 billion annually (Alzheimer's Association Facts and Figures 2015). While many preclinical studies in animal models have achieved great success in altering the disease course, translating these therapies to humans has been challenging. Most clinical trials targeting the principle neuropathological lesion, Aβ aggregates, have failed, suggesting a broader or more diversified approach will be needed to address AD. With the imminent rise in the 65+ aged demographic over the next two decades, now is the time, more than ever, to understand the basic underlying pathophysiology in order to better inform every possible therapeutic lead and ease the fiscal and personal impact of a devastating neurodegenerative disease.

Dementia due to plaques and tangles was first described by Dr. Alois Alzheimer in 1906 in his patient, Auguste Deter, a 51 year old women exhibiting symptoms of memory loss. Following her death, he examined her brain in detail and showed the histological features that today define dementia as Alzheimer's disease: the massive loss of cortical tissue and the presence of histopathological anomalies resembling plaques and tangles. By today's standards, Alzheimer's disease represents a complex, age-dependent and multi-faceted disease associated with Aβ "plaques" and tau "tangles." While these two neuropathological findings are the most widely studied aspects of the disease, it is well established that neuroinflammation also contributes to the disease process. The accumulation of Aβ and chronically upregulated pro-inflammatory cytokines, such as Interleukin-1β (IL-1β), places the brain at risk for

cognitive decline by compromising neuronal and synaptic function by mechanisms which have not yet been clearly defined.

The defining features of clinical Alzheimer's Disease is amyloid-β precursor protein (APP) proteolytic processing and generation of Aβ. APP can be cleaved by different enzymes that define whether the process is amyloidogenic or non-amyloidgenic. Amyloidogenic processing of APP by β-secretase and γ-secretase enzymes produces Aβ while non-amyloidogenic processing by α-secretase cleaves through the Aβ peptide region. While both types of APP processing occur, AD may represent a shift in relative rates of processing in favor of the amyloidogenic processing pathway (1,2). A number of studies suggest that APP is cleaved by secretases in the endosomal trafficking compartment (3). Thus, the shift toward amyloidogenic processing may be associated with changes to trafficking of endosomal-APP. Early endosomes contain βAPP-secretase cleaving enzyme 1 (BACE1) and have optimal pH for BACE cleavage of APP. The resulting 99 amino-acid APP fragment is trafficked to the endoplasmic reticulum and back to the membrane, where further amyloidogenic processing can occur to form Aβ. Thus, extracellular Aβ originates from trafficking and exocytosis of intracellular Aβ precursors.

The clinical manifestations of Aβ and a chronic immune response in the brain can lead to a characteristic and progressive loss of memory and cognitive function, likely owing to the progressive loss of synapses in the cortex. Synapse loss in Alzheimer's Disease was first described in the early 1990s using ultra resolution EM studies and immunostaining techniques, which elucidated major synapse loss in the frontal, temporal and entorhinal corticies, and the dentate of the hippocampus (4-7). A major

contribution stemming from these lines of work offered a now widely accepted hypothesis that synapse loss, rather than Aβ, remains the strongest correlate of cognitive decline. Many studies since then have proposed mechanistic explanations on the pathophysiological role for Aβ causing synapse loss. This link between Aβ and synapse loss originated mostly from animal models and cell culture studies showing that certain isoforms of Aβ can cause a loss of dendritic spines in cultures, disrupt LTP mechanisms and impair cognitive function in an otherwise normal animal. Furthermore, Aβ has been shown to affect AMPA receptors, NMDA receptors, and a number of signaling pathways including those of neurotrophic factors. Intriguingly, neurotrophic factor signaling pathways seem to be disproportionately diminished, likely due to their major role in synaptic plasticity. This lab and others have shown that Aβ adversely alters signaling of Brain-derived neurotrophic factor (BDNF). For example, sublethal levels of extracellular Aβ have been shown to downregulate BDNF expression (8); Aβ has been shown to decrease levels of BDNF mRNA via specific downregulation of BDNF transcripts IV and V (9) and Aβ oligomers alter BDNF-TrkB endosomal trafficking (10,11). These studies are interesting since they were conducted in neuronal culture models, suggesting that extracellular Aβ retains the capacity to cause pathological changes to the cell even in the absence of intracellular Aβ, and the pathological burden of extracellular Aβ can have profoundly adverse effects on growth factor signaling. In contrast, BDNF has been shown to exhibit a protective role against Aβ-induced toxicity by preventing Aβ-dependent impairment of LTP (12) and apoptosis (13). However, this protective role has significant limitations since accumulation of Aβ is progressive (spanning as much as 2 decades) and likely overcomes BDNF countermeasures.

Notwithstanding, the pathological burden on neurotrophic factors can have serious consequences on the maintenance of synapses and thus, cognitive function.

While a detrimental role for Aβ in the disease process is clear, the exact role of IL-1β in AD is less defined. Unlike Aβ, IL-1β is a more prevalent and pleiotropic protein with known functions as a potent stimulus for leukocyte recruitment to the CNS. Glia express and release cytokines in response to insult to initiate an immune response that, under normal circumstances, subsides in a timely and regulated manner. It seems however that IL-1β acts effectively within a narrow therapeutic window, suggesting that both time and levels of IL-1β can have serious consequences on neuronal function. Thus, IL-1β has been largely deemed the “double edged sword” as it seems to function as both as a facilitator of amyloid clearance and potent inducer of neuronal pathophysiology at chronic or significantly upregulated levels.

Constitutive expression of IL-1β occurs at very low levels in the normal brain and studies have shown that IL-1β has a direct role in some non-inflammatory processes, including synaptic plasticity (14). The involvement of IL-1β in synaptic plasticity was proposed on the basis that increased expression of IL-1β is seen after the induction of long-term potentiation (LTP) in the hippocampus, together with the finding that IL-1RA can also impair established LTP (15). In stark contrast, IL-1β can also be associated with inhibition of LTP, as many studies have shown that IL-1β is antagonistic to synaptic potentiation. In fact, IL-1β has been shown to be a potent inhibitor of LTP in the CA3 and CA1. Among the first evidence that IL-1β could directly modulate synaptic potentiation came from a study by Katsuki and colleagues, where most notably, they were able to show in the CA3-mossy fiber pathway that IL-1β negatively impacted LTP

in mouse slice culture (16). Later, Cunningham et al. showed in the rat perforant path afferents to the dentate that while low frequency synaptic transmission was unaffected by IL-1β, pre-treatment completely blocked LTP (17). Bellinger et al. demonstrated that exposure at 1 hour prior to tetanic stimulation was still enough to inhibit LTP, as measured by pEPSP and population spike amplitude (18). The resolution for the IL-1β dichotomy may be due to levels and the latency of immune response, whereby lower levels of IL-1β in an acute setting tend to function more in favor of plasticity while higher levels in a chronic setting tend to promote synaptic dysfunction. Notwithstanding, it is generally accepted that chronic and grossly upregulated IL-1β can negatively impact synaptic function and LTP.

BDNF signaling promotes synaptic plasticity, including the facilitation LTP (19). BDNF-dependent LTP promotes F-actin formation by providing cytoskeletal support and increases postsynaptic spine size (20,21). F-actin polymerization requires phosphorylation of the constitutively acti**v**e filament-severing protein cofilin and is modulated by expression of the immediate early gene product Arc (22), both of which are stimulated by BDNF signaling. IL-1β suppresses BDNF-dependent Arc induction and cofilin phosphorylation, prevented the formation of F-actin in spines, and impaired the stabilization of BDNF-dependent LTP in acute hippocampal slices (23). In addition, IL-1β compromises BDNF-dependent IRS-1 signaling and PI3K/Akt pathways, leading to diminished BDNF-dependent phospho-CREB levels (24). These results demonstrate that IL-1β impairs BDNF dependent plasticity and strongly suggests that a mechanism by which IL-1β impairs synaptic plasticity likely involves impaired endosomal trafficking.

The diversity with which IL-1β affects such a wide range of synaptic processes lends well to the idea of a common mechanism of endosomal dysfunction, since many, if not all, of the detriments are seen in mechanisms that are dependent on endosomal trafficking. For example, actin is a necessary component of synaptic vesicle trafficking and pooling. LTP depends heavily on receptor reconfiguration and insertion (25), which in turn, is dependent on endosomal trafficking from synaptic vesicles pools to the membrane. IRS-1 and Akt are activated by BDNF—yet if endocytosis is inhibited, the Akt pathways are also attenuated (26), suggesting that post-endocytic trafficking is a significant mechanistic component of signal transduction. And finally, the expression of plasticity genes is at least partially dependent on Erk5 which is activated by retrogradely transported BDNF and NGF endosomes . Thus, global endosome dysfunction proposes an attractively unified and underlying theme that accounts for such diversity in IL-1β (and even Aβ) induced anomalies.

BDNF Trafficking

The general mechanism for retrograde transport starts with ligand-dependent internalization of Trk receptors into signaling endosomes. Canonically, clathrin associated internalization is the first of many steps to commit the fate of a signaling endosome toward the retrograde pathway (27). Many co-factors and post-translational modifications are involved in the regulating its destination. Among these co-factors are: Ubiquitin, actin and Rab-family GTPases.

The distance between the site of receptor activation and its target effector can be remarkably vast in neuronal cells owing to the highly specialized architecture of the

neuron. Passive diffusion alone cannot account for such retrograde signaling kinetics, suggesting that an alternate mechanism is needed for the timely transport of intracellular cargo. Neurotrophins, such as NGF and BDNF, can activate presynaptic TrkA and TrkB to sustain survival via signaling and retrograde transport (28-32). The retrograde signaling model has been observed in multiple studies and states that following endocytosis, the signaling endosome is transported from distal axons to the cell soma to ultimately promote the expression of pro-survival signals. This phenomenon of endosome transport of both BDNF and NGF can be readily observed in living neurons (33,34). Our group has even characterized the rate of flow of BDNF vesicles tagged with GFP (11). Expanding on the hypothesis, studies have even shown that endosomes are typically associated with activated constituents of signaling cascades and that blocking trafficking can also block signaling. For example, endosomes isolated from PC12 cells exhibit phosphorylated NTRK, PI3K and other proteins that are known to be involved in the activation of ERK signaling cascades (31,35-38). In addition, studies that inhibit endocytosis or dynein-mediated transport by blocking dynamin in neurons cultured in compartmentalized chambers also blocks NTRK signaling (39), suggesting that endosome trafficking and signaling are interconnected. Collectively, contemporary views seem to suggest that both endocytosis and retrograde transport are significant component for activation of downstream signaling cascades.

Retrograde transport also has a clear role in neuronal developmental aspects including axonal outgrowth and pruning (40,41), synapse and circuit formation (42,43), and inducing the expression of factors necessary for plasticity (44,45). Normal transport

of neurotrophins is dependent on a variety of different factors such as actin and Rab family proteins. The importance of the actin cytoskeleton in retrograde NGF-TrkA endosome transport has been a well-studied example on the link between actin and trafficking. The landmark study demonstrating this association was by Harrington and colleagues, where they reported that actin depolymerization is essential for initiation of TrkA endosome trafficking and that a Rac1-cofilin signaling supports their maturation to retrograde transport-competent endosomes (46). This link between actin and early endosome trafficking was further demonstrated in Caenorhabditis elegans, whereby disruption of the Arp2/3 complex, which regulates branched actin dynamics, led to significantly larger early endosomes but did not significantly affect endocytosis (47). These results are not unlike our own. We have proposed that IL-1β too leads to a similar observable outcome of increased presynaptic endosomal content without measureable differences in endocytosis. Furthermore, our unpublished and ongoing studies strongly corroborate with the idea that F-actin dynamics are disrupted by IL-1β, which would stand as a viable mechanism for aberrant presynaptic Trk-endosome routing.

The Rab-family of proteins comprises over 70 different individual Rab proteins found in mammalian cells. Among the vast array of this superfamily of proteins, a few well-documented Rab proteins have proven to be central to Trk trafficking: Rab5 and Rab7. Rab5 regulates early endosome (EE) formation and fusion along the endocytic pathway while Rab7 regulates late endocytic compartments including fusion of the late endosome and lysosome (48-51). With respect to Trk-containing endosomes, NGF-TrkA complexes are found to be present in early endosomes positive for Rab5 in DRG

neurons (52) and we and others have shown that BDNF-TrkB endosomes associate with Rab-family proteins in axoplasmic trafficking (10,53). Following endocytosis, neurotrophin receptor complexes move into Rab5-positive early endosomes, as demonstrated in one study showing that treatment with NGF increased the amount of TrkA in Rab5 positive vesicles in PC12 cells (54). The function of Rab5-Trk endosome association is still an area of active research, although it has been proposed that Rab5 couples the local production of phosphoinositides to the selective recruitment of Rab5 effector proteins, (55), further supporting the role of Rab5 in mediating Trk signal transduction from endosomes in active transit. Among the first studies to demonstrate Rab5 to Rab7 progression showed that Rab5 and Rab7 act in a sequential manner in controlling an axonal retrograde transport pathway in motor neurons (56). Typically, Rab5 NTRK endosomes become Rab7 endosomes as they transit through the endosomal pathway, increasing the propensity for late endosomal characteristics such as presynaptic egress via retrograde axonal pathway or degradation. Importantly a disruption in this progression can lead to aberrant trafficking. In a study using WT and Alzheimer's Tg2576 primary neuronal cultures, the conversion to a Rab7-containing endosome was decreased by 37% in Tg2576 neurons when compared to WT, suggesting that Aβ is contributing to defects in the progression of endosomes through known trafficking stages (57).

Role of Ubiquitin in Trafficking

Ubiquitination is a characteristic and routine modification of the NTRK receptor family. Ub has been proposed to play a central role in endosomal trafficking and

endocytosis of NTRK receptors **(Figure 1.1)**. Ubiquitin is regulated by E3 ligases and deubiquitinating enzymes (DUBs), and the balance of each is critical to maintaining the proper regulation of ubiquitination in the cell. The following section outlines the roles and mechanisms of ubiquitin in endocytosis and trafficking.

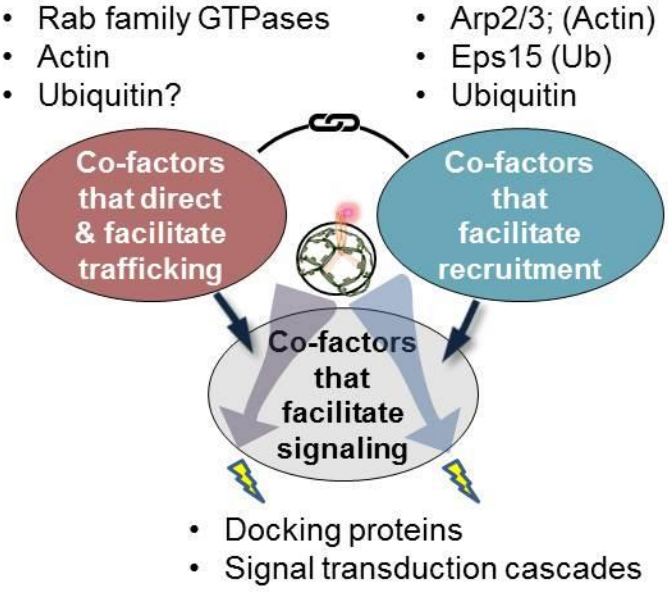

Figure 1.1 | Diagrammatic Representation on major classes of trafficking cofactors.
Among the hundreds of proteins that are known to associate with endosomes, three major class functions can be delineated: Co-factors that facilitate recruitment of necessary trafficking components (examples would be proteins that modulate actin or ubiquitination), Co-factors that direct or target endosomes to destinations, and co-factors that activate signal transduction cascades.

Mechanism of Ubiquitination

Ligation of Ub to protein effectors is a three tiered reaction involving Ub-activating enzyme, Ub-conjugating enzyme, and Ub-ligase. The multiple steps of Ub-ligation ensure a high level of specificity and regulation. Ub-activating enzyme, known

as E1, is the first and ATP-Dependent step in the initial reaction, where activation of the carboxyl terminus of Ub and conjugation of the Ub to an active-site cysteine occurs. In the second step, Ub is transferred to a similar cysteine residue in the active site of one of more than 20 known E2 enzymes (58). The third enzyme, known as E3, confers specificity to target proteins based on subunit assembly. There are two main classes of E3, HECT and RING-types which differ in mechanistic catalysis of Ub conjugation by only one step **(Figure 1.2)**.

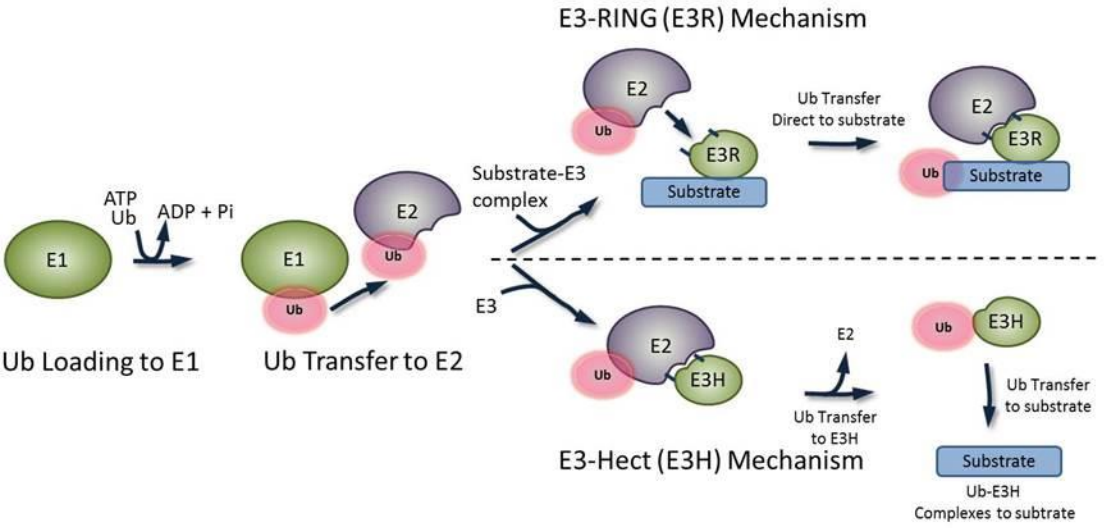

Figure 1.2 | Mechanism of Ubiquitination. Receptor Ubiquitination proceeds through a 3 strep mechanism. E1 is initially charged with the Ub peptide and transfers to active site Cys residue on an E2. E3-Ring domain enzymes bring the target substrate in close proximity to the Ub-E2 complex in a concerted mechanism whereby Ub is directly conjugated to the receptor. E3-Hect domain links Ub to the E3 ligase, after which, it is released to subsequently bind the target substrate.

Evidence of NTRK Ubiquitination by E3

The balance of E3 and DUBs remains an understudied phenomenon, especially in AD. This is likely due to there being many different enzymes, some likely yet to be discovered, that have intrinsic E3 ligase capabilities. Each E3 ligase usually targets a single receptor or a receptor family. Two prominent E3 ligases associated with NTRKs

are c-cbl and Nedd4. C-cbl seems to target many different kinds of receptor tyrosine kinases such as including EGFR (59,60), VEGFR (61,62), PDGFR (63), Insulin Growth Factor (64), and TGF-β (65). One notable study that showed a specific connection between c-cbl and TrkB found that glucocorticoids regulate TrkB protein levels via c-Cbl dependent ubiquitination, where c-cbl stabilized TrkB levels under stressed conditions (66). More studies are needed, however, to further establish the link between c-cbl dependent ubiquitination and TrkB trafficking. Another prominent E3 ligase associated with NTRKs is Nedd-4 (67). Nedd-4 (Neural precursor cell-expressed developmentally downregulated gene), first described as a developmentally regulated gene in the nervous system, is characterized by a distinct modular domain similar to Yeast Rsp5 (68), which has well known functions in regulating trafficking, sorting and degradation in yeast (69,70). The Nedd-4 E3 family is likely responsible for NTRK multimonoubiquitination and a few studies have been key in implicating its association with NTRKs and subsequent trafficking. The first report to show the Nedd-4-Trk receptor interaction demonstrated that neuron cell survival through Trk receptor pathways was regulated by Ubiquitination (71). A seminal finding showed that Trk receptors are multimonoubiquitiniated in response to neurotrophins by Nedd4, leading to targeted degradation of activated TrkA receptor levels (71). Corroborating the finding that NTRKs undergo ubiquitination following ligand binding, another study independently showed that TrkA and TrkB receptors undergo robust ligand-dependent ubiquitination that is dependent on activation of the endogenous Trk activity of the receptors (72). For TrkB, peak ubiquitiniation was found at 5 min post-BDNF treatment and was inhibited by K252a, a Trk phosphorylation inhibitor (72). The study did not conclude, however,

whether BDNF internalization or degradation was affected. It is apparent, however, that NTRK ubiquitination is associated with ligand binding through E3 ligases.

Role of Ubiquitin in Trafficking and Endocytosis

The framework of Ub-dependent NTRK endocytosis and sorting is partially dependent on the Ub code (which represents the various ways Ub can be conjugated to receptors) and ubiquitin binding proteins, which bind Ub to "read the code." Contemporary views on ubiquitin hold that Ub linkage type and soluble Ub-receptors that recognize Ub orchestrate the process of NTRK endocytosis and trafficking. Ub signals are linkage-specific and receptor-specific, meaning that different Ub polymer linkages can actually produce the same NTRK receptor fate. For example, multimonoubiquitination and Ub-Lys63 polyubiquitination have both been shown to be associated with NTRK internalization (73), while Ub-Lys48 polymers tends to increase proteasomal degradation and decrease the rate of deubiquitination (74). The Ub code is based on a sole peptide, ubiquitin, and ~6 different linkage types. Given the vast array of receptors and intracellular destinations that could ensue, interpretation of that minimalist code is largely conferred by Ub receptors (discussed in later sections). The role for Ub-dependent signaling in NTRKs was largely demonstrated in studies using NGF-TrkA. Much like TrkB, TrkA internalization is required for retrograde signaling to the neuronal soma, which ultimately controls neuronal survival, plasticity and differentiation (75). Two reports have shown that the TrkA receptor is ubiquitinated upon NGF stimulation (71,73). In one report, the authors proposed that ubiquitination is mediated by interactions with the p75 neurotrophin receptor and its associated E3

ligase, TRAF6, which catalyzes Lys63-linked polyubiquitination of TrkA (73). In the second report, Nedd4-2 was proposed as the E3 ligase for NGF-dependent multimonoubiquitination of TrkA. In both studies, receptor ubiquitination was shown to be critical for TrkA endocytosis and signaling. Thus, ubiquitination seems to regulate the internalization of NTRKs (76). There is consensus that Ub indeed regulates endocytosis of NTRK, but Ub-dependent post-endocytic trafficking still remains a largely unstudied phenomenon. Our line of work based on Poon and Carlos et al. (11) and ongoing studies is really the first to highlight the role of ubiquitin in axoplasmic trafficking, as this represents a much further downstream event from the more classical discussion on the role of Ub in endocytic sorting.

It is unlikely that a Ub signal itself is driving receptor endocytosis and sorting. Rather, it has been proposed that freely soluble Ub receptors containing Ub-binding domains are the determinant and rate-limiting step in NTRK trafficking. Commonly studied Ub receptors include cbl, Eps15, Hrs, Vps27, Vps9, Tollip and Vps23 and these share common architecture for Ubiquitin binding. Ubiquitin-dependent internalization of NTRK receptors requires the recognition of the ubiquitinated NTRK by UBD (Ubiquitin Binding Domain)-containing proteins, usually referred to as Ub receptors. Ub receptors contain Ub binding domains that recognize ubiquitin and some contain domains that recognize the NTRK receptor or other endocytic facilitators. The presence of UBDs allows the Ub receptor to either bind the variety of different Ub linkage-types, including mono-Ub moieties, which is more canonically associated with endocytosis. Further discussion of UBDs is out of the scope of this work but these topics have been reviewed in depth (77,78). Many of the Ub receptors that can also function as endocytic adaptors

underscore that Ub itself may be insufficient as a signal for endocytosis. Endocytic adaptors that also serve as Ub receptors may be preferentially targeted to ubiquitinated NTRK receptors by binding ubiquitinated-receptor and to the plasma membrane with phosphatidylinositol-binding domains (79). The mechanisms have largely been elucidated using EGFR (80). Epsin and Hrs are also well studied examples of this and are thought to participate in endocytic sorting of ubiquitinated proteins. As would be expected, they both contain a clathrin-binding domain (81), underscoring involvement in endocytosis. Only recently is Hrs becoming known as a regulator of TrkB endosome sorting, with one study having shown that Hrs plays an essential role in recycling of TrkB (82). However, no further studies on TrkB/Hrs from that group have been published. In the case of the much more widely established EGFR family, three specific UBD-containing proteins epsin-1/2, Eps15, and Eps15R are also involved in NTRK endocytic sorting. Binding of these proteins to ubiquitin moieties *in vitro* is well-established (83), and co-IP with EGFR has also been demonstrated (84-86). However, there have been studies that have argued both ways as to the extent to which these dual role "Ub receptor/endocytic adaptors" actually facilitate endocytosis. One study shows that knockdown of epsin-1 inhibited EGFR endocytosis (87), while other studies argue otherwise. It is clear, at least, that these Ub receptors are an integral component of the complex of proteins that surround NTRK endosomes and may partly contribute to endocytic sorting. It's important to note that no studies have ruled out whether these proteins also contribute to post-endocytic sorting for NTRK receptors, as in the case of the Ub receptor Hrs and TrkB.

Similar to the epsin1/2, Eps15 knockout reports, knockout of E3 ligases like c-Cbl by siRNA or overexpression of inactive Cbl mutants inhibited internalization of EGFR and several other RTKs (88-90). Since E3 ligases are responsible for NTRK ubiquitinaiton and knockout of E3's inhibits endocytosis, we know that Ub must be necessary for endocytosis. But based EGFR ubiquitin receptor studies, Ub but may not be sufficient to drive the process of endocytosis. By all accounts, it is clear that Ub-binding proteins are contributing cofactors in facilitating the endocytic sorting of would-be endosome transport complexes, but may not be the only regulator in internalization.

Ubiquitin C-terminal Hydrolase L1

Ubiquitin C-terminal Hydrolase L1 (UCH-L1) is the primary therapeutic target studied in this dissertation. Since UCH-L1 regulates one of the most important and pervasive mechanisms of growth factor signaling in the brain, targeting it may have lasting and impactful outcomes. While the potential unintended outcomes and side effects of treating such a target are possible, further studies are absolutely necessary to better comprehend the risks and the benefits *in vivo*.

UCH-L1 was first discovered as a deubiquitinating enzyme (DUB) that is widely distributed in neurons. Remarkably, UCH-L1 expression in the brain is remarkably high within the brain and it is an important regulator of brain ubiquitin levels (91-93). Canonically, it serves to cleave conjugated Ub to yield freely soluble Ub (94). Although ubiquitin can be synthesized *de novo*, the majority of the intracellular ubiquitin is derived from ubiquitin that is recovered from deubiquitinating enzymes (95). Thus, ubiquitin levels can be regulated by modulating deubiquitinating enzyme levels or activity of

deubiquitinating enzymes such as UCH-L1 (92,96). Further reports suggested that overexpression of UCH-L1 in SH-SY5Y neuroblastoma cell lines extended the half-life of mono-Ub proteins. UCH-L1 appears to stabilize signals more traditionally known for NTRK trafficking by redirecting proteins destined for degradation.

This enzyme is particularly interesting in neurodegenerative diseases for two main reasons: it is one of the most highly expressed proteins in the normal brain, and it is one of the most downregulated proteins in the Alzheimer's brain (11). Consistent with this finding, the brains of AD patients show an accumulation of ubiquitinated proteins (97). Moreover, analysis of proteasome function in postmortem human AD brains shows little change in the levels of mRNA or protein for the major proteasomal components but a strong inhibition of degradative activity (98). Numerous accounts have shown an overly-ubiquitinated protein state in the AD brain, likely owing to a compensatory response to over-accumulated Aβ (97). It is generally accepted that Aβ aggregation outcompetes and overwhelms normal protein clearance methods. Whether UCH-L1 follows Aβ accumulation or whether it exacerbates Aβ pathophysiology is unknown. It is clear, however, that even in the absence of neuropathology, downregulated UCH-L1 leads to overt degeneration. Examples of this can be seen in Gad mice, which exhibit an autosomal recessively inherited disorder caused by an in-frame deletion that includes exons 7 and 8 of UCH-L1, leading to a lack of UCH-L1 expression (99). These mice show sensory ataxia at an early stage, followed by motor ataxia at a later stage. Pathologically, the mutant is characterized by axonal degeneration and formation of spheroid bodies in nerve terminals, which resembles the phenotype we have proposed. In addition, gad mice show abnormal accumulation of

APP, Aβ, Ub, and proteasome subunit-positive deposits in the degenerating neuronal axons (99,100). These results clearly indicate that UCH-L1 is essential for the functional maintenance of neuronal axons and that a lack of Ub regulation can lead to sequestration or accumulation of proteins in axon terminal compartments.

The therapeutic potential of UCH-L1 is a widely debated and largely underdeveloped idea. With only a handful of studies in animal models, manipulating UCH-L1 in Alzheimer's is far from ready for clinical use. The first study to show positive outcomes of UCH-L1 in AD came from Gong and colleagues (96). The group reports that transduction of UCH-L1 protein fused to the transduction domain of HIV-transactivator protein (TAT) restores normal enzymatic activity and synaptic function both in hippocampal slices treated with oligomeric Aβ and in the APP/PS1 mouse model of AD. Moreover, intraperitoneal injections with the fusion protein improve the retention of contextual learning in APP/PS1 mice over time associated with restoration of normal levels of PKA and CREB phosphorylation. Further corroborating this finding was a more recent report showing that overexpression of UCH-L1 delays AD progression *in vivo* (101). The authors report that that UCH-L1 interacts with APP and regulates Aβ production by increasing free ubiquitin levels thus accelerating lysosomal degradation of APP. Furthermore, they demonstrated that overexpression of UCH-L1 by intracranial injection of UCH-L1-expressing rAAV even reduces Aβ production, a seminal finding that supports the idea of using UCH-L1 in conjunction with other therapies to treat AD.

Significance

The seminal finding of this dissertation supports the notion that Aβ and inflammation induce a neuronal state of uncoupling between BDNF and its downstream signaling targets. This phenotype can best be described as "Neurotrophic Resistance" **(Figure 1.3).** Furthermore, we have discovered that neurotrophic resistance can be

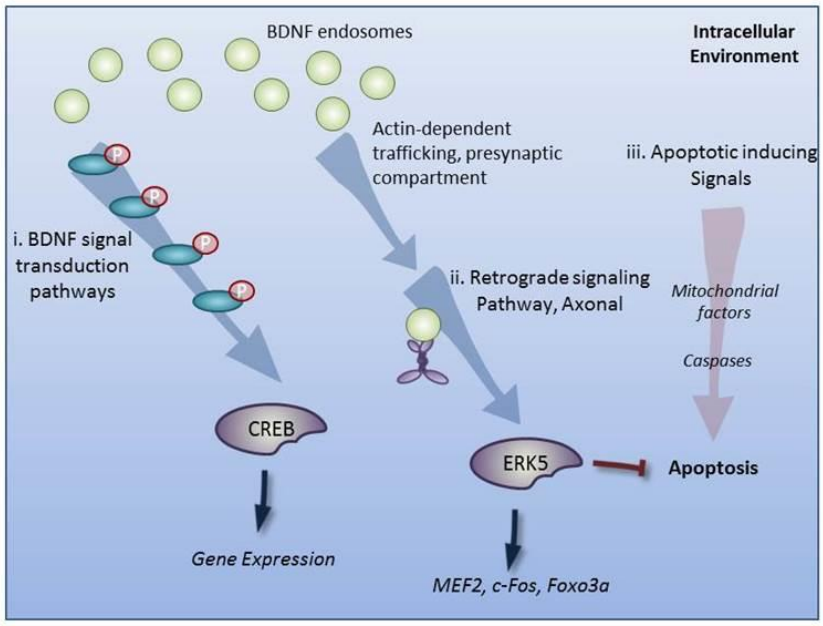

Figure 1.3 | Neurotrophic Resistance. Neuronal viability is dependent on steady state signals from both (i.) BDNF signaling pathways and (ii.) BDNF retrograde trafficking, leading to CREB and ERK5 activation. In this model, steady state signaling from BDNF competes with other cellular pathways that induce cell death (iii.). Any attenuation in the steady rate of signaling of BDNF tips the balance and increases the propensity for apoptosis. We have shown that Aβ and IL-1β uncouples BDNF from these signaling modalities, leading to a condition we refer to as neurotrophic resistance.

rescued by modulating only a single component of a vastly complex Ubiquitin regulation network that alters BDNF endosomal trafficking. This dissertation focuses broadly on the idea that in AD, endosomal dysfunction underlies neurotrophic resistance at multiple levels and ultimately leads to adverse outcomes on synaptic function and cell survival.

Steady-state endosomal flux is critical for signaling. Endosomes are nothing more than an organelle in the fluid-phase. Similar to blood flow kinetics in the cardiovascular system, endosomal transit thus recapitulates many elements of flow kinetics. Much like how infarction of cerebral arteries leads to attenuated perfusion and stroke, inhibiting endosome transport can have consequences on the cellular level: a so

called “cell stroke” **(Figure 1.4)**. We have proposed that Aβ and IL-1β alter the flow kinetics of BDNF endosomes, with intriguingly similar consequences to the subset of endosomes destined for retrograde transport. However, the mechanisms may differ. This is important because it describes a cellular phenotype whereby neurons are resistant to BDNF signaling and that even in the presence of BDNF, downstream targets remain uncoupled to signal transduction cascades. Further underscoring the importance, this study represents an early stage of the Global Endosome Dysfunction (GED) hypothesis, which I have devised. Follow up studies should examine this hypothesis in more detail, since pharmacologically targeting a cellular process like trafficking (which reaches many different subcellular systems and targets) may represent a viable paradigm in neurological disease therapy.

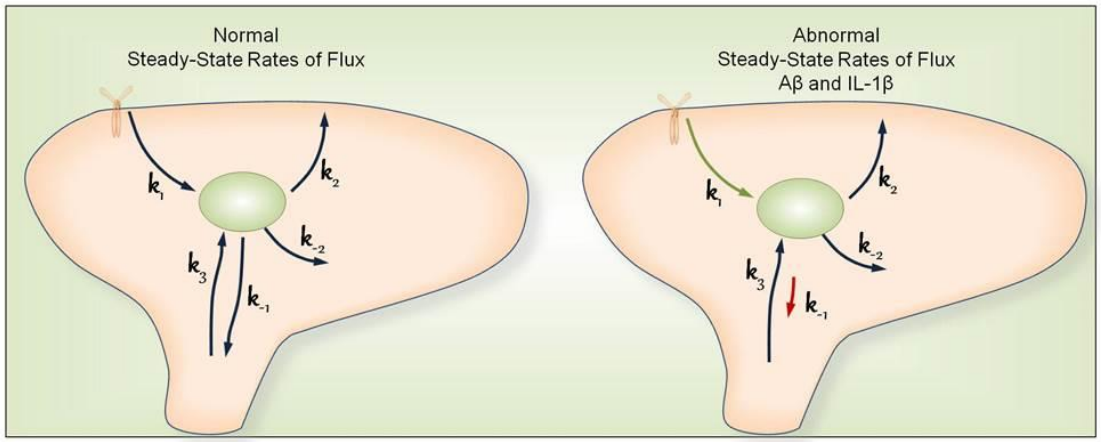

Figure 1.4 | Schematic representation on Steady-state rates of flux at the presynaptic terminal. Endosomal trafficking is a highly fluid process. Rates of net influx and efflux are a carefully regulated balance of multiple input and output stages roughly characterized by an aggregate rate constant, k, where k_1 represents an all forms of endocytosis, k_{-1} retrograde trafficking pathways, k_3 anterograde trafficking pathways, k_{-2} degradation, and k_2 recycling pathways. The left panel shows a representation of a theoretical normal and balanced flow in a presynaptic terminal. The panel on the right indicates the findings of this work. That is, Aβ and IL-1β act synergistically on retrograde trafficking pathways, k_{-1}, albeit by different mechanisms with no apparent effect on long-timecourse endocytosis, k_1.

Chapter 2

β-Amyloid (Aβ) oligomers Impair Brain-derived Neurotrophic Factor Retrograde Trafficking by Down-regulating Ubiquitin C-terminal Hydrolase, UCH-L1

Abstract

We previously found that BDNF-dependent retrograde trafficking is impaired in AD transgenic mouse neurons. Utilizing a novel microfluidic culture chamber, we demonstrate that Aβ oligomers compromise BDNF-mediated retrograde transport by impairing endosomal vesicle velocities, resulting in impaired downstream signaling driven by BDNF/TrkB, including ERK5 activation, and CREB-dependent gene regulation. Our data suggest that a key mechanism mediating the deficit involves ubiquitin C-terminal hydrolase L1 (UCH-L1), a deubiquitinating enzyme that functions to regulate cellular ubiquitin. Aβ-induced deficits in BDNF trafficking and signaling are mimicked by LDN (an inhibitor of UCH-L1) and can be reversed by increasing cellular UCH-L1 levels, demonstrated here using a transducible TAT-UCH-L1 strategy. Finally, our data reveal that UCH-L1 mRNA levels are decreased in the hippocampi of AD brains. Taken together, our data implicate that UCH-L1 is important for regulating neurotrophin receptor sorting to signaling endosomes and supporting retrograde transport. Further, our results support the idea that in AD, Aβ may down-regulate UCH-L1 in the AD brain, which in turn impairs BDNF/TrkB-mediated retrograde signaling, compromising synaptic plasticity and neuronal survival.

Introduction

Alzheimer disease (AD) is defined pathologically by the accumulation of extracellular Aβ plaques and intracellular neurofibrillary tangles, accompanying synaptic and neuronal loss in the AD brain. Although Aβ plaque accumulation is a clear risk factor associated with AD, cognitive decline precedes plaque pathology (1). Studies now suggest that soluble and/or oligomeric Aβ that accumulates early in the disease causes synaptic deficits and correlates more closely with cognitive dysfunction than Aβ plaque load (2–4). Consistent with these data, cerebral infusion of soluble Aβ oligomers impairs hippocampal long term potentiation, a form of synaptic plasticity associated with memory formation, and disrupts hippocampal-dependent learning (5, 6). Also, AD transgenic mice that accumulate soluble oligomers exhibit impaired hippocampal long term potentiation and hippocampal-dependent learning along with synaptic loss, prior to frank plaque deposition (7–10).

BDNF/TrkB signaling plays a major role in synaptic plasticity, learning, and memory (11). Similar to the deficits induced by oligomeric Aβ, reduced BDNF signaling also causes AD-like synaptic plasticity deficits (12–19). The parallels have given rise to the hypothesis that a potential mechanism underlying Aβ-mediated synaptic dysfunction involves disrupted BDNF signaling (20–22). Indeed, down-regulation of BDNF signaling may be an early and possibly primary event in AD, based on the finding that in early stages of AD (i.e., mild cognitive impairment), BDNF levels are decreased and correlate with cognitive decline (23).

Consistent with the hypothesis that Aβ-mediated synaptic dysfunction involves disrupted BDNF signaling, we have found that soluble Aβ impairs retrograde axonal

trafficking of the BDNF receptor, TrkB (22). Retrograde axonal transport of the BDNF-TrkB complex to the soma drives downstream signaling events important for neuronal health, survival, and plasticity, including CREB-dependent gene transcription (24). Retrograde axonal trafficking of the TrkB receptor involves multiple steps, including 1) TrkB internalization from the cell surface, 2) sorting/processing of TrkB to late endosomes/multivesicular bodies (MVBs), and 3) transport from the axon to the soma, mediated by dynein motors (24–29).

An important sorting signal that marks tyrosine kinase receptors for entry into the MVB pathway is ligand-induced ubiquitination, particularly monoubiquitination (30–33). Although the contribution of ubiquitin in TrkB retrograde trafficking has not been elucidated in detail, TrkB is multimonoubiquitinated in response to BDNF (34), suggesting that ubiquitin may be important for TrkB signaling. Taken together, with the recent finding that Aβ accumulation in neurons impairs the MVB sorting pathway in part by inhibiting the activities of deubiquitinating enzymes (35), one mechanism by which Aβ impairs TrkB retrograde trafficking may be via interfering with ubiquitin homeostasis.

Here we build on our previous finding that oligomeric Aβ results in a net decrease in TrkB retrograde transport and have identified a potential mechanism underlying this deficit. Oligomeric Aβ does not affect TrkB receptor internalization but impairs endosomal retrograde trafficking/signaling. Also, we demonstrate that oligomeric Aβ interferes with BDNF/TrkB signaling by impairing ubiquitin homeostasis. Specifically, Aβ-mediated trafficking/signaling deficits are mimicked by an inhibitor of the deubiquitinating enzyme ubiquitin C-terminal hydrolase L1 (UCH-L1). Furthermore, Aβ-mediated impairments are rescued by elevating intracellular UCH-L1 levels. UCH-L1

functions to maintain cellular ubiquitin homeostasis, and by manipulating this pathway, we show that the ubiquitin recycling pathway plays a role in neurotrophin-mediated retrograde signaling. These results suggest that in AD, soluble and/or oligomeric forms of β-amyloid disrupt BDNF-mediated retrograde signaling by altering ubiquitin homeostasis. This leads to deficits in neurotrophin-dependent gene expression that compromise synaptic plasticity and neuronal survival.

Experimental Procedures

Synthesis of Aβ Oligomers. Oligomers were prepared as described previously (36). Briefly, Aβ that was lyophilized as a hexafluoroisopropanol film (EMD Millipore) was dissolved in neat, sterile Me_2SO (5 mM) and diluted in PBS, pH 7.4, to 100 μM and aged overnight (4 °C). Aβ oligomer preparations were centrifuged (14,000 × g, 10 min, 4 °C); the supernatants were transferred to fresh Eppendorf tubes and stored at 4 °C until use. Confirmation of Aβ oligomers was carried out by Western analysis as described previously (22).

Purification of BDNF-GFP. Endotoxin-free BDNF-GFP plasmid (generous gift from Dr. Masami Kojima) was introduced by nucleofection (Lonza) into HEK cells followed by selection in DMEM containing 10% FBS and G418 (1 mg/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 medium was removed and concentrated with Amicon YM-30 centrifugal filters (5000 × g, 2 h) (30,000 molecular weight cutoff, Millipore). Pro-BDNF was converted to mature BDNF-GFP by treatment with plasmin (Sigma) as described previously (37).

Mature BDNF-GFP was further purified by size exclusion chromatography (Amicon YM-50) where the flow-through contained the protein of interest. BDNF-GFP is indistinguishable from BDNF both biochemically and biologically (38, 39), and we previously confirmed that our purified BDNF-GFP was biologically active (22). The BDNF-GFP concentration was determined by BDNF ELISA (Promega).

Assembly of Microfluidic Culture Chambers. The chamber was fabricated in PDMS using rapid prototyping and soft lithography similar to previously published procedures (40). Briefly, glass coverslips (24 × 40 mm, No. 1, Corning Inc.) 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)) in PBS (24 h, 5% CO_2, 37 °C incubator), rinsed, and allowed to air dry in a sterile hood. The chambers are noncovalently assembled by conformal contact. The chambers consist of two parallel microfluidic compartments, connected by inlet and outlet wells. The two compartments are separated by a solid barrier region with microgrooves embedded in the bottom of the connecting barrier. A slight volume difference between the two compartments (40 µl) is used to generate a fluidic resistance within the microgrooves, facilitating the isolation of BDNF to axons.

Primary Neuronal Cell Cultures. All of the procedures were performed under an Institutional Animal Care and Use Committee-approved protocol. Primary hippocampal or cortical neuron cultures were derived from rat embryo (embryonic day 18) as described previously (41). Briefly, dissected tissue was dissociated with trypsin, triturated, and either plated on poly-L-lysine-coated 6-well plates or plated in microfluidic chambers fitted with poly-L-lysine-coated glass coverslips in serum-free Neurobasal supplemented with B27 (Invitrogen). The cells were plated at a density of 5 ×

10^6cells/ml (for microfluidic chambers) and 5×10^5 cells/ml (for 6-well plates). Neuronal purity was assessed by immunostaining with a mouse monoclonal β-III-tubulin (1:1000; EMD Millipore) and rabbit polyclonal glial fibrillary acidic protein (1:4000, DAKO). Glial contamination was <5% (n = 6). Aβ oligomer treatments (1 μm) and the transduction with TAT-HA-UCH-L1 (transduction domain of HIV-transactivator protein and hemagglutinin fused to UCH-L1) (20 nM) were carried out at 7 DIV. The expression and purification of TAT-UCH-L1 were carried out as described previously (42). LDN was added for 24 h at a final concentration of 5 μM.

Cell Surface Biotinylation Assays. To assess TrkB internalization, (7 DIV) primary neurons were either treated with or without BDNF (50 ng/ml, 30 min) and then placed on ice to prevent further TrkB internalization. The remaining cell surface TrkB receptors were biotinylated with Sulfo-NHS-LC-Biotin (100 mg/ml, 30 min; Thermo Scientific) and then washed with 0.1 M Tris-HCl (pH 7.5), three times. The cells were lysed with radioimmunoprecipitation assay buffer containing a protease inhibitor mixture (Roche Applied Science), and biotinylated TrkB was immunoprecipitated with streptavidin-agarose beads that had been pre-equilibrated in radioimmunoprecipitation assay buffer. Immunoprecipitated proteins were incubated in sample buffer and processed for Western blot analysis using rabbit polyclonal TrkB (EMD Millipore).

Measuring the Velocity of BDNF-containing Endosomes. Time lapse microscopy was utilized to measure the rates of BDNF-GFP-containing endosomes within the microfluidic devices. Rat primary neurons (7 DIV) were imaged using an inverted Bio-Rad Radiance 2100 confocal microscope and a 60× oil emersion objective. Regions of interest from five axon segments from each chamber were randomly selected for time

lapse imaging. The images were acquired every 5 s for a total of 60 images (5 min). To determine velocity of BDNF-GFP particles within axons, kymographs were generated from the image stack of each time lapse experiment. The velocities of BDNF-GFP containing endosomes were determined in each kymograph, and statistical comparisons were performed using a Student's paired t test.

Quantification of Overall Retrograde Trafficking (BDNF-GFP) or Signaling (pERK5 and CRE-GFP) within Microfluidic Chambers. Cell culture medium (40 µl) was removed from each axonal well prior to the addition of BDNF-GFP. The resultant volume difference restricts BDNF-GFP to only the axonal compartment. After 2 h, somal compartments were analyzed for either net BDNF-GFP transport or p-ERK5 activation by immunocytochemical analysis as described previously (43). In brief, the microfluidic devices were removed, and the coverslips were rinsed with PBS, paraformaldehyde-fixed (4%), permeabilized in 0.25% Triton X-100 in PBS, (pH 7.4), and blocked with 5% goat serum. The cells were incubated in appropriate primary antibody overnight at 4 °C. GFP was stained with rabbit anti-GFP (Invitrogen) followed by anti-rabbit Alexa 488 secondary antibodies. p-ERK5 was stained with anti-p-ERK5 (1:1000; Cell Signaling) followed by anti-mouse Alexa 568. The cells were washed and then immunolabeled with TOTO-3 (Invitrogen) to identify nuclei. CREB-mediated gene expression was assessed in neurons that had been transfected with CRE-GFP (Stratagene) using Amaxa nucleofection (according to their protocol) prior to plating in the microfluidic devices. BDNF (50 ng/ml) was added to the axonal compartment following a similar protocol to the one for BDNF-GFP above.

Images were captured on a Bio-Rad Radiance 2100 confocal system using lambda strobing mode to avoid nonspecific cross-excitation or cross-detection of fluorophores. For each chamber device, three regions of interest were taken using the same settings. For each region of interest, five random areas were chosen and quantitated using ImageJ software (National Institutes of Health). The mean pixel intensity for each area was determined and normalized to TOTO-3. p-ERK5 translocation was also assessed by Western blot analysis. The lysates were prepared from each treatment group by aspirating the media from each well, and then removing the chamber from the coverslips. Next radioimmunoprecipitation assay buffer (100 µl) was added to the area of the coverslips, which contained neurons from the somal side, and then collected. Protein levels were determined by BCA, and equal protein amounts were separated by SDS-PAGE (10%) and processed for Western analysis with either p-ERK5 or total ERK5 antibodies (1:1000; Cell Signaling). Following secondary antibodies, the blots were developed with SuperSignal West Femto chemiluminescent substrate (ThermoFisher).

Microarray Methods. UCHL-1 gene expression changes in AD brain were assessed using a microarray database consisting of brain tissue from AD cases (n = 26; range, 74–95 years; mean age, 85.7 ± 6.5 years) and age-matched controls (n = 33; range, 69–99 years; mean age, 84.2 ± 8.9 years). The criteria for the selection of cases was described previously (44). RNA expression profiles were obtained from 40 hippocampal samples (AD, n = 17; controls, n = 23) and 43 superior frontal gyrus samples (AD, n = 20; controls, n = 23), using 83 Affymetrix HgU133 plus 2.0 arrays, based on the method described previously (44). Two probe sets were identified on the HgU133 plus 2.0 array

corresponding to UCHL-1 (Unigene Hs.518731), both of which had Present flags in all microarrays, indicating high expression reliability of the probes. Expression values were averaged across the probe sets to obtain an overall value for each case, followed by t test comparisons for each region and significance set at $p < 0.05$.

Preparation of Protein Samples from Brain Tissue. Transgenic mouse brain specimens were obtained from the University of California, Irvine Alzheimer Disease Research Center Tissue Repository. Wild-type and Tg2576 (an AD transgenic mouse line) mouse hippocampus or cortex (aged 15 months) was mechanically homogenized with a 1-ml syringe fitted with a 28 1/2-gauge needle (BD Biosciences) by repeated uptake in 200 μl of radioimmunoprecipitation assay buffer containing protease inhibitors (Roche Applied Science). The lysates were centrifuged (80,000 × g for 1 h), the protein concentration of the supernatant was determined by BCA, and samples were stored at −20 °C until analyzed.

Results

Aβ Oligomers Directly Disrupt BDNF/TrkB Axonal retrograde Trafficking by Impairing Vesicle Velocities. Recent evidence suggests that an aspect of neurodegenerative pathology is impaired neurotrophin-dependent retrograde transport (45, 46). In the case of AD, Trk retrograde trafficking deficits are likely Aβ-mediated (22, 47). To define the mechanism underlying the net decrease in BDNF/TrkB retrograde trafficking in the presence of Aβ oligomers (22), we investigated whether soluble Aβ interferes with 1) TrkB internalization at the membrane surface and/or 2)

translocation/transport of the BDNF/TrkB-containing endosome from the axon to the soma.

Cell surface biotinylation assays were employed to determine whether Aβ affected TrkB internalization. We found that Aβ did not impair TrkB receptor internalization in cultured rat primary neurons (7 DIV) (Fig. 1). In the absence of Aβ, BDNF treatment drove internalization of 38.3 ± 3.40% (**, $p < 0.001$) of cell surface TrkB. Similarly, in the presence of Aβ oligomers, BDNF treatment led to the internalization of 41.5 ± 9.8% (*, $p < 0.05$) of cell surface TrkB relative to Aβ-only treatment. No significant reduction in cell surface TrkB was observed with Aβ preincubation alone, in the absence of BDNF. The TrkB antibody detects both full-length TrkB and a truncated form of TrkB (48), enabling us to determine that soluble Aβ does not affect internalization of either full-length or truncated TrkB (Fig. 1). In addition, TrkB internalization was BDNF-specific because neural cell adhesion molecule was not internalized by BDNF treatment. Although previous studies have demonstrated that Aβ can alter cell surface receptor internalization (for example, of AMPA and NMDA receptors) (49, 50), our data demonstrate that Aβ does not affect the internalization of TrkB and suggest that Aβ-mediated trafficking deficits are downstream of TrkB internalization.

Next, we investigated whether Aβ oligomers impair retrograde trafficking by directly affecting the velocities of BDNF-containing endosomes. Vesicle velocities were measured using a novel microfluidics device developed in our laboratory that was described previously (51). This device allows axons to grow along a patterned surface and forces the separation of axons and soma within compartments, enabling the

isolated manipulation of axons, soma, or both. Thus, the device can be used to assess events occurring in the soma following axonal treatment and is ideal to study axonal retrograde transport and downstream events.

BDNF-GFP was added to the axonal compartment to allow for the visualization of axonal retrograde trafficking within neurons. We found that Aβ oligomers reduce BDNF-GFP-containing vesicle velocities within axons by 38.4 ± 13.4% (*, $p < 0.01$) relative to vehicle-treated neurons (Fig. 2A). The average vesicle velocity was 2.81 ± 0.253 μm/s in vehicle-treated axons, whereas the average vesicle velocity in Aβ-treated axons was 1.73 ± 0.378 μm/s. This is in agreement with our previous study that demonstrated that the velocities of BDNF-containing endosomes were markedly reduced in Tg2576 neurons when compared with wild-type neurons (22). Examination of the vesicle velocity distribution revealed that the presence of Aβ oligomers significantly decreased the percentage of endosomes with velocities >2 μm/s, with the majority of the

endosome velocities being <1 μm/s (Fig. 2B). Additionally, representative time lapse images reveal that in the presence of Aβ, the BDNF-GFP signal that can be visualized within trafficking vesicles is greatly reduced (Fig. 2C). These results suggest that Aβ oligomers can disrupt retrograde trafficking by affecting both the vesicle velocities of BDNF-containing endosomes and the amount of TrkB that is contained within the transported endosomes.

β-Amyloid Impairs BDNF-dependent Retrograde Signaling. The signaling endosome hypothesis implies that if the retrograde trafficking of BDNF-GFP-positive endosomes is impaired, then the propagation of BDNF retrograde signaling will also be impaired. To test this hypothesis, BDNF-GFP was added to the axonal compartment of

the microfluidic chambers, followed by assessment of ERK activation. ERK activation was determined by measuring the phosphorylation of ERK5 (p-ERK5) in the soma of neurons. ERK5 is the main ERK that is activated in response to axonally derived BDNF (28).

Representative images (Fig. 3) revealed that BDNF-GFP led to robust p-ERK5 activation within neuronal cell bodies located in the somal compartment. This is indicated by increased p-ERK5 labeling following BDNF-GFP, when compared with vehicle-only neurons (Fig. 3, A and B). However, p-ERK5 activation in response to BDNF-GFP is not readily apparent in neurons preincubated with Aβ (Fig. 3, C and D, respectively). Quantification of p-ERK5 immunoreactivity revealed that BDNF treatment increased p-ERK5 by 68.1 ± 8.4% (*, $p < 0.05$) when compared with vehicle only, whereas p-ERK5 levels were not significantly increased by BDNF treatment in neurons preincubated with Aβ oligomers, relative to Aβ-only treatment (Fig. 3J). These results are consistent with a retrograde signaling deficit caused by impaired BDNF/TrkB retrograde transport. At higher magnification, p-ERK5 (Fig. 3F) appeared to co-localize with the nuclear marker TOTO-3 (Fig. 3G) in a representative neuron, which suggests that p-ERK5 is translocated to the nucleus and is consistent with the literature (28). Additionally, BDNF-GFP immunoreactivity decorated the outer surface of the nucleus, indicating that axonally applied BDNF-GFP trafficked back to the soma (Fig. 3E). This observation supports the “signaling endosome” hypothesis for neurotrophin signaling, because it demonstrates that ligand-receptor complexes that originate from the axonal compartment undergo retrograde transport to the soma.

Next, we measured the extent of p-ERK5 translocation to the somal compartment by Western blot analysis. It revealed that somal p-ERK5 levels (normalized to total ERK5) increase almost 3-fold (*, $p = 0.05$) in response to axonally applied BDNF (Fig. 3, I and K). However, in cultures preincubated with Aβ, p-ERK5 translocation was not observed. Although p-ERK5 levels were lower following BDNF treatment in neurons preincubated with Aβ, it was not significant. The assessment of p-ERK5 translocation by Western blot analysis is consistent with our immunocytochemical results and together supports the notion that Aβ impairs BDNF-dependent retrograde signaling. Next, CREB-dependent gene transcription was assessed to further validate the hypothesis that soluble Aβ impairs BDNF-dependent retrograde signaling. CREB-mediated gene transcription was measured by quantifying GFP within neurons transfected with a CRE-GFP reporter plasmid (Stratagene). CRE-GFP is a cAMP response element (CRE) fused to GFP that is used to monitor downstream cAMP/PKA signaling. In neurons transfected with CRE-GFP, axonal BDNF treatment led to a robust increase in GFP immunoreactivity within soma (35.9 ± 4.73%; *, $p < 0.01$) when compared with vehicle only (Fig. 4, A, B, and E). GFP immunoreactivity was normalized with the neuronal marker, βIII-tubulin. In contrast, in neurons preincubated with Aβ oligomers, no significant increase in GFP immunoreactivity was observed following BDNF (Fig. 4, C–E). Thus, Aβ oligomers also impair axonal BDNF-mediated CREB-dependent gene activation. This impairment was also observed in APP (Tg2576) neurons (data not shown). Taken together, these results demonstrate that Aβ oligomers cause deficits in BDNF/TrkB retrograde signaling by affecting the trafficking of BDNF-GFP-containing endosomes, which in turn results in the decreased retrograde transport and activation of

ERK5 and CREB-dependent transcription that is necessary to maintain proper synaptic function and neuronal survival.

Impaired Deubiquitination Mimics the Effects of Amyloid on BDNF Retrograde Signaling.

Previous studies suggested that Aβ impairs proteasome function and deubiquitinating enzyme activity, which in turn can impair receptor sorting to MVBs and neurotrophin receptor trafficking (35, 52). Because MVBs represent the endosomal compartment that mediates sustained neurotrophin signaling from axon terminals to the soma (25, 53), it suggests that the BDNF/TrkB trafficking deficits may be caused by Aβ impairing deubiquitinating activity. To test this hypothesis, we assessed whether inhibiting deubiquitinating activity could mimic the effect of Aβ oligomers on retrograde transport. Deubiquitinating activity was inhibited with LDN (LDN-57444, EMD), a cell-permeable UCH-L1-specific inhibitor. As predicted, inhibiting deubiquitination with LDN impaired BDNF retrograde signaling as assessed by measuring p-ERK5 activation (Fig. 5,*A* and *B*). BDNF treatment led to a 45.7 ± 10.3% (*, $p < 0.05$) increase in nuclear p-ERK5 levels (Fig. 5*B*). However, in neurons pretreated with LDN, the addition of BDNF did not lead to an increase in p-ERK5 levels. Interestingly, basal p-ERK5 levels were lower when UCH-L1 was inhibited. We attribute this decrease to the importance of ubiquitin turnover to synaptic function. Nonetheless, these results support the hypothesis that Aβ oligomers may affect TrkB retrograde signaling by impairing UCH-L1 activity.

Similarly, LDN pretreatment impaired the translocation of p-ERK5 to the soma following BDNF as assessed by Western blot analysis (Fig. 3, *I* and *K*). Following BDNF treatment p-ERK5 translocation, the soma was not observed. These results were similar to Aβ-treated neurons and support the hypothesis that Aβ affects BDNF/TrkB signaling by impairing deubiquitinating activity. Additionally, like Aβ, LDN did not impair TrkB endocytosis. Using cell surface biotinylation assays, we show that the addition of BDNF led to a 45.7 ± 10.2% (*, $p < 0.05$) decrease in cell surface TrkB levels when compared with vehicle (Fig. 5, C and D). In neurons preincubated with LDN, we observed a similar decrease in TrkB (44.3 ± 8.56%; *, $p < 0.05$). Taken together, these results indicate that LDN mimics Aβ oligomers not by inhibiting TrkB internalization, but by impairing downstream BDNF retrograde signaling.

UCH-L1 Rescues TrkB Retrograde Trafficking Deficits Caused by Aβ.

Next, we investigated whether BDNF-mediated transport deficits induced by Aβ could be rescued by increasing UCH-L1 levels using a TAT-HA-UCH-L1 construct as described previously (42). UCH-L1 treatment rescued BDNF-GFP retrograde trafficking deficits caused by Aβ (Fig. 6). In the presence of Aβ oligomers, BDNF-GFP levels in the soma were 60.3 ± 7.1% (*, $p = 0.003$), lower than vehicle-treated neurons. However, in the presence of UCH-L1 alone, BDNF-GFP levels were 92.6 ± 12.0% of vehicle only. UCH-L1 rescued the Aβ-induced deficit in BDNF-GFP levels in the soma to 86.3 ± 14.1% of the vehicle-treated neurons (*, $p = 0.04$).

Thus, we demonstrate that Aβ-mediated deficits in retrograde transport can be rescued by UCH-L1. Taken together with our LDN data (Fig. 5), these results

demonstrate that modulating ubiquitin homeostasis via UCH-L1 impacts BDNF-mediated retrograde trafficking.

UCH-L1 Is Decreased in the Hippocampus in APP-Tg2576 Mice and in AD. Next, we determined whether UCH-L1 levels are also affected in an AD transgenic mouse model. We measured UCH-L1 protein levels in the hippocampus or cortex of 15-month-old wild-type or Tg2576 mice. We found that hippocampal but not cortical UCH-L1 levels were significantly decreased in Tg2576 mice relative to age-matched wild-type mice (Fig. 7, A and B) (*, $p < 0.03$). Lastly, we investigated whether the decrease in UCH-L1 translates to the in vivo condition in humans. We compared UCH-L1 gene expression levels in the hippocampus and superior frontal cortex (BA9/46) in AD brain versus age-matched cognitively intact controls. UCHL-1 gene expression data were

obtained from a microarray database as described previously (44) and revealed that UCH-L1 gene expression was significantly reduced in the hippocampal regions of AD cases versus aged-matched controls (Fig. 7B; $p < 0.05$). The decrease in UCH-L1 expression levels was hippocampus-specific, because we did not detect a difference in UCH-L1 expression in the frontal cortex.

Discussion

Overall, our results demonstrate that soluble Aβ impairs BDNF/TrkB retrograde axonal trafficking and signaling. Building on our previous findings that Aβ oligomers cause a net decrease in the amount of BDNF/TrkB trafficked back to soma (22), this is not due to Aβ oligomers affecting the internalization of TrkB. Although previous studies

have demonstrated that Aβ can alter cell surface receptor internalization (for example, of AMPA and NMDA receptors) (49, 50), our data demonstrate that Aβ does not affect the internalization of TrkB and suggested that Aβ-mediated trafficking deficits were downstream of receptor internalization.

Using a novel microfluidic chamber that facilitates the study of axonal transport, we found that Aβ oligomers cause a decrease in the amount of BDNF-GFP that is found within endosomes that undergo retrograde axonal transport. In addition, we found that the average velocity of BDNF-GFP-containing endosomes was decreased in the presence of Aβ, with the distribution of the vesicle velocities shifted to ones with lower velocities. These data suggest that soluble Aβ impairs the sorting of BDNF/TrkB to MVBs, the endosomal compartment that mediates TrkB retrograde axonal trafficking, and also reduces the velocity of trafficking MVBs. Together, these deficits contribute to impaired BDNF/TrkB retrograde transport. It is of note that early in the progression of AD and prior to Aβ deposition, when Aβ oligomers are likely present, enlarged endosomal structures can be detected in neurons (54). It is tempting to speculate that the decrease in vesicle velocity is attributed to enlarged MVBs. Because proteins sorted to MVBs are also degraded in a lysosome-dependent process (33), our data are also consistent with the finding that Aβ impairs lysosome-mediated degradation of TrkB (35).

We also demonstrated that BDNF/TrkB axonal trafficking deficits induced by Aβ were accompanied by impaired downstream BDNF/TrkB signaling, notably impaired nuclear translocation of p-ERK5, and down-regulated CREB-mediated signaling events. These results reveal that the presence of soluble Aβ impairs retrograde trafficking, resulting in diminished signaling between axons and cell bodies, supporting the

signaling endosome hypothesis that describes how cellular signals that are initiated at axon terminals undergo retrograde transport and are propagated back to the soma (55). Furthermore, the disruption by Aβ may be due to a generalized impairment in trafficking because the trafficking of other organelles/cargos is also impaired by Aβ (47, 56–59).

Our data reveal that a mechanism underlying the retrograde trafficking deficits in the presence of Aβ involves altered ubiquitin homeostasis. Ubiquitin is central for proteasome-dependent protein turnover as well as for intracellular trafficking of cargoes including many receptors (i.e., glutamate and neurotrophins) that are fundamental for synaptic remodeling and plasticity (60). Although ubiquitin can be synthesized de novo, the bulk of the cellular ubiquitin pool is derived from ubiquitin that is recovered from deubiquitinating enzymes (61). Thus, ubiquitin levels can be regulated by modulating deubiquitinating enzyme levels or activity of deubiquitinating enzymes such as UCH-L1 (42, 62). Remarkably, UCH-L1 represents 1–2% of the total protein within the brain and thus is an important regulator of brain ubiquitin levels (62–64).

Ubiquitination regulates many important processes including the targeting and delivery of receptors to MVBs, including trophic factor receptors (65–67). For example, ubiquitination mediates the delivery of the well-studied EGF receptor to MVBs for its degradation within lysosomes (33). In the case of Trk receptors, ubiquitination may regulate its endocytic trafficking to MVBs for sustained retrograde signaling (34, 53, 68).

Therefore, we tested whether disrupting the ubiquitin recycling pathway, which regulates cellular ubiquitin levels, can lead to BDNF-dependent retrograde transport deficits. Indeed, we found that BDNF/TrkB retrograde trafficking and signaling could be affected by manipulating deubiquitinating activity by either inhibiting or increasing UCH-

L1. Specifically, inhibiting UCH-L1 with LDN resulted in retrograde trafficking deficits parallel to those found induced by soluble Aβ, and retrograde trafficking deficits caused by Aβ could be rescued by increasing cellular UCH-L1 levels.

Although ubiquitination mediates the internalization of numerous receptors, we found that neither Aβ nor impairing ubiquitin recycling with LDN affected TrkB internalization. Our data support the finding that TrkB ubiquitination is not a prerequisite for its internalization (34) and may only regulate its endocytic fate (69, 70). Taken together, these results suggest that Aβ impairs the retrograde trafficking of TrkB by affecting ubiquitin homeostasis via UCH-L1 at a step that is downstream from receptor internalization. Further, our data suggest that ubiquitin homeostasis may be impaired in the hippocampus in AD, in both Tg2576 mouse model of AD and in the human brain. We demonstrate that in Tg2576 mice, hippocampal but not cortical UCH-L1 protein levels are reduced compared with wild-type littermates, similar to the findings in the brain of APP/presenilin 1 mice at 4–6 months of age, suggesting that the decrease in UCH-L1 follows the development of pathology (42). In parallel, we demonstrate that hippocampal but not cortical UCH-L1 gene expression is decreased in the AD brain relative to age-matched cognitively intact cases. The reduced availability of UCH-L1 in the AD brain likely impairs neurotrophin signaling in vivo, based on our in vitro data, which found that inhibiting UCH-L1 caused deficits in TrkB/BDNF retrograde trafficking and signaling. In support of our hypothesis that Aβ itself directly affects UCH-L1 levels, a decrease in monomeric ubiquitin levels caused by Aβ is reversed by increasing UCH-L1 levels in hippocampal slices (42). These data add to the growing evidence that disrupted ubiquitin homeostasis is an important aspect of AD pathobiology, with

previous studies demonstrating that impaired deubiquitination alters synaptic protein distribution and spine morphology and causes neurodegeneration (62, 71), which are salient features in AD. Altered ubiquitin homeostasis may contribute to generalized axonal transport deficits observed in AD. Inducing lysosome dysfunction impairs axonal retrograde transport of late endosomes and lysosomes and leads to AD-like axonal pathology (72). Because the sorting of proteins to lysosomes is ubiquitin-dependent (73), it suggests that by altering ubiquitin homeostasis, Aβ can trigger lysosome dysfunction and the observed transport deficits. Furthermore, we and others have found that Aβ directly affects mitochondrial transport and may be due to defective fission/fusion (58, 75), which is regulated by ubiquitination (76). Thus, it suggests that ubiquitination/deubiquitination plays a vital role in regulating axonal transport. Lastly, balancing ubiquitination/deubiquitination may also affect Aβ production because APP ubiquitination inhibits APP endocytosis and promotes the nonamyloidogenic processing (77).

Our data plus a growing body of evidence suggest that in AD there may be a general defect in intracellular trafficking. Our results describe a novel mechanism by which Aβ can impair ubiquitin homeostasis that leads to endosomal axonal retrograde transport deficits, impairs neurotrophin signaling, and contributes to impaired synaptic plasticity. As Aβ accumulates, one of the consequences may be impaired intracellular trafficking of cellular components that depend on ubiquitin conjugation for signal transduction and protein sorting and degradation. Defective trafficking in the etiology of AD is supported by the recent identification of GWAS-AD-linked polymorphisms that encode proteins linked to endosome function, e.g. PICALM and BIN1 (74). Therefore,

therapeutics aimed at modulating ubiquitin homeostasis may rescue intracellular trafficking deficits found in AD and improve cognition.

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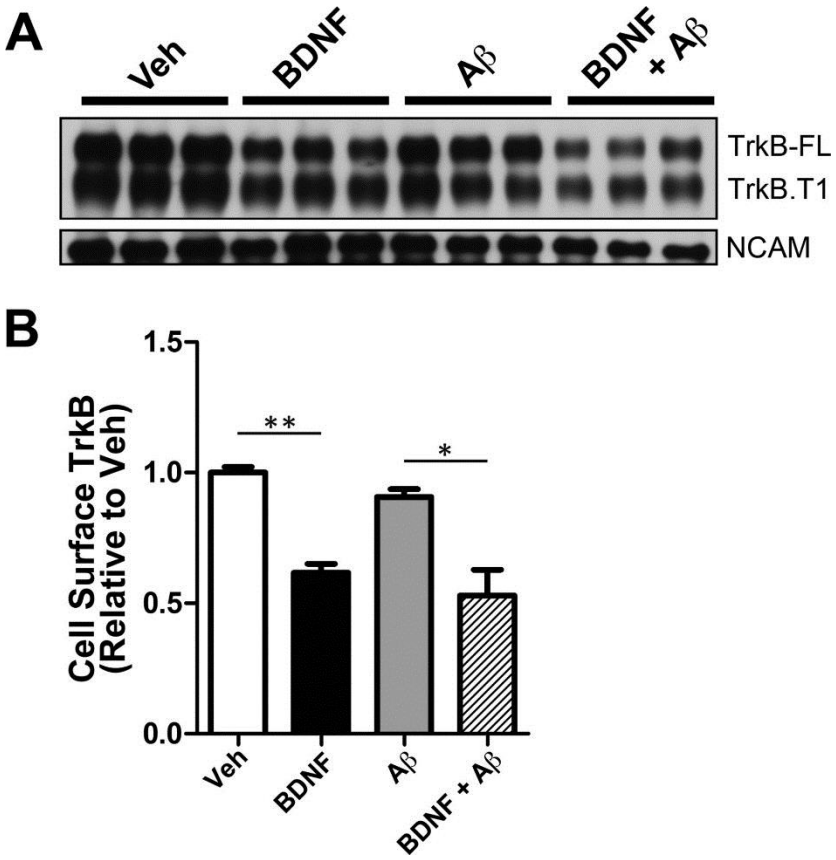
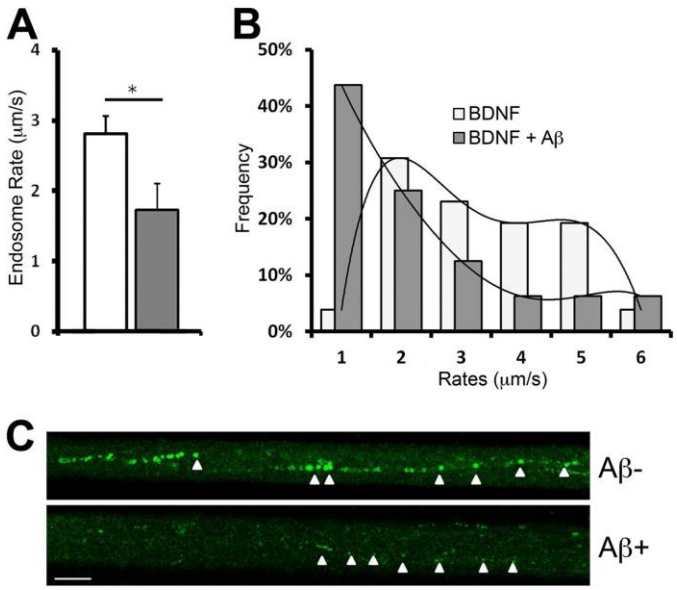
Figure 2.1



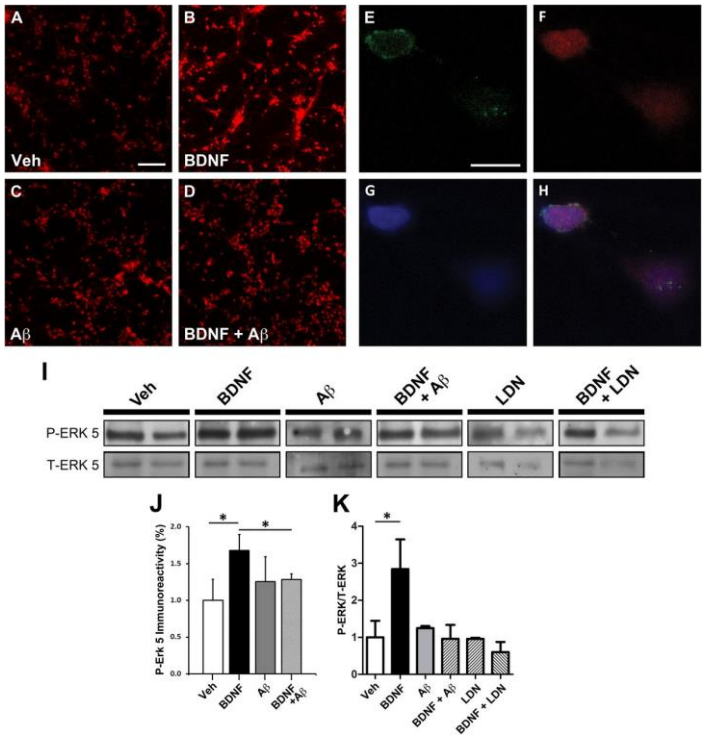
Aβ oligomers do not affect the internalization of BDNF receptors.
A, cell surface biotinylation was employed to measure TrkB levels at the cell surface following BDNF treatment (50 ng/ml, 30 min) as described under "Experimental Procedures." BDNF addition led to a decrease in cell surface levels of full-length TrkB (TrkB-FL) and truncated TrkB (TrkB.T1). Preincubation with Aβ oligomers (24 h) does not impair TrkB-FL or TrkB-T1 internalization. BDNF specifically caused internalization of BDNF receptors, but not the internalization of neural cell adhesion molecule (NCAM). B, cell surface TrkB-FL was quantitated using ImageJ (National Institutes of Health). The mean ± S.E. represents TrkB-FL levels normalized to vehicle-treated neurons (n = 3). Following BDNF treatment, we found that 38.3 ± 3.40% of TrkB-FL was internalized (black bar) when compared with vehicle (white bar) (**, $p < 0.001$). In the presence of Aβ, BDNF led to 41.5 ± 9.80% of TrkB-FL internalized (hatched bar) when compared with Aβ-only (gray bar) (*, $p < 0.05$). Veh, vehicle.

Figure 2.2



Aβ oligomers impair the trafficking of BDNF-GFP endosomes.
A, in the presence of Aβ, the average velocity of BDNF-GFP-containing endosomes was 1.73 ± 0.378 μm/s. This represented a 38.4 ± 13.4% (*, $p < 0.01$) decrease when compared with the average velocity of endosomes in the absence of velocities of BDNF-GFP-positive endosomes determined as described previously (22). B, distribution plot of the vesicle velocities reveal that in the presence of Aβ oligomers, the percentage of vesicle velocities >2 mm/s was greatly reduced (gray bars), and the majority of the vesicle velocity was <1 μm/s. C, representative time lapse image of BDNF-GFP containing endosomes demonstrates the amount of BDNF-TrkB complex that undergoes retrograde transport of the (from right to left). Scale bar, 10 μm.

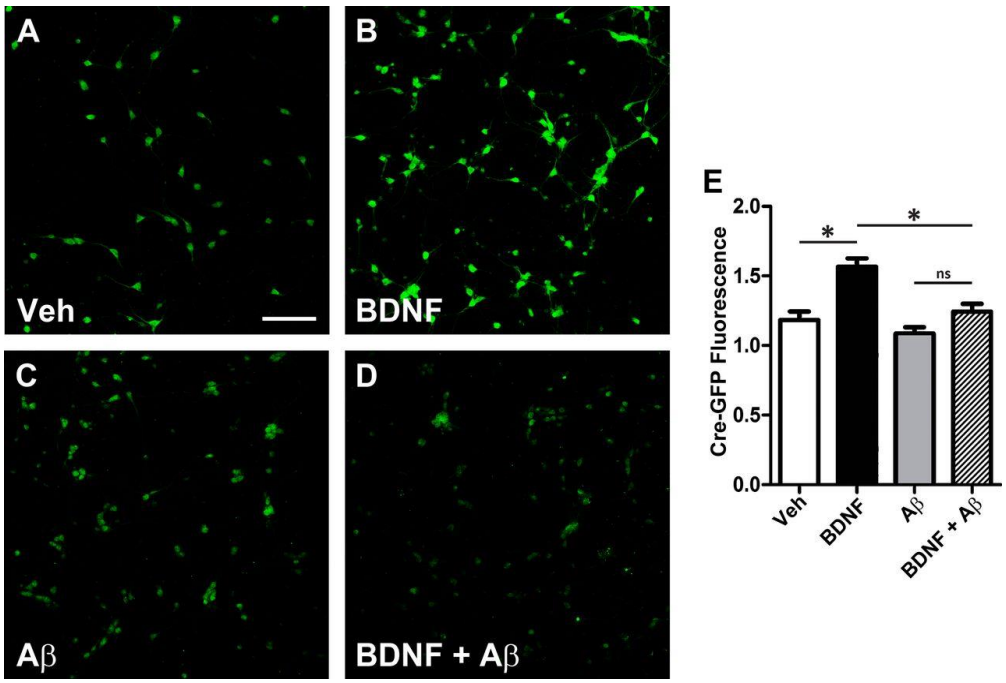
Figure 2.3



Aβ oligomers impair the trafficking of the signaling endosome complex including p-ERK5.

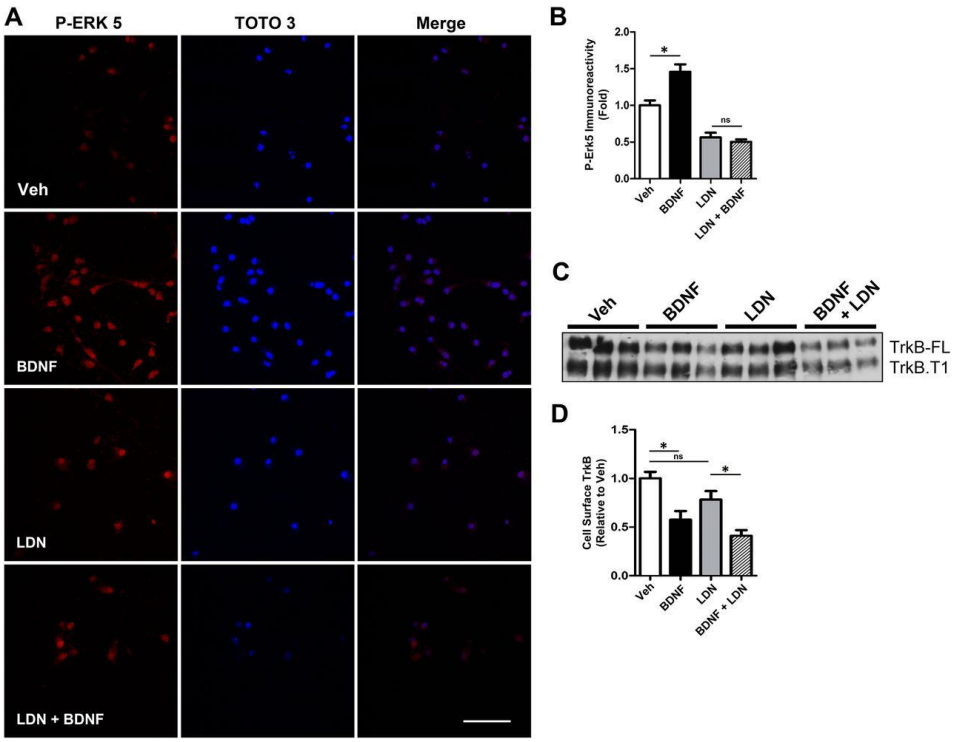
Microfluidic devices were employed to measure the retrograde transport dependent ERK5 activation. A–D, representative images demonstrating p-ERK5 levels (red) in the somal compartment of microfluidic chamber of vehicle-treated neurons (A) and following BDNF treatment (B), in the presence of Aβ oligomers only (C), and after BDNF treatment (D). Scale bar, 200 μm. E–H, representative images of a neuron demonstrating the co-localization of BDNF-GFP (green) on the outer surface of the nucleus (blue). Also, p-ERK5 (red) co-localized with the nucleus, suggesting that the BDNF-mediated retrograde signal, i.e., the signaling endosome, undergoes retrograde transport from the axonal compartment to the soma and specifically the nucleus. Scale bar, 20 μm. I, Western blot analysis of p-ERK5 and T-ERK5 isolated from the somal compartment of microfluidics devices as described under "Experimental Procedures." J, somal p-ERK5 was quantitated as described under "Experimental Procedures." BDNF leads to a 68.1 ± 8.4% (*, $p < 0.05$) increase in p-ERK5. However, in neurons preincubated with Aβ oligomers, p-ERK5 levels are not increased following axonal BDNF treatment. K, quantification of p-ERK5 relative to total ERK5 levels in the somal compartment following BDNF treatment. BDNF leads to a 284% increase in p-ERK5. However, in the presence of Aβ oligomers, BDNF does not lead to increased p-ERK5. Also, a UCH-L1 inhibitor (LDN-57444) was used to inhibit deubiquitinating activity and revealed that it could mimic the effect of Aβ oligomers in impairing BDNF-mediated retrograde signaling. Veh, vehicle; LDN, LDN-57444.

Figure 2.4



Aβ oligomers lead to decreased CREB-dependent gene expression.
Microfluidic devices were used to assess CREB-mediated gene expression. Rat primary neurons (embryonic day 18) were transfected with a CRE-GFP reporter construct to assess CREB-mediated gene activation. At 7 DIV, the axonal compartment was treated with BDNF (50 ng/ml, 2 h), and the chambers were processed for immunochemical analysis as described under "Experimental Procedures" using polyclonal anti-GFP (Invitrogen) to measure CRE-GFP levels and were normalized to the neuronal marker, BIII-tubulin (red). A, representative image of CRE-GFP levels (green) within the somal compartment and co-imaged with the neuronal marker, BIII-tubulin (red) in vehicle-treated neurons, and in neurons treated with BDNF. B and C, in the presence of Aβ oligomers, base-line levels of CRE-GFP are not significantly reduced when compared with vehicle. D, in the presence of Aβ oligomers, the increase in the amount of CRE-GFP is greatly reduced. E, CRE-GFP levels were quantified, and the means ± S.E. represent n = 4 and demonstrate that BDNF treatment to the axonal compartment led to a 35.9 ± 4.73 (*, $p < 0.01$) increase in somal CRE-GFP immunoreactivity when compared with vehicle (white bar). However, in the presence of Aβ oligomers, CRE-GFP was only increased by 14.6 ± 5.23%, when compared with Aβ oligomer only cells, but this was not significant ($p = 0.076$). Therefore, in the presence of Aβ, axonal BDNF leads to reduced CRE-GFP immunoreactivity (hatched bar) when compared with vehicle treated neurons (black bar) (*, $p < 0.01$). Scale bar, 200 μm. Veh, vehicle.

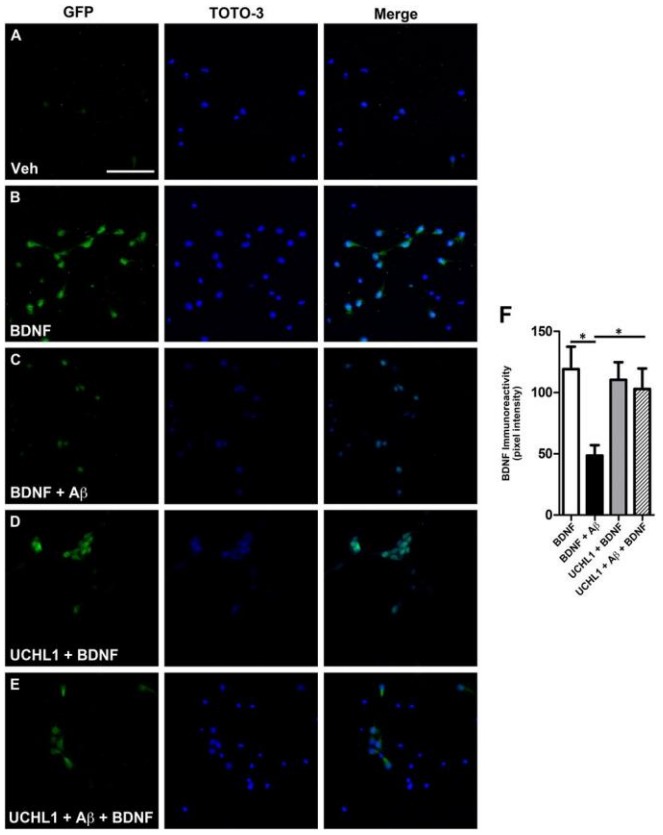
Figure 2.5



The UCH-L1 inhibitor LDN mimics the effect of Aβ oligomers on BDNF-dependent retrograde signaling.

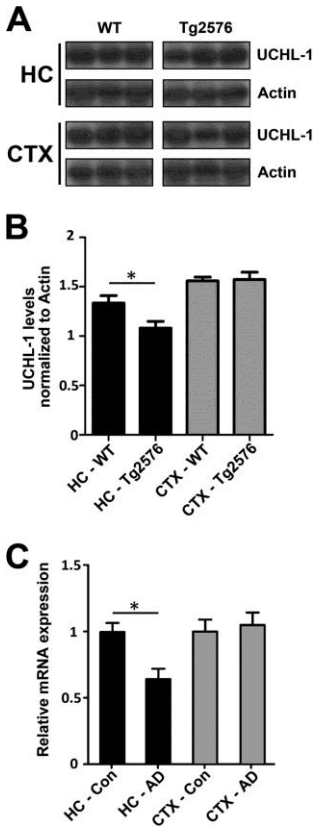
To assess the effect of LDN on BDNF-dependent retrograde signaling, we measured p-ERK5 activation in the presence of LDN. A, representative images that demonstrate that BDNF led to an increase in somal p-ERK5, but the UCH-L1 inhibitor, LDN, led to decreased basal somal p-ERK5, and in the presence of BDNF, the nuclear translocation of p-ERK5 is not detected. p-ERK5 immunoreactivity was normalized to the nuclear counterstain, TOTO-3. B, quantification of somal p-ERK5 levels demonstrate that although BDNF treatment (black bar) leads to a 45.7 ± 10.2% (*, $p < 0.05$) increase in p-ERK5 when compared with vehicle (white bar), in neurons preincubated with LDN (gray bar), we do not observe an increase in somal p-ERK5 following BDNF (hatched bar). C, cell surface biotinylation assays were employed to determine the effect of LDN on TrkB internalization and demonstrate that LDN does not affect the internalization of full-length TrkB (TrkB-FL) or truncated TrkB (TrkB.T1). D, quantification of cell surface TrkB-FL demonstrates that in vehicle-treated neurons (white bar), the addition of BDNF (black bar) led to a 45.7 ± 10.2% (*, $p < 0.05$) decrease in cell surface TrkB levels, whereas in the presence of LDN (gray bar), BDNF led to a 44.3 ± 8.56 (*, $p < 0.05$) decrease in TrkB-FL. The mean ± S.E. represents n = 3. Scale bar, 20 μm. Veh, vehicle; LDN, LDN-57444.

Figure 2.6



Transduction of UCH-L1 rescued Aβ-mediated retrograde transport deficits.
To assess whether increasing UCH-L1 could rescue Aβ-mediated transport deficits, we measured the extent of BDNF-GFP trafficking in neurons transduced with UCH-L1. A and B, representative images demonstrate that somal levels of BDNF-GFP are increased in neurons following BDNF-GFP treatment. GFP immunoreactivity was normalized to the nuclear marker, TOTO-3. C, pretreatment with Aβ led to decrease in BDNF-GFP when compared with vehicle-treated neurons. D, the addition of UCH-L1 alone led to increased somal BDNF-GFP immunoreactivity. E, UCH-L1 rescues the deficit in BDNF-GFP trafficking caused by Aβ. F, quantification of somal BDNF levels normalized to cell number (TOTO-3-positive nuclei) reveals that Aβ causes a 60.3 ± 7.1% (*, p = 0.003) decrease in the retrograde transport of BDNF-GFP back to soma compared with vehicle-treated neurons. In the presence of UCH-L1, BDNF-GFP levels were 92.6 ± 12.0% of vehicle plus BDNF. Importantly, UCH-L1 rescued the deficit in BDNF-GFP trafficking caused by Aβ. BDNF-GFP levels were 86.3 ± 14.1% of vehicle treated and revealed that UCH-L1 restored trafficking deficits caused by Aβ oligomers (*, p = 0.04). Scale bar, 200 μm. Veh, vehicle.

Figure 2.7



UCH-L1 is decreased in Tg2576 mice and in the Alzheimer disease brain.

A, levels of UCH-L1 are decreased in APP-Tg2576 hippocampus. Hippocampal lysates were prepared as described under “Experimental Procedures.” Protein was separated on SDS-PAGE, and Western blot analysis was carried out to determine the amount of UCH-L1 protein in wild-type and Tg2576 mouse brain. B, UCH-L1 protein levels are decreased within the hippocampus but not the cortex of 15-month-old Tg2576 mice (*, $p < 0.03$). UCH-L1 levels were quantitated and normalized to actin protein as described under “Experimental Procedures.” C, UCHL-1 gene expression is lower in AD brain. Expression profiles were obtained from a microarray database consisting of brain tissue from AD cases (n = 26; range, 74–95 years; mean age, 85.7 ± 6.5 years) and age-matched controls (n = 33; range, 69–99 years; mean age, 84.2 ± 8.9 years) and were generated using Affymetrix HgU133 plus 2.0 arrays as described previously (44). Two probe sets corresponding to UCHL-1 (Unigene Hs.518731) were identified on the HgU133 plus 2.0 array, both of which had Present flags in all microarrays, indicating high expression reliability of the probes. Expression values were averaged across the probe sets to obtain an overall value for each case, followed by t test comparisons for each region and significance set at $p < 0.05$ (*).

Chapter 3

Inflammation Induced attenuation of BDNF signaling is associated with reduced endosomal transport.

Abstract

Pro-inflammatory cytokines accumulate in the brain with age and Alzheimers disease (AD), which is linked to impaired neuron health and poor cognitive function. BDNF is a key growth factor signaling system for neuron health, function and synaptic plasticity. Proinflammatory cytokine IL-1β impairs BDNF signaling transduction pathways, but whether it affected the BDNF signaling endosome itself was not known. In this study, we elucidate the nature of impaired BDNF transport by IL-1β. We found that IL-1β attenuated the local dispersion of BDNF signaling endosomes throughout networks in primary hippocampal neuronal cultures. In IL-1β-treated cells, overall BDNF endosomal density was decreased and colocalization to presynaptic terminals was found to be more than two times higher than controls. Neurons cultured in microfluidic chambers also exhibited transport deficits. Selective IL-1β treatment to the presynaptic compartment in microfluidic chambers attenuated BDNF endosome flux, as measured by reduced BDNF-GFP endosome counts in the somal compartment. IL-1β impaired BDNF endosomal transport and also partially inhibited phospho-Erk5 (P-Erk5), a known BDNF retrograde trafficking target. Mechanistically, the deficiency in trafficking was not due to impaired endocytosis of the BDNF-TrkB receptor complex. Nor did the nature of the transport deficit seem to involve factors that affect the rate of transport, since BDNF endosomes traveled at the same rate in both controls and IL-1□ treatment groups. We suggest that the deficit resides in an impairment of endosomal trafficking from the terminal and its loading onto the retrograde transport system. Our work thus demonstrates a critical uncoupling between BDNF and downstream trafficking targets by IL-1β.

Introduction

Signaling by BDNF via the TrkB receptor is an important mechanism supporting neuronal survival and activity-dependent plasticity which is critical for cognitive function (1). Receptor trafficking, including endocytosis, sorting and transport, is key to BDNF signaling (2,3). BDNF follows many of the central tenets of the signaling endosome hypothesis (4). BDNF binds to its receptor, TrkB, and signal transduction is mediated though the receptor tyrosine kinase domain of the TrkB receptor. The activated complex is trafficked to both local and distal subcellular compartments in endosomal vesicles. If the signal initiates on the presynaptic terminal, the vesicle-bound neurotrophins retrogradely traverse intracellular compartments by dynein motors and are regulated by Rab GTPases (5-7). These endosomes therefore can carry complete signaling complexes capable of activating multiple intracellular cascades leading to plasticity and survival. In cultured neurons, retrograde trafficking of BDNF signaling endosomes originating in presynaptic compartments activates somal Erk5 (8).

Highlighting the significant role endosomal trafficking dysfunction may play in neurological diseases comes from the pathological enlargement of early endosomes in the Alzheimers (AD) brain and that many AD-related genes identified as risk factors by genome-wide association studies (GWAS) are involved in different stages of endosomal trafficking (*e.g.,* PICALM, SORL1) (9,10).

Interleukin-1β (IL-1β) is a key component of the microglial-mediated immune response in the brain and has deleterious effects on cognition and synaptic plasticity at high or chronic levels of exposure (11). In the mouse hippocampus, sustained expression of IL-1β impairs contextual and spatial memory (12). IL-1β suppresses LTP,

and BDNF-induced Akt, and CREB activation (13,14), while others have shown a link between acute neuroinflammation and disruption of specific neural circuit functions and cognitive impairment (15). Studies have also shown that blocking IL-1β can restore cognitive function (16). The effect of IL-1β is not only central, as even peripheral immune insults are enough to induce reversible cognitive decline (17-19). We hypothesize that the presence of IL-1β undermines proper endosomal function and compromises BDNF/TrkB signal transduction and synaptic plasticity, placing the brain at risk for cognitive decline and neurodegeneration.

Here we introduce a new mechanism in which IL-1β fundamentally alters BDNF-TrkB endosomal trafficking, leading to measurable deficits in downstream target Erk5. The nature of the trafficking aberration is not related to vesicle transport rate, but rather endosome flux. We suggest that in the presence of IL-1β, BDNF-TrkB endosome transport is impaired, leading to downstream signaling deficits. Since both inflammation and BDNF signaling pathway deficits are an invariant feature across multiple neurological diseases (20), our work underscores the importance of further research into understanding how BDNF signaling deficits could be fundamentally associated with underlying trafficking deficits.

Experimental Procedures

Primary Neuronal Cultures and Treatments. The use of all animals was approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Primary neuronal cultures were obtained from embryonic day 18 (E18) rat embryos. Dissociated cells were cultured on either pre-coated poly-L-Lysine 6-well plates (Biocoat

plates, BD Bioscience) or poly-L-Lysine coated glass coverslips affixed to microfluidic chambers at 1 x 106 cells/9.5 cm2 and 5 x 106 cells/ml (for microfluidic chamber), respectively. All cells were maintained in Neurobasal (Gibco) supplemented with B27, penicillin/streptomycin, and Glutamax at 37o, 5% CO2, 95% humidity. Rat recombinant Interluekin-1β (PeproTech) was used at a final concentration of 10 ng/ml. Human BDNF (PeproTech) was used at a concentration of 50 ng/ml and Ciliobrevin D (Sigma) was dissolved in Sterile Me2SO at 100μM.

Microfluidic Chambers. The chambers were fabricated in PDMS using rapid prototyping and soft lithography similar to previously published procedures (21). Glass coverslips (24 × 40 mm, No. 1, Corning) 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) in PBS (24 h, 5% CO2, 37°C incubator), rinsed, and allowed to air dry in a sterile hood. The chambers are noncovalently assembled and remained affixed to glass coverslips by conformal contact. The chambers consist of two parallel microfluidic compartments, connected by inlet and outlet wells. The two compartments are separated by a solid barrier region with microgrooves embedded in the bottom of the connecting barrier. A volume difference between the two compartments of 20 μl is used to generate a fluidic resistance within the microgrooves, facilitating the isolation of treatments.

Live Cell Trafficking Experiments. Endotoxin-free BDNF-GFP plasmid was introduced by nucleofection (Lonza Amaxa) into HEK293 cells followed by selection in DMEM containing 10% FBS and G418 (1 mg/ml, plasmid contains a neomycin cassette). BDNF-GFP was isolated from stable pre-pro-BDNF-GFP HEK293 lines after cells reached confluency. Secreted BDNF-GFP from the media was removed and

concentrated with Amicon YM-30 centrifugal filters (5000 x *g*, 2 h) (30,000 MW cutoff, Millipore). Pro-BDNF was converted to mature BDNF-GFP by treatment with plasmin enzyme (Sigma). Time lapse microscopy on live cells was utilized to measure the rates of BDNF-GFP-containing endosomes within the microfluidic devices. Rat primary neurons were imaged using an inverted confocal microscope and a 63x oil emersion objective. Regions of interest from axon segments from each chamber were randomly selected for time-lapse imaging. Prior to treatment, 20% of the cell culture media volume was removed from the axon terminal (treated) side of the chambers. BDNF-GFP or BDNF-GFP+IL-1β were then introduced to the wells. After 1 hour, transport rates were quantified on an inverted Olympus IX70 with a 63x oil emersion objective. The objective was focused on randomly chosen microgrooves of the chamber and imaged in a time-lapse in 5 sec intervals for 1 min. Image stacks from the time-lapse were imported into ImageJ and tracked using ManualTracker. These results were independently verified using GradienTech Particle Tracking Tool Software. For speed measurements, we used the Accumulated distance to time ratio. For velocity measurements we used the Euclidian Distance to time ratio.

Cell Surface Biotinylation Assays. To assess TrkB internalization, primary neurons (7 DIV) were either treated with or without BDNF (50 ng/ml) and then placed on ice to prevent further TrkB internalization. The remaining cell surface TrkB receptors were biotinylated with Sulfo-NHS-LC-Biotin (100 mg/ml, 30 min; Thermo) and then washed with 0.1 M PBS (pH 7.5), three times. The cells were lysed with radioimmunoprecipitation assay buffer containing a protease inhibitor mixture (Roche Applied Science), and biotinylated TrkB was precipitated with streptavidin-agarose

beads (Thermo, 50µl) that had been pre-equilibrated in radioimmunoprecipitation assay buffer. To elute, precipitated proteins were incubated in sample buffer and processed for Western blot analysis.

Western Blot Analysis and Antibodies*.* Following boiling or elution in Laemelli Sample Buffer lysates were run on a complete Bio-Rad Western Blotting System. Samples were electrophoretically separated on Bio-Rad 10% Tris-TGX PreCast Gels and blotted onto nitrocellulose membranes using BioRad Trans Turbo Dry transfer and buffer. All blots were blocked using 5% BSA. The following is a list of all primary antibodies used. Phospho-Erk5 (T218/Y220) (1:200, Cell Signaling), ERK5 (1:1000, Cell Signaling), TrkB (1:1000, EMD Millipore). Immunoreactivity was measured BioRad HRP conjugated secondaries. Blots were developed on a Bio-Rad Digital Detection System running ImageLab software, using Millipore Crescendo chemiluminescence development reagent. ImageJ was used for band densitometry analysis. ImageJ quantification metrics and western blots from the same lysate were ensured to be reasonably reproducible and all experiments were reproduced using completely independent cultures from different E18 litters.

Immunocytochemistry and Antibodies. Cells were immediately placed on ice and washed gently in ice-cold HBSS. Cells were then fixed in fresh, ice-cold 4% paraformaldehyde for 20 min. The microfluidic chamber devices were removed from the coverslip and fixed again in 4% paraformaldehyde. Cells were blocked (cold 5% BSA in 0.1M PBS) and permeablized (0.02% Triton-X100 in 0.1M PBS) followed by primary antibody, overnight. The following antibodies were used in this study (Anti-GFP 488 conjugate, Life Tech, 1:200; anti-synaptophysin, Millipore, 1:200; tau-1 (1:200), followed

by Alexa conjugated goat-anti-X secondary antibody (Life Tech, 1:200, X = primary host). All images were collected using a Leica confocal microscope. ROIs were randomly chosen and imaging proceeded serially and sequentially. Data was obtained from at least 2 independent experimental samples.

Vesicle Nanoparticle Tracking and Isolation. Rat neuronal cultures >12DIV were gently homogenized in 0.32 M sucrose. The extracts from individual wells, containing 1 x 106 neurons, were pooled. The extract of pooled vesicles was first centrifuged at 1000 x *g* for 10 min; the resulting supernatant was centrifuged at 13,000 x *g* for 20 min to obtain the crude P2 pellet. Following isolation, the particles were characterized on a Malvern NS300 running an NTA platform to obtain measures of homogeneity and size distributions. The vesicles were subjected to established immunolabeling protocols to for flow cytometry analysis according to a method published by Cell Signaling Technologies (www.cellsignal.com), reproduced here, in brief. Pellets were fixed in 2% paraformaldehyde and permeabilized in 90% Methanol. For short term storage, our samples remained in 90% methanol at -20^{o}C. Anti-Synaptophysin IgG was labeled directly with Alexa Fluor 647 (Cell Signaling) and used as a positive indicator of presynaptic compartmentalization within isolated P2 vesicles. After labeling, pellets were washed twice and then resuspended in PBS buffer (0.5 ml) for flow cytometry analysis. Data were acquired using a BD-LSR I analytic flow cytometer equipped with argon 488 nm and helium-neon 635 nm lasers. Twenty thousand particles were collected and analyzed for each sample. Debris was excluded by establishing a background flourescence threshold set by immunolabeling with host-IgG-647 isotype matched control on a non-GFP treated sample. Comparisons were made by from 3

independent pooled samples. Percent gated events positive for both synaptophysin and GFP were used for the analysis.

Results

IL-1β has been previously been shown to impair BDNF signaling pathways. The question remained whether IL-1β fundamentally affects the trafficking of the BDNF endosomal signaling complex itself. Here, we sought to define the nature and extent to which IL-1β could affect BDNF endosome trafficking.

Following endocytosis, we and others have observed that BDNF (and NGF) signaling endosomes are distributed from the presynaptic terminals in cultures (22) and ultimately delivered to the neuronal cell bodies to activate BDNF signaling effectors such as Erk5. BDNF trafficking is essential for general maintenance of neuronal networks, synaptic function, and cell survival (23). We propose that IL-1β induces BDNF trafficking deficits that may be associated with a reduced rate of BDNF endosome dispersion initiated from the presynaptic terminals in hippocampal neurons. Among the cells treated with IL-1β, 21% of all BDNF-GFP endosomes were found to be colocalized to presynaptic marker synaptophysin compared to only 11% of BDNF-GFP without IL-1β ($p < 0.05$, Fisher's Exact Test), suggesting there was a significant association between IL-1β treatment and localization of these vesicles (Figure 1A-G). To confirm and extend these observations and measure presynaptic BDNF-endosome clustering by another method, we isolated P2 fraction vesicles from neuron cultures with a defined size distribution shown in Figure 1H, obtained by Malvern's Nanoparticle Tracking Analysis technology and NS300. Using a synaptosome immunostaining protocol, we

examined the population of vesicles that were positive for presynaptic marker synaptophysin, as an indication that this population of isolated vesicles originated from a presynaptic compartment and would contain that synaptic BDNF-GFP at the time of collection. By this method, we found that size-gated events positive for both synaptophysin and BDNF-GFP were increased in cells exposed to IL-1β (Figure 1I), suggesting increased colocalization of BDNF-GFP at presynaptic terminals by two independent experimental methods.

Next, we wanted to know if the consequence of a lack of presynaptic terminal-initiated dispersion of BDNF endosomes observed in Figure 1 could be observed long range. For this experiment, we used microfluidic chambers we developed that allow the fluidic separation of axons and presynaptic boutons from cell bodies. This cell culture platform directs the growth of hundreds of aligned axons from one compartment through a barrier region into an adjacent fluidicially separated target compartment. Neurons introduced into a target compartment allow pre- and post-synaptic populations to be defined and independently labeled and manipulated. Thus, any BDNF-GFP introduced to the presynaptic terminal side can only arrive in the somal compartment via long range intra-axonal retrograde trafficking mechanisms (Figure 2A, C). Our experiment is therefore designed to assess long range trans-axonal flux of BDNF endosomes (Figure 2B). We selectively introduced BDNF-GFP or BDNF-GFP+IL-1β to presynaptic boutons for 1 h, after which BDNF-GFP cargo in the somal compartment was quantified as a measure of long range trans-axonal flux. We found that IL-1β treatment reduced BDNF-GFP endosome counts by 29 ± 10% relative to BDNF-GFP-only treatment controls (100

± 6%, n = 9, p < 0.05) somal compartments (Figure 2D-H), suggesting long-range BDNF endosome flux is attenuated by IL-1β.

If trafficking deficits are associated with IL-1β, then we would expect a long-range BDNF trafficking target to exhibit concomitant impairment. Erk5 is a well-established target kinase of BDNF-TrkB endosomes and a central signaling component of gene transcription related to neuronal survival. Evidence has shown that Erk5 becomes phosphorylated in the presence of BDNF and typically demonstrates somal localization and nuclear translocation upon neurotrophin-induced phosphorylation. We therefore tested whether IL-1β could cause downstream signaling deficits to BDNF endosome-induced phosphorylation of Erk5. In cultured neurons, our results indicate that BDNF treatment (50 ng/ml) increases phosphorylation of Erk5 by 210% baseline (± 16%, n = 4 independent cultures), while the BDNF+IL-1β group only increased P-Erk5 levels to 120% baseline (± 23%, n = 3 independent cultures, unpaired t-test, two tailed, $p < 0.05$) (Figure 3A). To mitigate other possible confounding factors such as BDNF dependent activation of Erk5 via non-trafficking pathways (somal TrkB, signaling cascades, etc), we inhibited the retrograde trafficking pawthway using Ciliobrevin D, a cell-permeable benzoyl dihydroquinazolinone derivative that acts as a reversible and specific blocker of ATPase motor cytoplasmic dynein. The remaining BDNF-induced P-Erk5 would indicate the contribution of non-trafficking dependent p-ERK5 activation. Figure 3B shows the western blot analysis of P-Erk5 levels in response to BDNF and BDNF+Ciliobrevin D treatment groups. BDNF-dependent P-Erk5 increased to 180% baseline (± 14%), while Ciliobrevin D attenuated the level of BDNF-induced P-Erk5 to 108% of baseline (± 25%, n = 5 independent cultures, t-test two tailed, $p < 0.05$). Since inhibiting trafficking

produced baseline P-Erk5 levels, we conclude that retrograde trafficking accounts for most, if not all, of the P-Erk5 activation in our cultures. Finally, we confirmed that the IL-1β effect on P-Erk5 is not restricted to *in vitro* cultures by testing using an acute brain slice assay from 3 month old rats. Coronal slices prepared fresh from sacrificed rats were treated with recombinant BDNF in the left hemispheres and were compared to BDNF+IL-1β treatments in the animal's corresponding right hemisphere. Thus comparisons of P-Erk levels as a function of treatment were between hemispheres from the same animal. Consistent with the data from our neuron culture experiments, we found BDNF-induced P-Erk5 in cultures treated with IL-1β by 14% (n = 4, t-test paired, two tailed, $p < 0.05$) (Figure 3C).

Given the association between IL-1β and endosome flux, we next sought to identify a link between IL-1β and changes to endosome flux. Since endosome dispersion/localization can be affected by the rate of transport, we quantified the velocity and speed in live-cell retrograde trafficking experiments to determine if IL-1β-induced endosomal trafficking deficits are directly caused by reduced endosome transport rate. BDNF-GFP particles exhibited a mean rate of 0.35 μm/sec (n = 10), compared to BDNF-GFP endosomes in cells treated with IL-1β, which exhibited a slightly faster but not significantly different mean rate of 0.5 μm/sec (n = 8). From these data, we conclude IL-1β does not reduce BDNF-GFP endosomal trafficking rates (Figure 4A). To further analyze the character of BDNF endosomal motility, velocity/speed ratios were used as a metric to elucidate endosome directedness, thus asking how much of a retrograde endosome's motion is actually concentrated in a retrograde direction. This metric would be especially sensitive to motile characteristics such as vesicle stalling. Again, we found

that the velocity/speed ratios of vesicles in both groups exhibited very similar mean values around 90% retrograde directedness for both groups (Figure 4B, BDNF: mean ± SEM = 93 ± 3%, n = 10, BDNF+IL-1: mean ± SEM = 87 ± 5%), collectively suggesting that neither rates nor transport characteristics are appreciably altered by IL-1β.

We next tested whether BDNF-TrkB endocytosis itself was impaired. If less BDNF-GFP endosomes were internalized, it could account, in part, for reduced BDNF-GFP counts. We used a cell surface biotinylation assay (EZ-Link LC-Biotin, Thermo) to measure whether BDNF-induced TrkB internalization was at all affected by IL-1β. We could find no evidence that IL-1β impaired endocytosis of BDNF (Mean ± SE =72 ± 9%, n = 2 independent time courses vs BDNF+IL-1β = 0.70 ± 10%, n = 2 independent time courses). In fact, to our surprise, IL-1β may be associated with a slight increase in endocytosis at earlier timepoints (Figure 5). We suggest that after internalization, BDNF-TrkB endosomes accumulate in the terminal and are not efficiently loaded onto the retrograde transport system to the soma in the presence of IL-1B.

Discussion

Our goal was to determine if IL-1β could cause BDNF signaling defects by altering retrograde transport of BDNF endosome trafficking, and to identify the nature of impairment. We found that the presynaptic initiated BDNF-GFP endosomes are less diffuse throughout neuritic networks in cultures following IL-1β treatment, consistent with the possibility that retrograde transport is disrupted. In addition, we show that neurons cultured in microfluidic chambers, that fluidically isolate axons from the cell somas, exhibited sustained long-range retrograde transport deficits that could be observed

when BDNF-GFP is added to the neurite compartment. The cultures that exhibited long-range trafficking deficits also demonstrated inhibition in the phosphorylation of BDNF endosome retrograde trafficking target, Erk5.

The long-range distance that neurotrophin-receptor complexes have to cover in addition to the highly coordinated and complex nature of retrograde transport represents a vulnerability in neurons. Previously we reported that Aβ applied to axons in microfluidic chambers caused an impairment in retrograde flow. Consistent with our data on IL-1β, Aβ resulted in long-range BDNF transport deficits leading to suppression of downstream Erk5 activity (24). However, different from IL-1β action, the effect of Aβ was associated with a reduced rate of BDNF endosomal transport. This suggests that Aβ and IL-1β, although mechanistically distinct, may be acting synergistically on the retrograde trafficking pathway.

Our study shows that while IL-1β reduces retrograde BDNF endosome trafficking, IL-1β does not measurably affect actual transport rates of BDNF endosomes or endocytosis of BDNF. This data suggests that the deficit resides in an impairment of endosomal trafficking from the presynaptic terminals and its loading onto the retrograde transport system. There are several possible mechanisms that may contribute to the effect of IL-1β on BDNF endosome trafficking, including alterations to trafficking accessory protein complexes such as Rab/Rac family, retromer complexes, changes to Actin dynamics. Many of these mechanisms would lead to accumulation of BDNF endosomes in presynaptic terminals.

Actin dynamics has been shown to play a key role in the regulation of early endosome dynamics in Caenorhabditis elegans (25). In this paradigm, disruption of

Arp2/3 complex, which regulates branched actin dynamics, led to significantly larger early endosomes but did not affect endocytosis (ref. b). Our previous studies along with our current unpublished results strongly suggest that F-actin dynamics are disrupted by IL-1β, which would stand as viable mechanism for presynaptic misrouting of endosomes.

Our results are significant in the context of previous studies that have implied disrupted vesicle transport. One of the earliest pathological features in the AD brain is the accumulation of vesicles, a finding that has been observed in both animal and cell-based models (26). The etiological mechanism of enlarged presynaptic vesicles in disease remains largely unknown. Studies have shown early endocytic changes like increased volume of early endosomes in early stage neurological disease (27), which may reflect the capacity of IL-1β to redirect delivery of endosomal cargo to autophagic pathways (28,29). Even in studies on other neurological disorders, the phenotype that implicates fundamental changes to endosomal trafficking have been observed. One study reports that endosomal accumulation of APP in motor neurons reflects impaired vesicle trafficking in amyotrophic lateral sclerosis (30), while other reports have shown that α-synuclein multimers cluster synaptic vesicles and attenuate trafficking (31). Taken in context with our findings, neuropathology and chronically upregulated inflammation may act synergistically to incapacitate the steady-state vesicle flow throughout the cell. It follows that the lack of steady state endosomal circulation would lead to adverse outcomes.

Endosomal dysfunction can be interrelated to a number of signaling deficits, as well. For example many neurotrophic pathway deficits can be elicited by IL-1β, including impaired activation of Akt, CREB. Since signaling cascades depend on ligand-receptor activation and since activated receptors are distributed intracellularly via the endosome transport, it follows that proper endosomal transport is a vital component of many signaling cascades. Endosomal transport dysfunction is a cell-wide problem, since virtually all classes of cell-surface receptors that undergo endocytosis, recycling or sorting depend heavily on proper trafficking. Global endosomal dysfunction as an underlying mechanism of singling deficits thus warrants further investigation.

To our knowledge, our study is the first to establish a link between IL-1β and BDNF retrograde trafficking deficits. The hypothesis that the IL-1β and Aβ impair endosomal trafficking is an exciting and significant possibility, particularly considering that dysregulated endosomal sorting is thought to contribute to a wide variety of neurodegenerative diseases including AD, Huntington's disease and Parkinson's disease (32).

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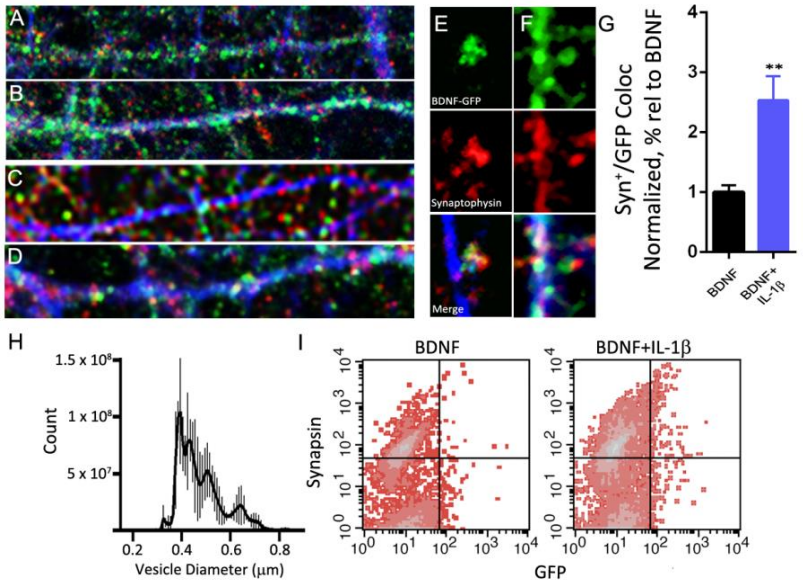
32. Aridor, M., and Hannan, L. A. (2002) Traffic Jams II: An Update of Diseases of Intracellular Transport. *Traffic* 3, 781-790 *Inflammation-induced attenuation of BDNF endosome transport*

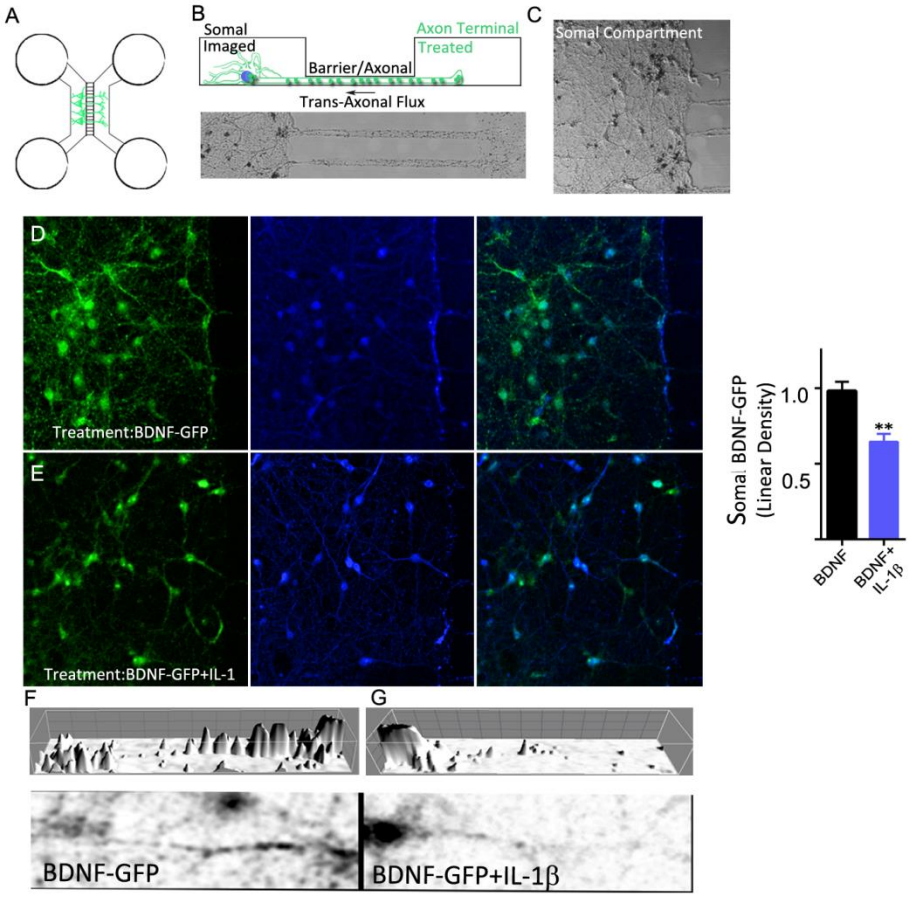
Figure 3.1



Increased presynaptic colocalization of BDNF-GFP caused by IL-1β as an indicator of reduced endosome transport.

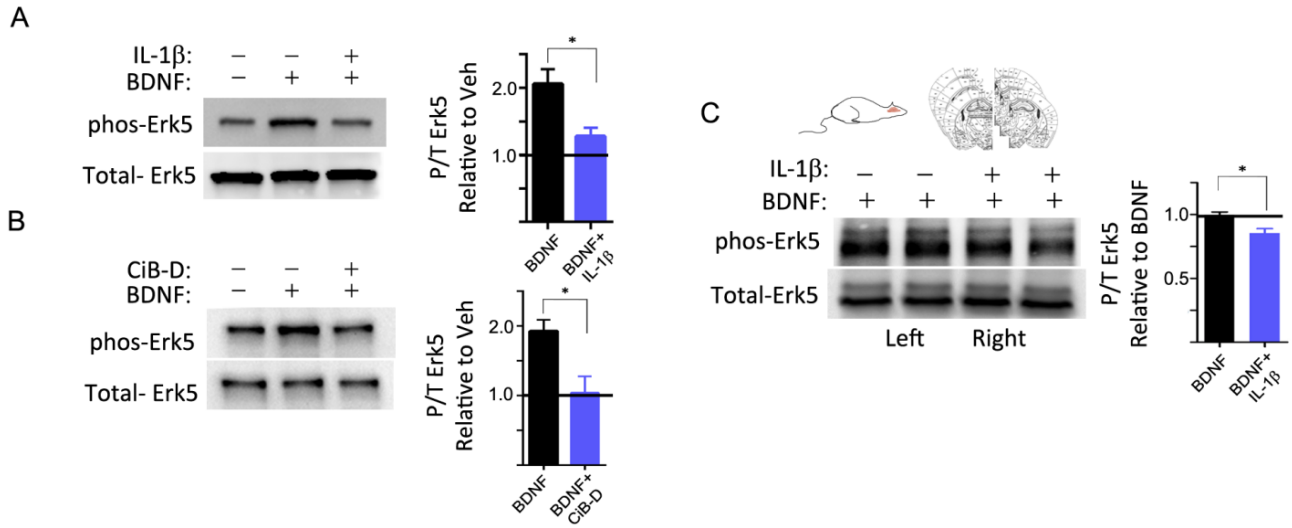
Neurons treated with (A,B) BDNF-GFP or (C,D) BDNF-GFP+IL-1β were fixed and analyzed after 1 hour post-treatment to assess BDNF-GFP endosome dispersion through neurites in culture (E-G) We measured the extent to which BDNF-GFP was found colocalized to presynaptic compartments. After surveying thousands of presynaptic boutons, we found that IL-1β treatment was associated with a 2.6-fold increase in BDNF-GFP colocalization. Graph depicts Mean ± SE (as fold relative to BDNF); BDNF = 2.6 ± 0.4, n = 3 vs BDNF+IL-1β = 1.0 ± 0.2, n = 3 independent cultures. P2 fraction vesicles were isolated from three independent cultures and pooled as an alternate means of colocalization. (H) The vesicles were characterized using Malvern Nanosight NS300 Nanoparticle Tracking Analysis to obtain size distributions and (I)The population of vesicles that were positive for presynaptic marker synaptophysin was then probed for the presence of BDNF-GFP by flow cytometry, as an indicator BDNF-GFP retained in presynaptic compartments. BDNF+IL-1β groups exhibited twice as many GFP events colocalized with synapsin (n = 3 independent cultures pooled). IgG isotype-matched conjugated to Alexa 647 was taken as control for non-specific background and used for gating synapsin.

Figure 3.2



IL-1β reduces trans-axonal long range BDNF endosome volume flow.
(A) Top-down schematic of neurons in microfludic chambers. (B) Edge on schematic (upper panel) and top-down microscope image (lower panel) of the three compartments in the microfluidic chambers. (C). Representative neuron culture in microfluidic chambers. (D,E). Presynaptic boutons in axon terminal compartment were treated with BDNF-GFP or BDNF-GFP+IL-1β, after which BDNF-GFP endosomal linear density in the somal compartment were analyzed for as an indicator of BDNF-GFP that arrived via long range trans-axonal transport mechanisms. Images are of somal compartments. (F,G) Neurites showing a train of BDNF-GFP endosomes in 3D (upper) and 2D (lower). Three-dimensional images represent intensity peaks and were used for quantification. (H) BDNF+IL-1β treatment resulted in a 34 ± 9% decline in GFP linear density (unpaired t-test, $p < 0.05$), relative to BDNF. Mean ± SE; BDNF = 1.0 ± 0.08, n = 9 vs BDNF+IL-1β = 0.72 ± 0.08, n = 6, unpaired two-tail t-test, $p < 0.05$).

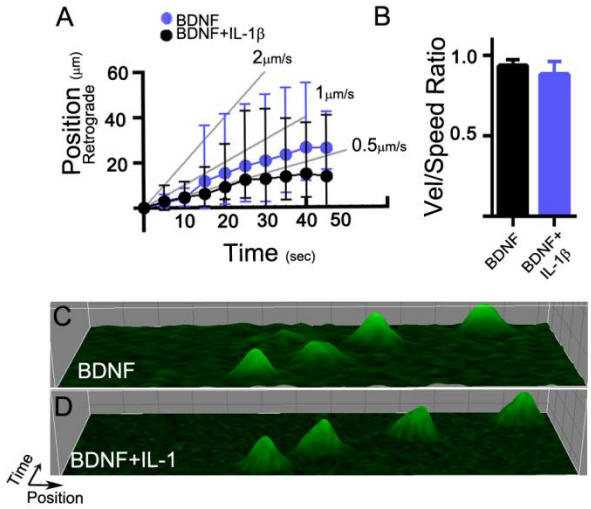
Figure 3.3



IL-1β attenuates BDNF-induced phosphorylation of retrograde signaling target ERK5.

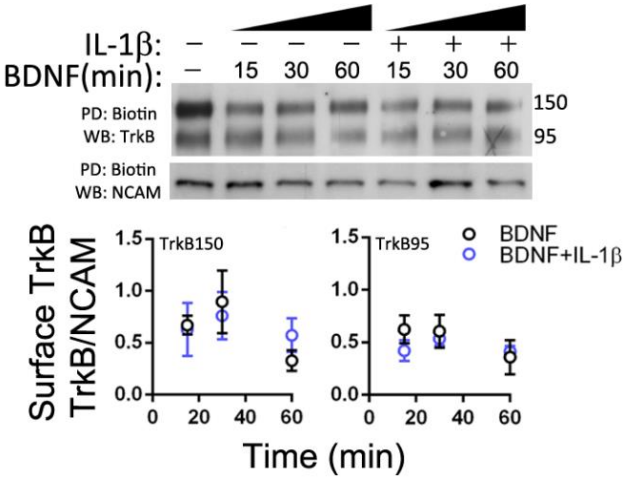
(A) Levels of P-ERK5 were measured in cultured neurons with BDNF or BDNF+IL-1β treatments. Neurons treated with BDNF exhibited 2-fold increase in P-ERK5 levels over baseline non-treated controls while BDNF+IL-1β treatment exhibited only a 1.3-fold increase in P-ERK5 levels over baseline, suggesting IL-1β attenuates BDNF-induced phosphorylation of ERK5. Mean ± SE; BDNF = 2.0 ± 0.2, n = 4 independent cultures vs BDNF+IL-1β = 1.1 ± 0.2, n = 3 independent cultures, unpaired two-tail t-test, $p < 0.05$) (B) Levels of P-ERK5 in response to BDNF or BDNF + Ciliobrevin D, a trafficking motor inhibitor, were reduced to baseline: Mean ± SE; BDNF = 1.9 ± 1.4 fold, n = 5 independent cultures vs BDNF+CiB-D = 1.1 ± 0.25 fold (by unpaired t-test $p <0.05$) (C) Levels of P-ERK in *ex vivo* slices with BDNF (left hemisphere) or BDNF+IL-1β (right hemisphere) compared in adult rats exhibited 14 ± 2% decrease in P-ERK levels after treatment with BDNF+IL-1β when compared to BDNF only treatment. Graph rescaled such that $\overline{x}_{BDNF} = 1.0$. Mean ± SE; BDNF = 1.0 ± .02 fold, n = 4 animals vs BDNF+IL-1β = 0.84 ± .02 fold, n = 4 animals, paired t-test: $p < 0.05$)

Figure 3.4



Signaling deficits not associated with a decline in the rate of transport.
(A) Displacement vs. Time of retrograde vesicles in BDNF or BDNF+IL-1β conditions. Endosomes traveled at an average of 0.35 (n = 10) and 0.5 (n = 8) μm/s, respectively. Graph is Mean ± Range of all values. IL-1β does not reduce the rate of transport of BDNF-GFP vesicles. (B) Ratio of endosomal velocity to speed, indicating directedness of motion. There is a very minimal difference between the means. Mean ± SE; BDNF = 0.93 ± 0.3, n = 10 vs BDNF+IL-1β = 0.87 ± 0.5, n = 8. IL-1β does not alter the character of vesicle locomotion. (C) 3D kymographs of BDNF and (D) BDNF +IL-1β treatment groups.

Figure 3.5



IL-1β does not measurably alter cell surface TrkB expression.
(A) Cell surface TrkB was measured in a 1 hour timecourse in response to BDNF or BDNF+IL-1β. Following treatment, endocytosis was arrested and cells were biotinlyated (See Methods). Cell surface TrkB levels did not differ significantly among groups over 1 hour. The average rate of endocytosis per 1 hour was 53% for TrkB150 after treatment with BDNF vs 47% following treatment with BDNF+IL-1β, suggesting that IL-1β does not significantly alter endocytic kinetics of TrkB at 1 hour. Rates ΔCS-TrkB95/hr: BDNF = –72 ± 9%, n = 2 independent time courses vs BDNF+IL-1β = –0.70 ± 10% , n = 2 independent time courses). Rates ΔCS-TrkB150/hr: BDNF = –72 ± 9%, n = 2 independent time courses vs BDNF+IL-1β = –0.70 ± 10%).

Chapter 4 Discussion

The research presented herein is the first to discover a connection between aberrant retrograde trafficking and the presence of IL-1β. Using microfluidic chambers, we have shown distinct mechanisms by which Aβ and IL-1β alter neurotrophic factor signaling and trafficking. Since both Aβ and chronic inflammation are pathophysiological hallmarks of the AD brain, our work strongly suggests that a singular targeting strategy may not be sufficient to alleviate adverse outcomes to neurotrophic factor signaling.

One significant aspect of this work was the approach to *in vitro* culture models in microfluidic isolation devises (chambers), invented by Noo Li Jeon, Anne Taylor and

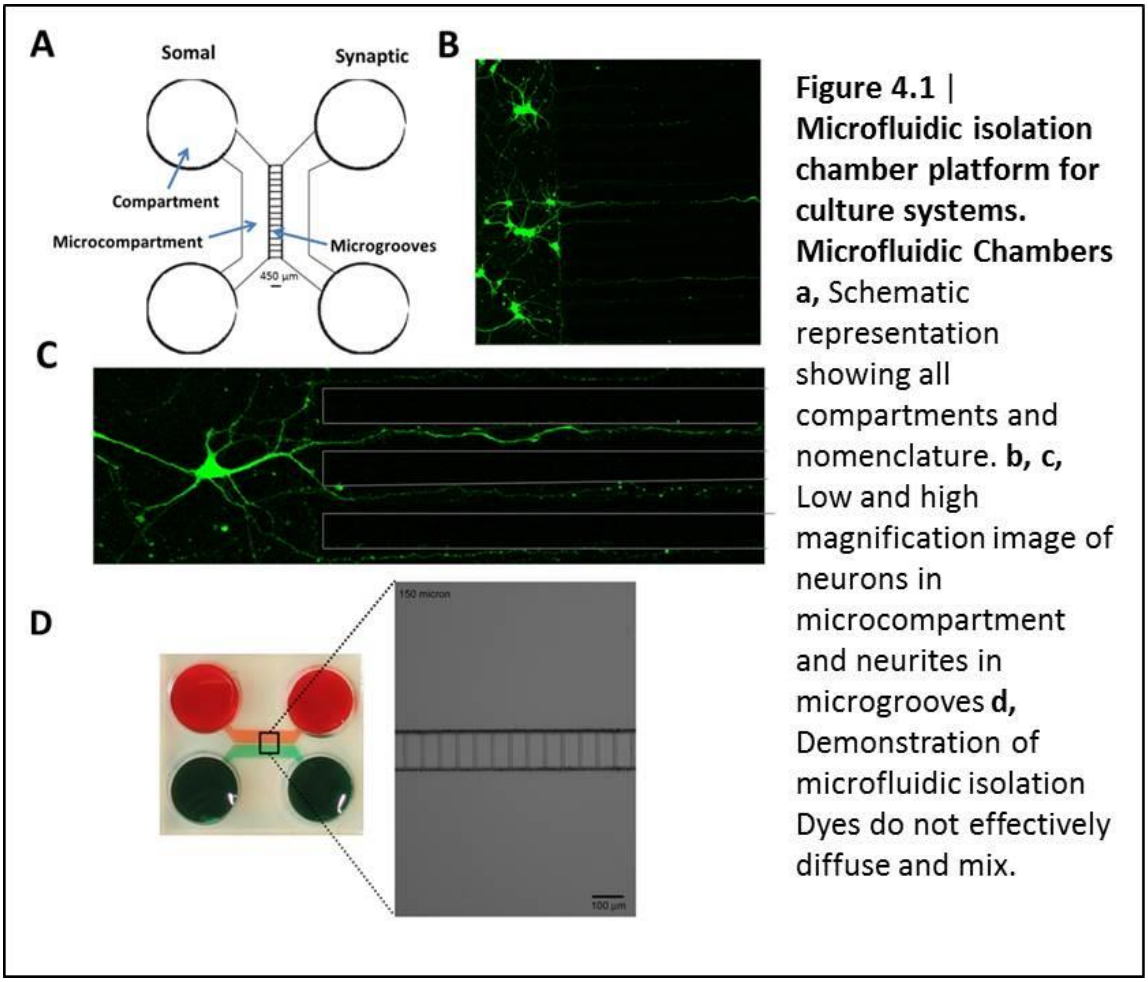


Figure 4.1 | Microfluidic isolation chamber platform for culture systems. Microfluidic Chambers a, Schematic representation showing all compartments and nomenclature. **b, c,** Low and high magnification image of neurons in microcompartment and neurites in microgrooves **d,** Demonstration of microfluidic isolation Dyes do not effectively diffuse and mix.

Carl Cotman**.** Microfluidic chambers isolate and direct growth of pure axons and enable selective treatment of axon terminals without also treating proximal axon segments and cell somas. They are ideal platforms for trafficking studies since BDNF-GFP can be applied distally and traced to somal compartments. The compartmentalization is an added advantage over traditional cell cultures in our design, since it allows our analysis to be solely focused on axoplasmic mechanisms, without confounding measures from other simultaneous trafficking mechanisms. Given the microfluidic dynamics that spatially restrict treatments and the ordered growth of axons, chambers offer the best platform to assess intracellular trafficking and mobility **(Figure 4.1)**.

Our studies using Aβ demonstrated that downstream BDNF trafficking phospho-targets like CREB and Erk-5 were found to be in decline relative to controls and that this decline was likely due to loss of fast axonal transport, mediated by the microtubule/dynein molecular motor complex system. This corroborates previous findings on Aβ induced trafficking deficits. One study of particular interest found that Aβ alters trafficking of internalized acetylcholinesterase and dextran without affecting endocytosis (102). When surface acetylcholinesterase was tagged with FITC-conjugated FasII, fluorescence gradually accumulated in intracellular particles. Similar to our observations, the presence of extracellular Aβ increased this accumulation and altered trafficking patterns. Specifically, vesicles shifted from the juxtanuclear zone to more peripheral cytoplasm. The FasII- structures were positive for LAMP1, identifying them as late endosomes and surface acetylcholinesterase trafficked into the lysosomal compartment. Aβ also affected the transport of fluorescent dextran which caused a 60% increase in intracellular accumulation (102), suggesting that Aβ does alter trafficking

and, based on our work, altering steady-state flux can lead to an observable accumulation phenotype. Further illustrating the phenotype *in vivo, a*nother study further proposed that abnormal accumulation of vesicles correlates with axonal and synaptic pathology in young Alzheimer's mice hippocampus (103). It will be interesting to learn whether the observation of endosomal accumulation is a functional, compensatory or pathophysiological outcome and whether there is a targeted effort to shunt accumulated vesicles toward degradation.

Our experiments using IL-1β were based on the precedent of research showing pervasive and diverse IL-1β-induced deficits to neuronal function. In the mouse hippocampus, sustained expression of IL-1β impairs contextual and spatial memory (104). IL-1β suppresses LTP, and BDNF-induced Akt, and CREB activation (17,24), while others have shown a link between acute neuroinflammation and disruption of specific neural circuit functions and cognitive impairment (105). Studies have also shown that blocking IL-1β can restore cognitive function (106). The effect of IL-1β is not only central, as even peripheral immune insults are enough to induce reversible cognitive decline (107-109). We show that IL-1β was associated with impairment in retrograde vesicular efflux (bottleneck effect) **(Figure 4.2)**, which, like our results with Aβ, lends a mechanistic explanation to a number of trafficking-based studies that have observed an accumulation of vesicles.

This is both interesting and relevant since one of the earliest pathological features in the AD brain is the accumulation of vesicles, a finding that has been observed in both animal and cell-based models (110). The etiological mechanism of enlarged presynaptic vesicles in disease remains largely unknown. Studies have shown

early endocytic changes increased volume of early endosomes in early stage neurological disease (111), which may reflect the capacity of IL-1β to redirect delivery of endosomal cargo to autophagic pathways (112,113). In many neurological disorders, trafficking is a prevalent but perhaps understudied phenomenon. For example, there are reports that endosomal accumulation of APP in motor neurons reflects impaired vesicle trafficking in amyotrophic lateral sclerosis (114), while other reports have shown that α-synuclein multimers cluster synaptic vesicles and attenuate trafficking (115), setting the precedent that neuropathology can sequester synaptic vesicles leading to endosomal dysfunction.

Endosomal trafficking dysfunction can be interrelated to a number of signaling deficits, as well. Many neurotrophic pathway deficits can be elicited by IL-1β, including impaired activation of Akt and CREB. Since signaling cascades depend on ligand-receptor activation and since activated receptors are distributed intracellularly via the endosome trafficking mechanisms, it follows that proper endosomal transport is a vital component of many signaling cascades. Intriguingly, it has been shown that inhibitors of endocytosis following ligand induced activation attenuate receptor signaling, suggesting that trafficking is at least partially linked to signal transduction (116,117).

Endosomal transport dysfunction is a cell-wide problem, since virtually all classes of cell-surface receptors that undergo endocytosis, recycling or sorting depend heavily on proper trafficking mechanisms. The idea that the IL-1β and Aβ impair endosomal trafficking is an exciting and significant possibility, particularly considering that dysregulated endosomal sorting is thought to contribute to a wide variety of

neurodegenerative diseases including AD, Huntington's disease and Parkinson's disease (118).

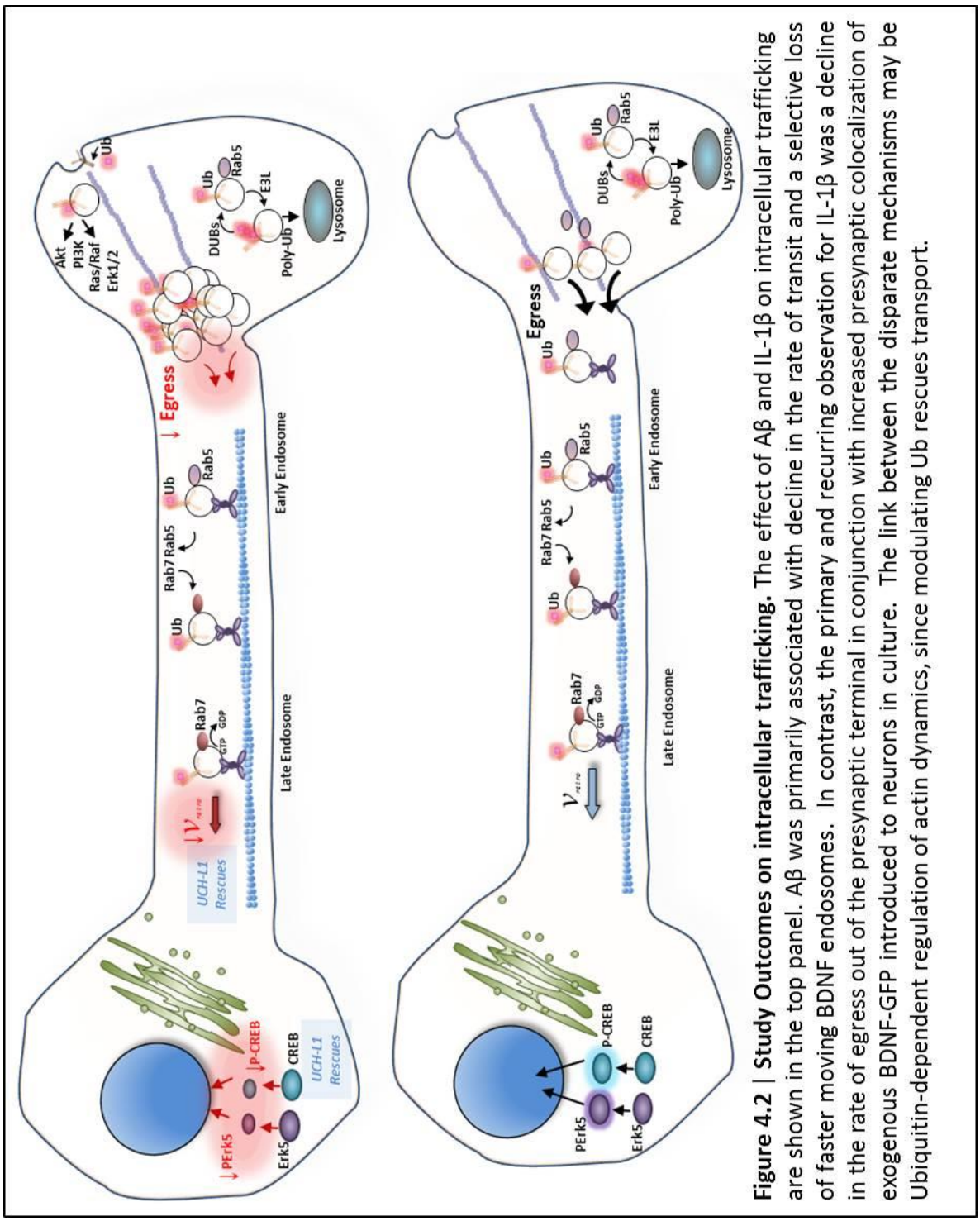


Figure 4.2 | Study Outcomes on intracellular trafficking. The effect of Aβ and IL-1β on intracellular trafficking are shown in the top panel. Aβ was primarily associated with decline in the rate of transit and a selective loss of faster moving BDNF endosomes. In contrast, the primary and recurring observation for IL-1β was a decline in the rate of egress out of the presynaptic terminal in conjunction with increased presynaptic colocalization of exogenous BDNF-GFP introduced to neurons in culture. The link between the disparate mechanisms may be Ubiquitin-dependent regulation of actin dynamics, since modulating Ub rescues transport.

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