Modulation of Neuronal Insulin Signaling
Rescues Axonal Transport Defects in an Alzheimer’s Disease Model

by

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B.Sc. (Chemistry), University of Northern Colorado, 2011

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

in the
Department of Molecular Biology and Biochemistry
Faculty of Science

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Abstract

Defective brain insulin signaling contributes to the cognitive deficits in Alzheimer's disease (AD). Oligomeric amyloid-β peptides (AβOs), the neurotoxin implicated in AD, induce a variety of cellular insults, including dysregulation of intracellular signaling cascades and disruption of fast axonal transport. I show that modulation of insulin signaling prevents AβO-induced defects of brain-derived neurotrophic factor (BDNF) transport in wild type (tau+/+) and tau knockout (tau−/−) primary hippocampal mouse neurons. Tideglusib, an inhibitor of glycogen synthase kinase-3β (GSK3β), an insulin signaling intermediate implicated in AD, rescues BDNF transport in tau+/+ and tau−/− neurons. Furthermore, Exendin-4, an anti-diabetes agent, activates the insulin signaling pathway through glucagon like peptide-1 receptor stimulation to also rescue BDNF transport defects similarly to Tideglusib. These results indicate a protective link between insulin signaling and tau-independent transport regulation. By establishing links between insulin signaling and AβO action, my results allow for establishing novel directions for AD therapeutics.

Keywords: Alzheimer’s disease; axonal transport; diabetes; glycogen synthase kinase-3β; amyloid beta oligomers
Dedication

To my parents, Aniko and Peter, my supervisor, Michael, my lab partners, Elisa, Kathlyn Lesley, Mat, and Trevor, and, my friends, Dana, Eric, and Seth. Thank you so very much for your support and encouragement throughout this adventure.
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<th>Description</th>
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<td>Amyloid-β</td>
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<tr>
<td>AβO</td>
<td>Amyloid-β Oligomers</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic</td>
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<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>APS</td>
<td>Adaptor Protein with pleckstrin homology and Src homology 2 domains</td>
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<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
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<tr>
<td>ca-AKT</td>
<td>Constitutively Active AKT</td>
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<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
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<td>CAP</td>
<td>Cbl-Associated Protein</td>
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<td>Cyclin-Dependent Kinase 5</td>
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<td>CNS</td>
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<td>cAMP response element-binding protein</td>
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<td>c-Jun N-terminal Kinase</td>
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<td>Kinesin Heavy Chain</td>
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<td>Kinesin-3</td>
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<td>LTD</td>
<td>Long-term depression</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<td>MAP</td>
<td>Microtubule associated protein</td>
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<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mTOR</td>
<td>Mammalian Target Of Rapamycin</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
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<tr>
<td>PAD</td>
<td>Phosphatase-Activating Domain</td>
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<tr>
<td>PDK-1</td>
<td>Phosphoinositide-Dependant Kinase 1</td>
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<td>Pleckstrin Homology</td>
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<td>TNF-a</td>
<td>Tumor Necrosis Factor Alpha</td>
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1. Introduction

1.1. Alzheimer’s disease

Alzheimer’s disease (AD) is the most prevalent form of dementia that affects aging populations. AD’s neurodegenerative processes affect individuals by depriving them of important neurological functions, such as, memory, reasoning, judgment, and language. Unfortunately, there is little available by way of therapeutics for this ultimately deadly disease. Presently, an estimated 5.3 million people in North America are suffering from AD. One in eight individuals aged 65 years or older are affected by AD, yielding an incidence rate of 13%. Furthermore, the rate of incidence increases to 33% for individuals over the age of 85. A report recently published by the American Association of Alzheimer’s disease estimated that there were 36 million people living with AD, worldwide, in 2010 (World Alzheimer’s Report, 2012). As life expectancy increases, it is estimated that this figure will double to 66 million by year 2030, and to 115 million by 2050. This growing population of AD patients will continue to be a heavy burden on not only healthcare, but the economy as well, thus finding a cure has never been more paramount.

In 1906, Dr. Alois Alzheimer reported the first case of AD, describing his patient, Auguste D., who presented oscillating states of significant memory loss and lucidity. The histopathological post-mortem analysis of Auguste’s brain revealed the presence of neuropathological lesions, which lead Dr. Alzheimer to hypothesize that the reason for these dramatic personality changes resided in these lesions. After approximately 80 years of this initial characterization, these neuropathological lesions were identified as neurofibrillary tangles and amyloid plaques. The neurofibrillary tangles are intracellular aggregates, composed of hyperphosphorylated tau protein. In AD, tau loses its physiological function to associate with microtubules and stabilize them, thus forming paired helical filaments that aggregate within neuronal cells. On the other hand, the amyloid plaques are extracellular deposits, mainly constituted of aggregates of amyloid-
β (Aβ) peptide, generated from the cleavage of amyloid precursor protein (APP), through the action of enzymes known as β- and γ-secretases\textsuperscript{11, 12}.

Today, 107 years after the first description of AD, definitive diagnosis of this disease can only be made post-mortem by detecting histopathological markers of AD. In clinical practice of AD diagnosis, specific neuropsychological tests are performed\textsuperscript{13}, which aim to exclude other possible causes of dementia, thus pointing to the probable diagnosis of AD, but can only be confirmed after the patient’s death.

1.2. Amyloid beta

Since the identification of Aβ as the major component of the amyloid plaques\textsuperscript{8, 9}, there have been an increasing number of studies supporting a central role for Aβ in neuronal dysfunction and the neurodegeneration that affects individual patients\textsuperscript{14-17}. Aβ is formed when APP is misprocessed and undergoes a gain of function. Normally, APP undergoes posttranslational processing, involving several different secretases and proteases, via two major pathways. In the non-amyloidogenic pathway, APP is cleaved by α-secretase followed by γ-secretase. α-cleavage, cuts APP at the 17th amino acid inside the Aβ peptide sequence (Fig. 1.1), releases a large secreted extracellular domain (sAPP-α) and a membrane-associated C-terminal fragment consisting of 83 amino acids (C83). APP C83 is further cleaved by γ-secretase to release a P3 peptide and the APP intracellular domain (AICD), both of which are degraded rapidly. In the amyloidogenic pathway, APP is primarily processed by β-secretase (Fig. 1.1), shedding sAPP-β and generating a C-terminal fragment consisting of 99 amino acids (C99)\textsuperscript{18}. γ-secretase further cleaves C99 to release AICD and the amyloidogenic Aβ peptide. In normal aging, the peptide remains as soluble Aβ. It is released in the brain and is progressively degraded and removed by the proteases neprilysin or insulin-degrading enzyme. These regulate steady state levels of Aβ\textsuperscript{18}. Neprilysin, a membrane-anchored zinc endopeptidase, degrades the monomeric and oligomeric form of Aβ (AβO)\textsuperscript{19}. It was shown that a reduction in neprilysin caused an accumulation of cerebral Aβ\textsuperscript{20}. Insulin-degrading enzyme degrades small peptides such as insulin and the monomeric form of Aβ\textsuperscript{21}. Deletion of insulin-degrading enzyme in mice, reduced Aβ degradation by 50\%\textsuperscript{22}. Additionally, overexpressing insulin degrading enzyme or neprilysin prevented plaque
formation. In AD, Aβ peptide becomes present in large quantities, aggregates forming amyloid fibrils that are deposited progressively in the extracellular environment, accumulating in the form of amyloid plaques.

![Figure 1.1](image)

**Figure 1.1. Processing of the amyloid precursor protein (APP).**

Nonamyloidogenic and amyloidogenic processing of APP occurs in the plasma membrane. In the nonamyloidogenic pathway (left side of figure), cleavage of APP by α-secretase releases a soluble ectodomain (sAPPα) into the extracellular space and generates a residual fragment of 83 amino acids C-terminal (C-83) in the membrane. The C-83 is then cleaved by γ-secretase releasing the peptide p3. In the amyloidogenic pathway (right side of figure) APP is cleaved by β-secretase, resulting in the secretion of the slightly truncated molecule sAPPβ and retaining the 99 residues C-terminal membrane fragment (C-99). The C-99 cleaved by γ-secretase generates the Aβ peptide. Cleavage of both fragments, C83 and C99, releases the intracellular fragment AICD. Used with permission from Querfurth and LaFarla, 2010.

Aβ1-40 is the major soluble Aβ species secreted from the cell. However, the major component of AOs and amyloid deposits in the brains of AD patients is the 1-42 Aβ peptide. Although not completely characterized, the shift in production from Aβ1-40 to Aβ1-42 is driven by mutations, either familial or sporadic, in APP itself and/or the secretases that process APP. During the progression of AD, Aβ shifts towards the misprocessed Aβ1-42 form. Interestingly, Aβ1-40 can prevent neuronal death induced by Aβ1-42. These findings suggested that the ratio of Aβ1-42/Aβ1-40 is important as Aβ1-40 showed inhibitory effect of fibril formation of Aβ1-42. At the earliest stage of AD there most likely is a shift from a ratio favoring Aβ1-40 to Aβ1-42, thus Aβ1-40 losing it’s protective effect, exacerbated by the brain’s reduced capacity for Aβ clearance.
Additional evidence for Aβ’s role in neurodegeneration has come from genetic studies of familial forms (hereditary) of AD, which represent approximately 5% of AD cases\textsuperscript{28, 29}. In such cases, patients are affected by the disease earlier in life, around age 40 or 50, and have mutations in APP or the enzymes that cleaves APP, accelerating the formation of Aβ. However, there is yet to be an identified genetic determinant factor for this condition\textsuperscript{28, 30}. Most cases of AD are sporadic, with aging being the largest determinant of the disease\textsuperscript{31}.

The commonality of the accumulation of Aβ in all cases of AD, including sporadic and hereditary, led to the amyloid beta hypothesis, which was accepted by most scholars in the field, as an explanation of the etiology of the disease \textsuperscript{32}. Hardy and Higgins proposed that the Aβ fibrils found in amyloid plaques that accumulated in the diseased brain initiate a cell-signaling cascade that leads to cellular dysfunction and subsequently death, resulting in a state of dementia. However, Hardy and Selkoe developed a newer version of the proposal, after a decade of additional studies, on the impact of small, soluble oligomeric forms of Aβ (AβO) in neuronal synapses \textsuperscript{33, 34}, not the amyloid plaques on their own (Fig. 1.2) \textsuperscript{35}. 


Figure 1.2. Updated amyloid cascade hypothesis.
Hypothetical sequence of events triggered by the Aβ peptide. Note that this is an alternative mechanism of memory loss based on the impact of small, soluble AβOs in neuronal synapses. Adapted from Haass and Selkoe, 2007.

With the help of many studies, evidence has been mounting that oligomeric forms of Aβ are neurotoxic and are present in the brain of AD patients. This new AD hypothesis is called the AβO hypothesis of AD. In fact, studies have identified AβOs in concentrations of up to 70 times higher in extracts of AD-affected brains, compared with brains of non-demented individuals. Studies suggest that AβOs bind directly or very closely to N-methyl-D-aspartate (NMDA) receptors. Furthermore, AβOs induce an imbalance of physiological functions regulated by activation of NMDARs, including deregulation of calcium homeostasis. Several studies have also shown that AβOs reduce levels of surface NMDARs, either by inducing endocytosis or blocking the recruitment of the receptor to the plasma membrane.
Previous work suggests other possible targets for AβOs, such as cellular prion protein (PrP(C)), which has been identified as a possible receptor for binding AβOs. Another possible candidate, is the glutamatergic receptor subtype AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic). Results in cultured hippocampal neurons have shown that pharmacological inhibition of AMPA receptors, especially its type 2 subunit (GluR2) inhibits the binding of AβO on the dendritic spines. Furthermore, results demonstrate that AβOs interact preferentially with synaptic complexes containing GluR2 and calciuneurin, a calcium-dependent protein phosphatase. Additionally, GluR2 regulates the endocytic process responsible for the rapid AMPA receptor internalization linked to AβOs. Furthermore, the interaction of AβOs with the extracellular domain of the p75 neurotrophin receptor (p75NTR) seems to be involved in AβO-induced in vitro and in vivo neuritic dystrophy. Other possible receptors that AβOs bind may include receptor advanced glycation end products (RAGE) and the protein Frizzled, which induces inhibition of the Wnt/β-catenin signaling pathway. This data reinforces and validates the clinical relevance of the use of oligomers of β-amloid peptide (AβO) as an experimental model and also as a therapeutic target for AD (Fig. 1.3).

**Figure 1.3.** The latest AD hypothesis states that Aβ oligomers are the neurotoxin in AD

Right side of the picture depicts beta-amyloid oligomerizing to form AβOs, that then bind to and damage neurons. On the left beta-amyloid, aggregates into fibrils and then amyloid plaque, but remain less toxic than AβOs. Used with permission from the Health and Human Services Progress Report on Alzheimer's Disease, 2004-05.
One finding showed that nanomolar concentrations of AβOs could potentially impair synaptic plasticity mechanisms, to inhibit long-term potentiation (LTP), a form of synaptic plasticity widely used as an experimental paradigm for studying the processes of memory formation in the hippocampus. Furthermore, the oligomers inhibit an initial process of synaptic depression that culminates with the elimination of the dendritic spine \(^{50}\). A dramatic reduction in the number of dendritic spines was observed after a long incubation period with oligomers\(^{42, 44, 50}\). As the spines are specialized protrusions, which occur on dendrites of excitatory synapses, the impact of oligomers in the synapses suggests a basis for the loss of neuronal connectivity and therefore the cognitive deficits observed in patients with AD. These deleterious effects on the synapses are probably a result of oligomers' ability to bind with high specificity to excitatory synaptic sites\(^{51}\), an event of particular relevance to the mechanism of memory loss (Fig. 1.4).

**Figure 1.4.** AβOs binding to various membrane receptors on a dendritic spine and inducing toxic signals that ultimately end in synaptic dysfunction and memory loss.

AMPAr = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor; NMDAr = N-methyl-D-aspartate receptor; IR = insulin receptor.

Recent studies investigated the mechanisms by which oligomers are able to impair synaptic plasticity with glutamatergic transmission being considered the basis for
the excitatory stimuli associated with learning and memory. The NMDA receptors and AMPA subtypes of glutamate receptors perform a central role in glutamatergic transmission. They are required for the induction of LTP and synaptic strength. Additional work showed a massive reduction in the levels of AMPA and NMDA on the neuronal plasma membrane. Significantly, the post-synaptic density 95 (PSD-95), a protein that forms a fundamental framework for anchoring and stabilizing the AMPA and NMDA receptors, is also reduced after treatment with Aβ oligomers. Among the receptors mentioned above, it is important to note that the number of insulin receptors, which play a crucial role in neuronal plasticity, is dramatically reduced in the neuronal plasma membrane after exposure to oligomers (Fig. 1.4).

It is important to emphasize that the pathological loss of dendritic spines and accumulation of substantial levels of Aβ oligomers is well described in cases of AD and transgenic mouse models of AD. These Aβ oligomers interfere with important synaptic processes by causing aberrant tau phosphorylation characteristically found in the brains of AD patients, inducing oxidative stress, and triggering neuronal influx of excessive calcium through NMDA receptor dysregulation. The combination of these findings suggests that synapses are the main target of oligomers. AβO binding leads to morphological changes in the composition of receptors resulting in synaptic dysfunction and could be responsible for the initial cognitive decline affecting individuals with AD.

1.2.1. Tau

Neurofibrary tangles (NFT) are one of the hallmarks of AD. NFT are made up of hyperphosphorylated tau, a microtubule associated protein (MAP). The tau protein is one of several MAPs that regulate the dynamics of microtubules (MTs) and in recent years several studies relate their function to transport-related pathologies. Tau protein has both physiological and pathological role in the cells. When the phosphorylation state of tau is properly coordinated tau exercises control in the regulation of neurite outgrowth, in axonal transport, and the stability and dynamics of MTs. However, under pathological conditions, a shift towards tau hyperphosphorylation leads to a loss of tau association with MTs and formation of NFTs, ultimately contributing to cell death.
Tau knockout mice have been a useful tool to assess the role of tau in AD pathogenesis. In tau-knockout mice, the absence of tau was associated with a 2-fold increase in the microtubule associated protein 1A (MAP1A) expression in 7-day-old mice. It was also shown that a 1.3 fold increase exists in adult tau-knockout mice, possibly compensating for the absence of tau\textsuperscript{75}. The tau knockout mouse also presented without overt phenotype when young\textsuperscript{75, 76}. Ittner et al., was able to show that the tau knockout mice were protected from Aβ-induced premature mortality and memory deficits, as the tau knockout mice were less susceptible to excitotoxicity\textsuperscript{77}. GSK-3β overexpression in the brain of mice resulted in degeneration of the dentate gyrus, but when crossed with tau-deficient mice the degeneration was significantly ameliorated\textsuperscript{78}. Morris et al., suggests that tau regulates neuronal excitability is required for Aβ and other excitotoxins to cause neuronal deficits, aberrant network activity and cognitive decline\textsuperscript{79}.

1.3. **Insulin signaling**

1.3.1. *Insulin signaling in peripheral tissue*

A problem faced by all animals is a period of fasting after meals, but energy may be required continuously. Mammals solve this problem through the storage of nutrients, so that such stock may be used as an energy source during the long periods of fasting. An important agent that regulates this process is insulin, a hormone released by β-cells of pancreatic islet cells in response to high levels of nutrients in the blood supply, such as glucose. By binding to its receptor on responsive tissues, insulin triggers the activation of signaling pathways, whose main function in peripheral tissues, is to stimulate the transport of nutrients such as glucose, fatty acids, and amino acids from the blood into the tissues. In these tissues, insulin promotes the formation of macromolecular storage forms such as glycogen, triglycerides, and proteins.

Furthermore, insulin signaling gives rise to a broad spectrum of mammalian physiological effects, such as being able to promote somatic growth\textsuperscript{80, 81}, or, after birth, promoting cell growth and survival of many tissues, including pancreatic β-cells, neurons, bone, and the retina\textsuperscript{82-84}. Thus, the action of insulin in peripheral organs is
considered to be a major anabolic hormone, being fundamental to the development, growth, glucose homeostasis, and metabolism of fatty acids and proteins.\(^{85}\)

On the molecular level, the insulin receptor belongs to the tyrosine kinase receptor superfamily, and is a transmembrane receptor with two α- and two β-subunits.\(^{86-88}\) Insulin binds to the extracellular α-subunits, resulting in the autophosphorylation of specific tyrosine residues in the intracellular β-subunits through changes in the receptor configuration.\(^ {89}\) Once activated, insulin receptor substrate proteins, IRS-1 to 4, are recruited to the insulin receptor and bind to the trans-phosphorylated receptor at tyrosine docking sites.\(^ {90}\) In addition to these four IRS proteins, there are at least five more intracellular substrates of the IR that are adapter proteins, such as Shc, APS, sh2b, Gab1/2 and DOCK1/2.\(^ {90-92}\) Although the role of each of these proteins merits attention, recent studies suggest that the majority of responses to insulin, especially those associated with the growth and metabolism of carbohydrates, are mediated largely through two isoforms of the insulin receptor substrate, IRS-1 and IRS-2 (Fig. 1.5).\(^ {93}\)

Subsequently, the IRS proteins become phosphorylated at multiple tyrosine residues, via the activated receptor kinase. The IRS proteins have no intrinsic catalytic activity themselves, but the tyrosine phosphorylation provides docking sites for signaling molecules with a Src homology 2 (SH2) domain.\(^ {94}\) Growth factor receptor-bound protein (Grb2) and phosphatidylinositol 3-kinase (PI3K) are SH2 domain-containing signaling molecules that interact with tyrosine phosphorylated IRS. Grb2 can bind to the active IR, stimulating interaction between two other proteins, SOS and Ras, resulting in the induction of GTPase activity of Ras signaling for triggering cell division through the mitogen-activated protein kinase (MAPK).

One of the key players in the IR signaling cascade is PI3K, which plays a central role in mitogenic and metabolic actions of insulin.\(^ {95}\) PI3K is a heterodimeric protein, consisting of two subunits: a p85 adaptor subunit and a p110 catalytic subunit, that contains two SH2 domains that have to be occupied with phosphotyrosine residues of the IRS for PI3K activation.\(^ {96}\) This interaction leads to the translocation of PI3K to the plasma membrane and positions PI3K near its substrate, phosphatidylinositol-(4,5)bisphosphate (PIP\(_2\)). PI3K then catalyzes the conversion of PIP\(_2\), a plasma membrane localized phospholipid, to the second messenger phosphotidylinositol-
(3,4,5)triphosphate (PIP₃)⁹⁷, ⁹⁸. PIP₃ can recruit various serine kinases that contain a pleckstrin homology (PH) domain that is needed to interact with the phospholipids within the plasma membrane⁹⁹, ¹⁰⁰. These molecules include phosphoinositide-dependant kinase (PDK-1), protein kinase B (PKB or also known as AKT) and atypical protein kinase C (PKC) ζ and λ.

PDK-1 is able to phosphorylate and activate other serine kinases, like AKT¹⁰¹. AKT becomes important in the transmission of insulin signaling by inhibiting the protein glycogen synthase kinase-3 (GSK-3), as well as activating the forkhead transcription factors and the cAMP responsive element (cAMP response element-binding protein, CREB)¹⁰². Other subsequent effectors of AKT, such as mTOR (mammalian target of rapamycin), p70-S6 kinase, as well as the atypical isoforms of PKC lead to increased glucose transport, protein synthesis, and glycogen levels¹⁰³.

Other cascades that are involved, and through which insulin signaling is propagated, include the mitogen-activated protein kinase (MAPK) pathways and the Cbl/Cbl-associated protein (CAP). The activation of the MAPK induces cell growth and mitotic activity. On the other hand, Cbl and phosphorylated tyrosine residues in the kinase activity of the insulin receptor insulin receptor kinase (IRK), mediate the activation of glucose transport through activation of TC10 and its interaction with a complex leading to exocytosis¹⁰⁴, ¹⁰⁵. Thus, insulin action covers several signaling pathways, which collectively propagate signals of paramount importance for homeostasis (Fig. 1.5)⁹³, ¹⁰⁶-¹⁰⁸.
1.3.2. Insulin signaling in the central nervous system

It was long been believed that the central nervous system (CNS) was not sensitive to peripheral insulin levels, until the presence of insulin receptors (IR) was observed in the CNS\textsuperscript{109-111}, in neuronal and glial cells, cortex and hippocampus\textsuperscript{112}. Additional studies demonstrated that the insulin receptors are located in the synapses of hippocampal neurons\textsuperscript{113}. The presence of insulin was also detected in the brain\textsuperscript{114-116}. IRs have are widely expressed throughout the brain, especially in the olfactory bulb, hypothalamus, hippocampus, and cerebral cortex\textsuperscript{117}. 

Figure 1.5. The signaling cascade of insulin and insulin growth like factors 1 and 2 (IGF 1 and 2).

IRS bind to five different isoforms of cytoplasmic receptor triggering signaling: two isoforms of the insulin receptor, IRa and IRb (red), the insulin-like growth factor 1 receptor, IGF1R (blue) and the hybrid forms of receptors, IRb::IRa::IGF1R. Receptor activation induces phosphorylation of cellular proteins IRS-1 and IRS-2, however, other substrates can be activated, transmitting the insulin signal through different pathways. The phosphorylation in lilac represent inhibitory phosphorylation, and those that are in green represent stimulatory phosphorylation. In β-cells, GLP-1 stimulates increased glucose, calcium, and cAMP-mediated signaling, as well as inducing increased expression of the IRS-2 through CREB and TORC-2. Figure used with Nature Publishing Group's permission from Taguchi and White, 2008.
Insulin production in the CNS is a question that still remains controversial. Some work has demonstrated the expression of the gene encoding insulin in the brain of mouse embryos\textsuperscript{118}, suggesting a role for insulin during brain development. Other groups, analyzing rabbit brains demonstrate that a single gene synthesized insulin locally, in neuronal cells\textsuperscript{119, 120}. Additional evidence has pointed to the synthesis of insulin in neuronal cells, and for its release after depolarization of these cells\textsuperscript{121}. However, there is evidence to contradict these studies, showing that there were no signs of insulin mRNA detected in brain homogenates\textsuperscript{122}. There is also a large body of evidence indicating that insulin derived from the brain and peripheral system are transferred through a transport system mediated by a receptor present in the blood-brain barrier\textsuperscript{123, 124}. Acute elevations of plasma insulin are followed by cerebral spinal fluid insulin increases, suggesting that insulin levels in the CNS are linked to the peripheral tissues. It has also been demonstrated that insulin may participate in neurodevelopment as insulin can induce direction of axonal growth in \textit{Drosophila} and \textit{Caenorhabditis elegans}. The authors suggest that the insulin receptors present on the neuronal growth cone recognize a gradient of insulin and respond by induction of cell proliferation and migration, in addition to inducing cell differentiation into functional neurons in the appropriate context\textsuperscript{125, 126}. However, the authors were not clear if this insulin gradient is present in the nervous system of vertebrates.

An extensive amount of data has suggested that insulin promote neuronal survival\textsuperscript{127}. In slices of rat cerebellum, insulin reduced apoptosis of neurons of the external granular layer\textsuperscript{128}. Interestingly, the application of insulin in cultured cortical neurons had an anti-apoptotic effect against serum deprivation\textsuperscript{129}. These initial studies, that identified the effect of insulin to promote neuronal survival, have been extended with studies that investigated the signaling pathways involved.

The binding of insulin to its receptor in neurons, as well as in peripheral organs, can activate two parallel signal transduction cascades, the PI3K/AKT and MAPK cascade. Mice that did not express insulin receptors in neurons were treated with insulin, resulting in inhibition of PI3K/AKT in cultured neurons, as well as a reduced protection against apoptosis induced by potassium chloride\textsuperscript{130}. These findings confirm that the action of insulin can inhibit apoptosis, and that this response is completely dependent on the presence of the insulin receptor. The brains of these mice did not change in
structural development. However, the authors argue that the absence of IR, in vivo, can be compensated for by neurotrophic factors\textsuperscript{130}. Additional evidence suggests that AKT is fundamental in order to mediate effects on neuron survival. PI3K activates the expression of AKT and protected the cells from apoptosis, contrary to what was observed when dominant negative, or inactive AKT was expressed\textsuperscript{131}. In vivo data show that active AKT protects against hypoxic stress and nitric oxide toxicity\textsuperscript{132}. Once activated, AKT protects against toxic stimuli by inhibiting proteins that belong to the apoptotic machinery such as the Bad protein, caspase 9, and GSK3 protein kinases and further block the transcriptional activity of p53\textsuperscript{133}.

1.3.3. \textit{Insulin and synaptic plasticity}

Currently, a new role for insulin in the CNS and synaptic plasticity is being considered as administration of insulin in healthy individuals, with constant glucose levels, improved memory formation\textsuperscript{134, 135}. These observations may have been due to a direct effect of insulin on the hippocampus, as memory formation increased insulin receptor mRNA in the hippocampal area CA1 of rat\textsuperscript{117}. Additionally, memory formation also elevated the protein levels of the insulin receptor in the synaptic membrane fractions of hippocampal cells\textsuperscript{117}. Furthermore, insulin was able to induce the strengthening of synapses\textsuperscript{112, 136}.

The study by Chiu et al., 2008 confirmed the hypothesis that insulin signaling can regulate the number and maintenance of synapses and dendritic plasticity in vivo. The authors observed that insulin contributed not only to the processing of sensory information, but also to the plasticity of structural cells, which is necessary for the functioning of neurons in brain circuits.

High-frequency stimulus and a large influx of calcium in the synapse induce LTP\textsuperscript{137} (Stanton, 1996). The influx of calcium functions as a second messenger signaling pathways involved in modulating the strengthening of the synapse. Insulin signaling has been implicated in the mechanisms of LTP and LTD to influence NMDA receptor activity\textsuperscript{138}. In addition, AMPA receptors were recruited to the membrane through insulin signaling and increased LTP\textsuperscript{138-140}. During the excitatory transmission depression (LTD), insulin facilitates the AMPA receptor internalization of the GluR2 subunit. Stimulation of
rat hippocampal slices with insulin induced a PI3K-dependant increase in the expression of PSD95\textsuperscript{141}. PSD95 is essential for anchoring a number of important proteins and receptors of the synapse, and the regulation of its expression could provide an explanation of an additional molecular mechanism for the modulation of synaptic plasticity by insulin.

1.4. Alzheimer's disease and insulin resistance

The deficiency of the insulin signaling pathway in peripheral tissue and the brain has been implicated in AD, diabetes, and aging\textsuperscript{142-144}. Aging is associated with reduced levels of insulin and IR in AD patients\textsuperscript{145, 146}. Additionally, AD brains show reduced mRNA of IRs, PI3K, AKT and GSK-3\(\beta\), thus proposing that Alzheimer's might be a new form of diabetes, known as Type 3 diabetes\textsuperscript{147}.

As previously described, A\(\beta\)Os are potent neurotoxins that bind synapses triggering a series of events that lead to profound synaptic dysfunction. As IRs are present in the synapses and reduced in AD brains, some studies have sought to investigate the impact of A\(\beta\)Os on the insulin receptor. A recent study demonstrated that in cultured hippocampal neurons short, low dose treatments of A\(\beta\)Os were able to induce the redistribution of IRs from the dendrites to the cell body\textsuperscript{44}. Consistent with the removal of IRs from dendrites, insulin responsiveness also decreased in cells treated with A\(\beta\)Os, as they showed a profound reduction in IR tyrosine kinase activity\textsuperscript{57}. Interestingly, another group showed that A\(\beta\)O induced dysfunction of IRs, suggesting that this disorder is the main factor in the mechanism leading to the inhibition of LTP\textsuperscript{148}. Therefore, the dysfunction of insulin signaling appears to be an important aspect of the oligomer-induced toxicity. This suggests that insulin resistance in the brain of AD patients occurs in response to the action of these neurotoxins, which can cause a specific form of diabetes in the brain.

The mechanism by which insulin resistance in the brain arises in individuals with AD is largely unknown. A possible mechanism was proposed by a study that investigated whether insulin could provide a physiological mechanism of protection against the toxicity of A\(\beta\)Os. Using mature cultured hippocampal neurons, the study
demonstrated that insulin blocked the binding of oligomers to neurons, and that this effect was wholly dependent on the tyrosine kinase activity of the insulin receptor. This suggests that insulin leads to internalization of binding sites of AβOs, which results in preventing the removal of insulin receptors in the membrane of neurons. Furthermore, treatment of neurons with insulin prevented the excessive production of reactive oxygen species. Interestingly, the deterioration of synaptic spines induced by oligomers was completely prevented by insulin. In addition, serine phosphorylation of IRS-1 (IRS-1pSer), which inhibits insulin signaling and is found in both AD and diabetes, was found in primary hippocampal neuron cultures following AβO application. The study also showed elevated IRS-1pSer levels in the hippocampi of a transgenic AD model mice and cynomolgus monkeys following intracerebroventricular injection of AβOs. Furthermore, human AD affected brains also show elevated levels of IRS-1pSer and activated JNK. Bomfim et al., 2012 proposed that AβOs accumulate in the brains of AD patients, activate the JNK/TNF-α pathway, which induces IRS-1 phosphorylation at multiple serine residues, and inhibits physiological IRS-1pTyr. This inhibition of the insulin signaling pathway may add to the deregulation of the downstream effector, GSK-3β, that plays a central role in AD.

The deregulation of GSK-3β accounts for many of the pathological hallmarks of AD in both sporadic and familial cases. Studies have found that GSK-3β is closely involved in the hyper-phosphorylation of tau, memory impairment, and the increased production of Aβ. In addition, GSK-3β is a key mediator of apoptosis and could be directly contributing to neuronal loss in AD. If GSK-3β really is important to AD pathogenesis, then one would expect evidence for increased activity of the enzyme in AD. However, the evidence is lacking as it is very difficult if not impossible to measure enzymatic activity in post-mortem brain tissue. But there have been a few studies that show indirect evidence that does support a role of GSK-3β in AD, such as GSK-3β co-localizing with dystrophic neurites and NFTs. Active GSK-3β appears in neurons with pre-tangle changes and there is an increase in GSK-3β activity in the frontal cortex in AD. Other studies have found that GSK-3β expression is upregulated in the hippocampus of AD patients. Furthermore, Mate et al., reported that a polymorphism in the GSK-3β promoter is a risk factor for late onset AD. Collectively, these findings suggest that GSK-3β activity might be increased in AD, through changes in its
phosphorylation state as well as expression levels, although the direct evidence for this is still limited at present and some studies find no change in GSK-3β activity\textsuperscript{153} or reduced GSK-3β activity\textsuperscript{158} in AD. As insulin signaling is capable of modulating GSK-3β, therefore, insulin treatment may have important therapeutic implications for AD, by preventing the aberrant GSK-3β signaling that occurs during AD. Furthermore, drugs that increase insulin sensitivity could serve as a powerful tool in the search for new therapeutic approaches for AD.

1.5. Exendin-4 and the glucagon-like peptide-1 receptor

A complex network of neural and endocrine signals occur in response to meals regulating digestion, absorption, and storage of nutrients ingested. Many of these signals are generated by physico-chemical properties of the ingested nutrients as they pass through the intestine. The importance of these signals was first described by McIntyre et al., 1964, which hypothesized that the absorption of glucose would be higher due to elevation of insulin levels and elevation oral administration of glucose, rather than the intravenous administration of the same glucose dose in humans. According to the author, the increased secretion of insulin was due to the production of hormones in the intestine, in response to elevated levels of glucose stimulated by nutrient loading. The first such hormone described was glucose-dependent insulinotropic polypeptide (GIP), followed a decade later by the glucagon-like peptide 1 (GLP-1)\textsuperscript{159}. Both are secreted into the intestine, GIP by K-cells of the duodenum and GLP-1 by L-cells in the same region.

GLP-1 results from the post-translational cleavage of the glucagon gene product, preproglucagon, by the enzyme PC1/3\textsuperscript{160}. GLP-1 (7-37) is the predominant biologically active form found in humans, although small quantities of another active form, GLP-1 (7-36), have also been detected. The action of GLP-1 has been extensively studied for more than two decades, because of its ability to reduce plasma glucose levels and increase insulin levels, which is extremely important for patients with type II diabetes. Thus, therapeutic strategies for the activation of GLP-1 have been developed. This peptide is very rapidly degraded by the enzyme dipeptidyl peptidase. This enzyme, present in serum and cell membranes, rapidly cleaves GLP-1 (7-37) at its N-terminal portion, producing an inactive fragment of GLP-1, GLP-1 (9-36). Therefore, great efforts
have been made to increase the biological half-life of GLP-1, thus improving its effectiveness in vivo\textsuperscript{161}. Exendin-4 (Ex-4; also known as exenatide), is an agonist of GLP-1, which was initially characterized in the saliva of a Gila monster lizard. It has a vastly greater half-life than GLP-1, due to lacking the site of action of dipeptidyl peptidase\textsuperscript{162}. Thus, due to its potent and prolonged insulinotropic effect, this peptide has recently been approved for the treatment of type 2 diabetes.

The GLP-1 receptor (GLP-1R) is a transmembrane G protein-coupled receptor (GPCR) that was initially cloned from rat pancreatic islets cells and later identified in human pancreas. In addition to GLP-1, glucagon can also bind to the GLP-1R, but with a thousand-fold lower affinity than GLP-1\textsuperscript{163-165}. Receptor binding of GLP-1 promotes activation of adenylate cyclase, increasing the production of cyclic AMP, the main mediator of action of GLP-1 stimulated insulin secretion in β cells\textsuperscript{166}.

Another molecule activated by GLP-1 is PI3-K, an enzyme implicated in multiple events of β cells such as growth, survival, and metabolism. Additionally, GLP-1r agonists can directly activate IRS-2 and recruit the regulatory subunit of PI3K\textsuperscript{167}. The expression of IRS-2 can also be induced by GLP-1 through the activation of the transcription factor CREB by cAMP-dependent protein kinase (PKA)\textsuperscript{168}. As described, there are many paths by which GLP-1 can stimulate insulin secretion and glucose homeostasis (Fig. 1.5).

1.5.1. **GLP-1 in the CNS**

The production and secretion of GLP-1 can occur in a small population of neurons\textsuperscript{169}. The identification of GLP-1 receptors in different regions of the brain prompted various studies to better understand the functions of this peptide. A large body of evidence supports the hypothesis that a central role for learning and memory is exerted by GLP-1\textsuperscript{170}. Recent data shows that intracerebroventricular infusions of GLP-1 were able to improve memory and learning in rats\textsuperscript{171}. Although the molecular basis of the mechanisms for learning and memory are still the focus of many studies, there are clear results that show structural and functional changes in the synapse, due to signaling cascades of calcium, cAMP, and the transcription factor CREB.
Recent work has demonstrated that the peptide GLP-1 also plays a neuroprotective role, as it was able to prevent excitotoxic neuronal death caused by the excessive activation of glutamate receptors\textsuperscript{172}. In addition, this protective effect was also observed against excitotoxic brain damage caused by seizures in mouse models\textsuperscript{171}.

The potent GLP-1r agonist, exendin-4, was able to prevent oxidative and metabolic insults in cell and animal models of ischemia and Parkinson’s disease\textsuperscript{173}. Moreover, these agonists of GLP-1r reversed the inhibition of LTP induced by AβOs\textsuperscript{174}. Further evidence for the central role of GLP-1r in synaptic plasticity is supported by data that demonstrates that cognitive deficits are present by animals that lack the expression of GLP-1 receptors\textsuperscript{175}. Therefore, these data suggests a promising role for GLP-1, as a therapeutic agent for the treatment of various neurodegenerative conditions, including AD.

1.6. Axonal transport

Due to the polarity of neurons intracellular transport is particularly crucial for neurons to maintain their synapses and synaptic function\textsuperscript{176}. A typical neuron is a highly polarized cell consisting of a soma with many short, tapered dendrites and a long, thin axon of even caliber. Vertebrate axons of the CNS lack the protein synthesis machinery necessary to make proteins that are required for the maintenance and function of the synaptic terminal. Most proteins localized at the axonal synaptic terminal are synthesized in the cell body and then transported along the axon in membranous or proteinaceous complexes\textsuperscript{177}. There are several specific mRNAs that are transported to the dendrites to support protein synthesis locally, as they contain protein synthesis machinery\textsuperscript{178}. Besides proteins, cellular components such as endosomes, mitochondria, synaptic vesicles, and dense core vesicles (DCV) are also transported by active cytoskeletal-based transport, as delivery by diffusion would be too slow to maintain cell functions. These components are formed in the cell body and then travel long distances through the axons and dendrites, until they reach their pre- or post-synaptic sites, where they finally release their contents of neuropeptides and proteins\textsuperscript{179}. The distance traveled by the vesicles in neurons are longer compared with other cell types, as the axon of a human motor neuron can measure over 1 meter long\textsuperscript{180}.
Microtubules run along the dendrites and axons and serve as rails upon which organelles and macromolecules can be transported (Fig. 1.6). Microtubules are composed of polymers of α- and β-tubulin heterodimers, which when assembled, form protofilaments. Protofilaments then associate to form hollow cylinders of approximately 25 nm in diameter. These microtubules are highly dynamic as they often cycle between states of polymerization and depolymerization. The dynamic assembly and disassembly of microtubules is controlled by microtubule-associated proteins (MAP) in vivo and in vitro. The microtubule end, with β-tubulin exposed, is called the plus-end, while the α-tubulin exposed end is known as the minus-end. These features give microtubules polarity, which enables motor proteins, such as kinesins and dyneins, to “know” directionality as they transform chemical energy into mechanical motion.

Both kinesins and dyneins are ATPases, containing a microtubule binding domain and a cargo-binding domain, with kinesins generally moving toward the plus end of microtubules, or away from cell body (anterograde). Dyneins, however, generally move toward the minus end of the microtubules, or toward the cell body (retrograde). Dyneins are comprised of two heavy chains, two light chains and two intermediate light chains. It is believed that the dynein-mediated transport is regulated by its interaction with the dynactin complex, which consists of several proteins.

The mechanisms of regulation of kinesin and dynein activity, as well as the regulation of axonal transport, as a whole, remain largely unknown. Data demonstrates that the motility of soluble kinesin-1 in the cell is inhibited, but when cargo binds to the motor it becomes active. Binding partners identified for anterograde and retrograde motor proteins seem to be involved with a regulatory mechanism. These partners include amyloid precursor protein (APP), c-Jun N-terminal kinase (JNK) and proteins that interact with it (JIP1, JIP2, JIP3/Sunday driver). Additional evidence suggests the important role of protein phosphorylation in regulating motor function, involving glycogen synthase kinase 3β kinases (GSK-3β) and cyclin-dependent kinase 5 (CDK-5).
1.6.1. Alzheimer’s disease, axonal transport, and tau

Studies suggest correlation between aging and the emergence of axonal transport defects, as there is a reduction in the quantity of microtubule tracks, as well as changes in the distribution of the microtubule associated proteins, such as tau and the neurofilament protein. Another study observed an apparent accumulation of proteins, such as APP, along the axon. Furthermore, manganese enhanced MRI studies in mice supported these observations by demonstrating reductions in axonal transport in vivo correlated with age. The understanding of these age-related changes in the structure and function of axons remains unclear, especially since it is unknown...
whether aging affects all transport proteins or if only a few specific signaling pathways are impaired.

The disruption of neuronal transport is considered an early pathological manifestation in multiple neurodegenerative diseases, including Huntington's disease, amyotrophic lateral sclerosis, and AD. In particular, the involvement of defective axonal transport was found in the brains of patients with AD and in animal models of AD, such as, transgenic mice overexpressing human APP or induced mutations in presenilin-1 or protein tau also resulted in defective transport. In addition, manganese enhanced MRI studies in mutant APP transgenic mice and APP/PSI/tau triple transgenic mice supported these observations by demonstrating reductions in axonal transport prior to deposition of neuropathological inclusions in vivo. Collectively, these independent observations provide strong evidence that axonal transport deficits represent a common feature among the diverse pathologies associated with AD.

The precise mechanisms underlying axonal transport deficits in AD are largely unknown, but multiple pathways are likely involved. For instance, some reports suggested that increasing microtubule stability reduces tau-mediated axonal transport deficits. Studies have led to the proposal that tau protein reduces axonal transport by physically interfering with the binding of kinesin to microtubules. However, physical blockade of kinesin by tau was not supported by other studies. Tau also was proposed to block microtubule binding of kinesin and to revert cytoplasmic dynein directionality, but other reports contradict this possibility. Recent studies indicate that AβOs may inhibit axonal transport through multiple mechanisms including activation of N-methyl-D-aspartate receptors and activation of specific kinases (see 1.6.2). While all of these are important mechanisms for kinesin regulation, my work focuses on motor regulation by protecting or reverting abnormal activation of kinases and phosphatases, a major AD hallmark.
1.6.2. **Cellular signaling and axonal transport**

Basic neuronal functions depend upon the regulated delivery of cargos to specialized axonal compartments. For example, effective delivery of cargoes containing sodium channels at the nodes of Ranvier is necessary for saltatory conduction. Similarly, in order to have effective neurotransmission there has to be delivery of synaptic vesicle precursors to hundreds of “en passant” and terminal synapses in neurons\(^\text{176}\). This argues for the existence of regulatory mechanisms of axonal transport as these local cargo delivery systems are highly specific. Multiple mechanisms have been proposed to regulate axonal transport in vivo. Some are based on regulation of motor protein activities by autoinhibition\(^\text{215}\), phosphorylation\(^\text{189, 216}\), calcium-dependent interactions with cargo-associated binding partners, or recruitment of specific adaptor proteins\(^\text{217}\). Dysfunction in any or all these regulatory mechanisms may be important to AD pathogenesis as aberrant signaling cascades are widely recognized\(^\text{214}\).

While mutations in molecular motor subunits are not known in AD patients, alterations in regulatory mechanisms for axonal transport might underlie the abnormalities in axonal transport characteristic of AD\(^\text{189}\). Several studies indicate that phosphorylation of motor proteins represents a major mechanism for the regulation of axonal transport. Several kinases that are abnormally activated in AD that can affect axonal transport, are glycogen synthase kinase 3 (GSK-3β), cyclin-dependent kinase 5 (Cdk5), and casein kinase 2 (CK2)\(^\text{189}\). A variety of protein kinases regulate specific functional activities of kinesin, e.g. c-Jun amino-terminal kinase 3 (JNK3) was recently identified as a mitogen activated protein kinase (MAPK) that phosphorylates serine 176 within the motor domain of kinesin heavy chain (KHC). This phosphorylation impaired the binding of kinesin to microtubules and its translocation along axons in vivo\(^\text{218}\). The protein kinases CK2 and GSK-3β regulate the binding of kinesin to cargoes through a mechanism involving phosphorylation of KLCs\(^\text{219, 220}\). Additionally, the inhibition of Cdk5, through an unknown mechanism, increased activation of PP1, activates GSK-3β, and phosphorylates of KLCs, among other substrates\(^\text{221, 222}\).

Recent work identified a signaling cascade by which pathological forms of tau activate axonal PP1. Active PP1 in turn activates GSK-3β via dephosphorylation at serine 9, ultimately increasing KLC phosphorylation and dissociation of kinesin-1 (KIF5)
from cargoes\textsuperscript{223}. Further studies identified a phosphatase-activating domain (PAD) within the N-terminus of tau that, when abnormally exposed, promotes activation of the PP1-GSK3 signaling cascade, leading to inhibition of kinesin-based anterograde axonal transport\textsuperscript{224}. New studies showed that increased PAD exposure represents an early molecular event that precedes formation of NFTs in sporadic AD\textsuperscript{224}. Collectively, these data suggest that early pathological changes in tau protein lead to increased PAD exposure, which induces aberrant activation of a PP1-GSK3 cascade, phosphorylation of KLCs and inhibition of conventional kinesin-based anterograde axonal transport\textsuperscript{224, 225}. Most of these previous experiments were performed on KIF5 leaving the mechanisms of regulation of the primary motor for DCVs, KIF1A, largely unknown. One of my aims of this thesis was to understand the signaling that might influence KIF1A motility.

I hypothesize that the insulin signaling pathway, ultimately via GSK3\textbeta\ dysregulation, modulates BDNF axonal transport defects induced by A\beta Os in primary cultured mouse hippocampal neurons independent of tau. The specific objectives of my thesis were:

1. To investigate the role of tau in insulin receptor stimulation’s protective effect in maintaining physiological axonal transport from A\beta Os.
2. To elucidate the role of AKT, through an insulin receptor independent manner, in the prevention of axonal transport defects in tau \textsuperscript{+/+} and tau \textsuperscript{-/-} neurons.
3. To test whether GSK-3\textbeta inhibitors and Exendin-4 rescue the impact of A\beta O in axonal transport defects in tau \textsuperscript{+/+} and tau \textsuperscript{-/-} neurons.
2. Methods and Materials

2.1. Hippocampal cell culture and expression of transgenes

Primary hippocampal neuronal cultures from E16 wild type (tau<sup>+/+</sup>) and tau knockout (tau<sup>-/-</sup>) mice (The Jackson Laboratory) were prepared as described by Kaech and Banker<sup>226</sup>. Briefly, the neurons were plated onto poly-L-lysine (Sigma-Aldrich) pre-treated glass coverslips and then the plated coverslips with the neurons facing down were placed into a dish containing a monolayer of astrocytes. The neurons with the astrocyte layer were kept in Neurobasal/B27 (Invitrogen) or primary neuron growth media (PNGM) (Lonza). At 10-12 days in vitro (DIV), cells were co-transfected with 1.0 µg pmUBa-eBFP, pβ-actin-BDNF-mRFP (received from Gary Banker, Oregon Health and Sciences University, Portland, OR), and/or ca-AKT-HA (Addgene Plasmid # 16228) using Lipofectamine 2000 (Invitrogen). Prior to transfection 0.5 µM kynurenic acid (Sigma-Aldrich) was added to decrease excitotoxic damage. Cells expressed the plasmids for 24-36 hours before live imaging.

2.2. Aβ, insulin, and inhibitor treatments

Aβ1-42 (Bachem Inc., Torrance, CA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Merck, Darmstadt, DE) over ice followed by incubation at room temperature for 60 min. The solution was then placed on ice for 5-10 min and aliquoted into Eppendorf tubes. The tubes were left open in the laminar flow hood for 12 hours at room temperature to allow evaporation of HFIP. The complete elimination of HFIP was performed by centrifugation for 10 min with a SpeedVac. Aliquots containing thin films and transparent Aβ were stored at -80 °C for later use. AβOs preparations were made by dissolving an aliquot of Aβ film in fresh anhydrous DMSO (Sigma) to obtain a 5 mM solution. This solution was then diluted to 100 mM in sterile PBS and incubated at 4 °C
for 24 hours. After incubation, the preparation was centrifuged at 14,000 g for 10 minutes at 4 °C. The supernatant was collected after centrifugation, leaving behind insoluble aggregates of Aβ, and kept at 4 °C until use. A Bradford assay was done to determine the concentration of AβOs. AβOs were applied to cells at a final concentration of 500 nM for 18 hours. For the insulin experiment, 1 hour prior to AβO or vehicle exposure, cells were incubated with 1.0 µM Insulin (Sigma) or equivalent volumes of vehicle (PBS). For the GSK-3β inhibitor VIII, Tideglusib, and Exendin-4 experiments, 18 hours following AβO or vehicle exposure, cells were incubated with either 0.3 µM Exendin-4 (Tocris), 0.5 µM GSK-3β VIII (Calbiochem), or 2.5 µM Tideglusib (Sigma) or equivalent volumes of vehicle (DMSO) for 1-5 hours prior to imaging of transport.

2.3. Live imaging and analysis of BDNF-mRFP transport

BDNF-mRFP Transport was analyzed using a standard wide-field fluorescence microscope equipped with a cooled CCD camera and controlled by MetaMorph, according to Kwinter et al., 2009. Briefly, cells were sealed in a heated imaging chamber, and streaming recordings were acquired from double transfectants at an exposure time of 250ms for 25s. This captured dozens of transport events per cell in 100-μm segments of the axon. Axons were identified based on morphology by using BFP expression to determine the orientation of the cell body relative to the axon, and thus to distinguish between anterograde and retrograde transport events. Vesicle flux, velocity, and run lengths were obtained through tracing kymographs in MetaMorph. Vesicle flux was defined as the total distance traveled by vesicles standardized by the lengths and duration of each movie (see below). Kymographs were traced by hand by selecting all anterograde (positive slope) and retrograde movements (negative slope) separately within each kymograph. The trace information was analyzed using custom software that calculated the values of flux, velocity, and run length. This moving fraction analysis method gives us comparable population statistics based on parameters reproducible between many independent cultures, as plasmid expression is variable between cultures and could affect the number of organelles labeled in any one experiment.
2.4. Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde and blocked with 0.5% fish skin gelatin\textsuperscript{227}. To confirm A\textbeta\textgreek{O} binding after 18 hours in culture, cells were stained with an A\textbeta\ antibody (1:1000; 6E10, Millipore) or an A\textbeta\O\ structure specific antibody (1:100; 11A1, Immuno-Biological Laboratories, Japan). To assess ca-AKT-HA plasmid expression, neurons were stained with anti-HA (1:500; Boehringer Mannheim). Neurons were subsequently incubated with compatible secondary antibodies conjugated to Cy5 (1:500; Jackson ImmunoResearch Laboratories) and Alexa 488 (1:500, Invitrogen).

2.5. Statistical analyses

All videos were processed using MetaMorph software (Universal Imaging), which was used to generate a time-distance graph for each video with the kymograph option. Diagonal lines on each kymograph were traced and the information was analyzed by custom-made software\textsuperscript{227} that calculated the values of flux, velocity and run lengths based on the calibration that at a magnification of 630X, 1 pixel = 0.160508 \textmu m (vertical axis). Vesicle flux was defined as the total distance traveled by vesicles standardized by the length and duration of each movie (in micron-minutes): 

\[ \sum_{i=1}^{n} \frac{d_i}{\ell \times t} \] 

where \( d \) are the individual DCV run lengths, \( \ell \) is the length of axon observed and \( t \) is the duration of the observation. Vesicles traveling less than 2 \textmu m were not included in the analysis as distances this short could be accounted for by diffusion based on the formula that root-mean square displacement is \( \sqrt{2Dt} \) where \( D \) is the diffusion coefficient (\( D=0.01 \ \textmu m^2/s \) for DCVs) and \( t \) is the duration of the observation (\( t=50 \) s)\textsuperscript{227}. Runs were considered terminated if a particle remained in the same position for 4 frames (1s). A one-tailed student t-test, using equal or unequal variance based on F-tests, was used to determine significance between pair wise comparisons of control and experimental conditions in Microsoft Excel.

Statistical analyses were performed using Excel (Microsoft). Data are presented as mean \( \pm \) SEM. Significant differences between treatments were analyzed by t-tests
with equal or unequal variance at a 95% confidence interval. For live imaging experiments, a minimum of 12 cells from 3 independent cultures (n=3) were analyzed.
3. Results

3.1. Insulin prevents AβO-induced DCV transport defects in a tau-independent manner

Insulin signaling plays a critical role in neuronal homeostasis\textsuperscript{135, 228}. Zhao et al., 2008 has shown that AβOs cause insulin receptor internalization via a non-competitive mechanism with respect to insulin that blocks insulin pathway activation. One consequence of the internalization of the insulin receptor is the eventual downstream hyperactivation of GSK-3β, a well-described characteristic of AD\textsuperscript{44, 229}. De Felice et al., 2009 was able to protect against AβO-induced insulin receptor internalization with insulin pre-treatment. Our lab has shown that applying insulin prior to AβO treatment protects against brain-derived neurotrophic factor (BDNF) transport defects in primary hippocampal neurons from wild type (tau\textsuperscript{+/+}) mice\textsuperscript{149}. GSK-3β is a tau kinase, which in a hyperactive state leads to an increase in p-tau, where the accumulation of p-tau potentially disrupts transport due to microtubule destabilization. Yet, GSK-3β may have other substrates that affect the transport apparatus, for example, motor proteins. In addition, other studies have found that the PAD within the N-terminus of tau, when abnormally exposed, promotes activation of the GSK-3β signaling cascade, leading to inhibition of kinesin-based anterograde axonal transport\textsuperscript{224}. I sought to determine if insulin could protect against transport defects induced by AβOs in a tau-independent manner by imaging organelle transport in tau\textsuperscript{−/−} mice (Fig. 3.1). To determine if AβO-induced transport defects occur in a tau-independent manner, I expressed BDNF-mRFP, a DCV cargo, in tau\textsuperscript{−/−} neurons, and imaged after 18 hours of exposure to 500 nM AβOs (Fig. 3.1A-D). Retrospective immunocytochemistry was used to confirm AβO binding to dendrites (Fig. 3.1B, D). Total axonal flux of AβO treated neurons was significantly reduced relative to vehicle control (59% decrease, Fig. 3.2, 3.3A and Table 3.1). However, in cells treated with 1.0 µM insulin before the addition of the 500 nM AβOs axonal flux was unchanged when compared to vehicle control (Fig. 3.2, 3.3A and Table...
3.1). Average velocity for total, anterograde, and retrograde transport was significantly increased with insulin pre-treatment when compared to the AβO condition (Fig. 3.3B and Table 3.1). Furthermore, average run length of retrograde transport was significantly longer in the insulin pre-treatment condition comparing to AβO treated cells (Fig. 3.3C and Table 3.1). This result showed that tau is not required for AβO-induced transport defects and can be prevented through stimulation of insulin receptors.
Neurons were pre-incubated with 1.0 µM insulin for 30 min and treated for 18 hours with 500nM AβOs. Representative images of BDNF-mRFP (A) and soluble BFP (B) expression in tau+ neurons. (C) AβO binding after 1.0 µM insulin treatment followed by 18 hours of 500nM AβO application. (D) Overlay image of soluble BFP and 500nM AβO. (E) A series of frames of a recording of BDNF-mRFP transport. Particles can be observed moving in both anterograde (green arrow) and retrograde (red arrow) directions. The recording is then transformed into a kymograph (F) with the blue highlighted region visualized in the series of frames in E. Arrows indicate the axon; arrowheads indicate dendrites. Scale bar = 25 µm

Figure 3.1. AβO binding retrospectively confirmed with immunocytochemistry and an example of kymograph generation.
Figure 3.2. **Insulin prevents DCV transport defects in tau -/- neurons.**

Neurons were pre-incubated with 1.0 \( \mu \)M insulin (or vehicle) for 30 min and then treated for 18 hours with 500 nM AβOs (or vehicle). Representative kymographs comparing the effects of AβOs, insulin, and insulin + AβOs on DCV transport in tau-/- neurons compared to control vehicle.
Figure 3.3. **Flux, velocity, and run length comparisons of DCV transport defects prevented by insulin.**

Effects of treatments on DCV flux, average velocity, and average run length. Cultures were pre-incubated with 1.0 uM insulin (or with vehicle) for 30 min and then treated for 18 hours with 500 nM AβOs (or with vehicle). A) AβO treatment decreased bidirectional flux compared to vehicle control; in comparison insulin pre-treatment of AβOs prevented the flux decrease and was similar to vehicle control. B) AβO treatment decreased bidirectional average velocity compared to vehicle control whereas Ins + AβOs had similar velocity to vehicle. C) AβO treatment decreased bidirectional and retrograde average run length compared to vehicle control, with Ins + AβO having no change compared to vehicle control. A minimum of 12 cells from 3 different cultures was analyzed per condition. * p<0.05, ** p<0.01, *** p<0.001, all columns relative to vehicle control. + p<0.05, ++ p<0.01, +++ p<0.001, all columns relative to AβO.
3.2. AKT overexpression prevents AβO-induced transport defects

AKT, an insulin signaling intermediate, can inhibit GSK-3β activation by direct phosphorylation thus perhaps preventing the blockade of BDNF transport\(^{230}\). To determine if active AKT in the presence of AβOs would ameliorate axonal transport defects, I transfected neurons with a plasmid containing a constitutively active form of AKT (ca-AKT) and BDNF-mRFP (Fig. 3.5). Ca-AKT expression prevented transport defects of BDNF, as axonal flux was similar to vehicle control in tau\(^{+/+}\) and tau\(^{-/-}\) (Fig. 3.4, 3.6, and 3.7; Table 3.2). Additionally, average velocity for all events and anterograde transport significantly increased with AKT overexpression when compared to AβOs in tau\(^{+/+}\) neurons (Fig. 3.7B and Table 3.2), while only retrograde velocities in tau\(^{-/-}\) were found to be significant (Fig. 3.6B and Table 3.2). Furthermore, average run length of transport was found to be significantly longer in the AKT + AβOs condition than in AβO.
control (Fig. 3.7C, and Table 3.2) in tau+/-. In tau+/+ neurons only the AKT + AβOs retrograde average run length was significant longer compared to AβO control (Fig. 3.6C and Table 3.2). The reduction in flux, without corresponding changes in velocity and run length might be explained by “all-or-none” inhibition of individual transport complexes. If there is an inhibition of kinesin or dynein it will lead to in a bidirectional inhibition of transport not just a single direction change (see Discussion)231-234. Overexpression of AKT was capable of preventing BDNF transport defects by presumably inhibiting GSK-3β cascade overactivation in both tau+/+ and tau-/− neurons. However, once transport defects are already initiated, could inhibiting the GSK-3β cascade lead to re-establishing normal transport physiology?

Figure 3.2. AKT overexpression prevents DCV transport defects.

Representative kymographs comparing DCV transport effects of AβOs, AKT, and AKT + AβOs in tau+/+ and tau-/− neurons. Cultures were co-transfected with CA-AKT plasmid and treated for 18 hours with 500nM AβOs (or vehicle). AKT overexpression was able to prevent AβO-induced transport defects. Representative kymographs comparing DCV transport effects of AβOs, AKT, and AKT + AβOs in tau+/+ and tau-/− neurons compared to vehicle control.
Figure 3.3. AβO binding and CA-AKT expression in tau<sup>−/−</sup> neurons.

Neurons were co-transfected with CA-AKT (yellow) and BDNF-mRFP (red), and treated for 18 hours with 500 nM AβOs. After live imaging, cells were retrospectively stained by immunocytochemistry to confirm expression of CA-AKT and AβO binding. Representative images of soluble BFP (A), BDNF-mRFP (B), and CA-AKT (D) expression in tau<sup>+/+</sup> and tau<sup>−/−</sup> neurons. (C) AβO binding after 18 hours of 500 nM AβO application. Arrows indicate the axon; arrowheads indicate dendrites. Scale bar = 25 µm
Figure 3.4. Flux, velocity, and run length comparisons of DCV transport defects prevented by AKT overexpression in tau+/+ neurons.

Effects of treatments on DCV flux, velocity, and run length. Neurons were co-transfected with CA-AKT plasmid and treated for 18 hours with 500 nM AβOs (or vehicle) in tau+/+ neurons. A) AβO treatment decreased bidirectional flux compared to vehicle control; in comparison AKT overexpression prior to application of AβOs prevented the flux decrease. B) AKT overexpression with AβOs increased bidirectional and anterograde average velocity compared to AβOs. C) AKT + AβO had longer average run length when compared to vehicle and AβO in the retrograde direction. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, ***p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
Figure 3.5. **Flux, velocity, and run length comparisons of DCV transport defects prevented by AKT overexpression in tau⁻/⁻ neurons.**

Effects of treatments on DCV flux, velocity, and run length. Cultures were co-transfected with CA-AKT plasmid and treated for 18 hours with 500 nM AβOs (or vehicle) in tau⁻/⁻ neurons. A) AβO treatment decreased bidirectional flux compared to vehicle control; in comparison AKT overexpression prior to application of AβOs prevented the flux decrease. B) AKT overexpression with and without AβOs increased retrograde average velocity compared to vehicle control and AβOs. C) Average run length was significantly greater in the AKT treated condition over vehicle control; AKT + AβOs also had greater bidirectional average run length than the AβO control. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, ***p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
## Table 3.2. Quantitative Analysis: DCV Transport Defects Prevented by AKT overexpression

<table>
<thead>
<tr>
<th>Flux (min⁻¹)</th>
<th>All events</th>
<th>Anterograde</th>
<th>Retrograde</th>
<th>All events</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau +/-, vehicle</td>
<td>11.9±0.89</td>
<td>6.42±0.8</td>
<td>5.49±0.57</td>
<td>100±7.43</td>
</tr>
<tr>
<td>tau +/-, AβOs</td>
<td>5.17±0.66***</td>
<td>2.37±0.46***</td>
<td>2.81±0.29***</td>
<td>43.42±5.42***</td>
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<tr>
<td>tau +/-, AKT</td>
<td>16.1±1.63*</td>
<td>8.66±1.2</td>
<td>8.02±0.78*</td>
<td>135.28±13.69*</td>
</tr>
<tr>
<td>tau +/-, AKT + AβOs</td>
<td>14.06±0.97***</td>
<td>6.03±0.62***</td>
<td>8.04±0.9***</td>
<td>118.12±8.09***</td>
</tr>
<tr>
<td>tau -/-, vehicle</td>
<td>12.44±1.14</td>
<td>6.72±0.91</td>
<td>5.73±0.70</td>
<td>100±9.14</td>
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<tr>
<td>tau -/-, AβOs</td>
<td>5.02±0.59***</td>
<td>2.7±0.39***</td>
<td>2.32±0.36***</td>
<td>40.3±4.68***</td>
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<tr>
<td>tau -/-, AKT</td>
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<td>6.86±0.9</td>
<td>1.71±1.09</td>
<td>120.72±11.55</td>
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<tr>
<td>tau -/-, AKT + AβOs</td>
<td>14.06±1.39**</td>
<td>8.28±1.13**</td>
<td>8.04±0.9</td>
<td>118.12±11.55</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Velocity (µm/s)</th>
<th>All events</th>
<th>Anterograde</th>
<th>Retrograde</th>
<th>All events</th>
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<tr>
<td>tau +/-, vehicle</td>
<td>1.82±0.08</td>
<td>1.8±0.11</td>
<td>1.78±0.08</td>
<td>100±4.34</td>
</tr>
<tr>
<td>tau +/-, AβOs</td>
<td>1.5±0.12*</td>
<td>1.41±0.12*</td>
<td>1.54±0.13</td>
<td>82.49±6.52*</td>
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<tr>
<td>tau +/-, AKT</td>
<td>1.96±0.08</td>
<td>1.98±0.12</td>
<td>1.92±0.08</td>
<td>107.87±4.26</td>
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<tr>
<td>tau +/-, AKT + AβOs</td>
<td>1.86±0.12*</td>
<td>1.85±0.14*</td>
<td>1.83±0.11</td>
<td>102.13±6.36*</td>
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<td>1.66±0.06</td>
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<td>tau -/-, AβOs</td>
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<td>tau -/-, AKT</td>
<td>2.12±0.11</td>
<td>2.13±0.12</td>
<td>2.05±0.11**</td>
<td>111.54±5.58</td>
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<tr>
<td>tau -/-, AKT + AβOs</td>
<td>2.07±0.13</td>
<td>2.06±0.15</td>
<td>2.03±0.11**</td>
<td>109.13±6.54</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Run length (µm)</th>
<th>All events</th>
<th>Anterograde</th>
<th>Retrograde</th>
<th>All events</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau +/-, vehicle</td>
<td>6.1±0.44</td>
<td>6.65±0.58</td>
<td>5.46±0.35</td>
<td>100±7.11</td>
</tr>
<tr>
<td>tau +/-, AβOs</td>
<td>5.76±0.42</td>
<td>6.33±0.72</td>
<td>5.45±0.3</td>
<td>94.4±6.84</td>
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<td>6.52±0.41</td>
<td>6.72±0.49</td>
<td>6.26±0.42</td>
<td>106.93±6.61</td>
</tr>
<tr>
<td>tau +/-, AKT + AβOs</td>
<td>6.82±0.39</td>
<td>6.97±0.61</td>
<td>6.6±0.42*</td>
<td>111.7±6.28</td>
</tr>
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<td>tau -/-, vehicle</td>
<td>4.96±0.27</td>
<td>5.11±0.34</td>
<td>4.9±0.26</td>
<td>100±5.27</td>
</tr>
<tr>
<td>tau -/-, AβOs</td>
<td>4.85±0.28</td>
<td>4.9±0.36</td>
<td>4.46±0.28</td>
<td>97.81±5.59</td>
</tr>
<tr>
<td>tau -/-, AKT</td>
<td>6.18±0.49*</td>
<td>6.01±0.68</td>
<td>6.16±0.47*</td>
<td>124.74±9.74*</td>
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<tr>
<td>tau -/-, AKT + AβOs</td>
<td>6.25±0.41**</td>
<td>6.34±0.51**</td>
<td>6.03±0.43**</td>
<td>126.08±8.29**</td>
</tr>
</tbody>
</table>

---

* p<0.05, ** p<0.01, *** p<0.001, when compared with tau +/- vehicle (from each tau +/- column)
+ p<0.05, ++ p<0.01, +++ p<0.001, when compared with tau +/- AβOs (from each tau +/- column)
# p<0.05, ## p<0.01, ### p<0.001, when compared with tau -/- vehicle (from each tau -/- column)
§ p<0.05, §§ p<0.01, §§§ p<0.001, when compared with tau -/- AβOs (from each tau -/- column)

Additional details:
- tau +/-, vehicle n=15 kymographs (15 cells, 1161 vesicles), tau +/-, AβOs n=13 kymographs (13 cells, 454 vesicles), tau +/-, AKT n=14 kymographs (14 cells, 1213 vesicles), tau +/-, AKT + AβOs n=18 kymographs (18 cells, 1363 vesicles), tau -/-, vehicle n=15 kymographs (15 cells, 1388 vesicles), tau -/-, AβOs n=15 kymographs (15 cells, 528 vesicles), tau -/-, AKT n=16 kymographs (16 cells, 1235 vesicles), tau -/-, AKT + AβOs n=13 kymographs (13 cells, 1125 vesicles).
3.3. GSK-3β inhibition rescues AβO induced transport defects

To determine if once axonal transport defects are initiated could the transport defects be rescued, I used reversible and irreversible specific GSK-3β inhibitors: GSK-3β Inhibitor VIII and Tideglusib, respectively. After transfecting soluble BFP and BDNF-mRFP, I applied 500 nM AβO to induce transport defects. 18 hours after AβO application, with transport defects initiated, the GSK-3β inhibitors were added and transport was measured. The thiazole-based GSK-3β inhibitor VIII (Calbiochem) experiments showed that total axonal flux was restored and significantly increased (126% in tau+/−, 159% in tau−/−) when AβO treated cells had 500 nM GSK-3β inhibitor VIII applied for 1 hour (Fig. 3.8, 3.9A, 3.10A and Table 3.3). Additionally, AβOs + GSK-3β inhibitor VIII had significantly higher average velocity for anterograde and retrograde transport relative to AβO control in tau−/− neurons (Fig. 3.10B and Table 3.3). AβOs in tau+/− neurons induced significant decrease of average velocity in total and anterograde transport compared to vehicle control (Fig. 3.9B and Table 3.3), and AβOs + GSK-3β inhibitor VIII was similar to vehicle control. In summary, my data show that GSK-3β inhibitor VIII application reversed AβOs-induced transport defects in a non-tau dependent manner.

Tideglusib (Sigma), an irreversible thiadiazolidinone GSK-3β inhibitor, significantly restored total axonal flux (134% in tau+/−, 129% in tau−/−) when compared to AβO treated cells (Fig. 3.11, 3.12A, 3.13A and Table 3.4). Tideglusib may have inhibited GSK-3β past a physiological balance, as Tideglusib significantly increased total and anterograde flux values over vehicle control (Fig. 3.12A and Table 3.4). Additionally, average velocity for total and retrograde transport significantly recovered with Tideglusib application when compared to AβOs in tau+/− neurons (Fig. 3.12B; Table 3.3). Tideglusib rescued average velocity in total and anterograde transport of tau−/− neurons (Fig. 3.13B and Table 3.4). Tideglusib was able to reverse AβO induced transport defects in both tau+/− and tau−/− neurons further suggesting that GSK-3β signaling to be able to regulate transport in the absence of tau.
Figure 3.6. **GSK-3β Inhibitor VIII rescues DCV transport defects.**

Cultures were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then GSK-3β inhibitor VIII (or vehicle) was applied. Representative kymographs comparing DCV transport effects of AβOs, GSK-3β inhibitor VIII, and AβOs + GSK-3β inhibitor VIII in tau+/+ and tau−/− neurons compared to vehicle control.
Figure 3.7. **Flux, velocity, and run length comparisons of DCV transport defects rescued by GSK-3β Inhibitor VIII in tau+/− neurons.**

Effects of treatments on DCV flux, velocity, and run length. Cultures of tau+/− neurons were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then GSK-3β Inhibitor VIII (or vehicle) was applied. A) AβO treatment decreased bidirectional flux compared to vehicle control; GSK-3β Inhibitor VIII application to AβOs treated cells reverted bidirectional flux to control levels. B) AβOs decreased average velocity compared to vehicle control. C) Average run length was unaffected by treatments. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, ***p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
Figure 3.8. Flux, velocity, and run length comparisons of DCV transport defects rescued by GSK-3β Inhibitor VIII in tau<sup>+</sup> neurons.

Effects of treatments on DCV flux, velocity, and run length. Cultures of tau<sup>+</sup> neurons were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then GSK-3β inhibitor VIII (or vehicle) was applied. A) AβO treatment decreased bidirectional flux compared to vehicle control; GSK-3β inhibitor VIII application to AβOs treated cells reverted bidirectional flux to control levels. B) AβOs decreased average velocity compared to vehicle control and was reverted back to vehicle control levels with GSK-3β inhibitor VIII application. C) Average run length was unaffected by treatments. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, ***p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
Table 3.3. **Quantitative Analysis: DCV Transport Defects Rescued by GSK-3β inhibitor VIII**

<table>
<thead>
<tr>
<th></th>
<th>Flux (min⁻¹)</th>
<th>Run length (µm)</th>
<th>Velocity (µm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All events</td>
<td>Anterograde</td>
<td>Retrograde</td>
</tr>
<tr>
<td><strong>BDNF transport</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flux (min⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tau +/+, vehicle</td>
<td>19.36±2.00</td>
<td>10.73±1.79</td>
<td>8.64±0.87</td>
</tr>
<tr>
<td>tau +/+, AβOs</td>
<td>8.81±1.14***</td>
<td>3.61±0.7**</td>
<td>5.20±0.65**</td>
</tr>
<tr>
<td>tau +/+, GSK inh.</td>
<td>17.67±1.29</td>
<td>9.49±1.01</td>
<td>8.18±0.87</td>
</tr>
<tr>
<td>tau +/+, AβOs + GSK inh.</td>
<td>15.04±1.42**</td>
<td>8.29±0.85***</td>
<td>6.76±0.97</td>
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<tr>
<td>tau +/−, vehicle</td>
<td>20.44±1.03</td>
<td>11.89±0.94</td>
<td>8.55±0.90</td>
</tr>
<tr>
<td>tau +/−, AβOs</td>
<td>7.72±1.17###</td>
<td>3.89±0.57***</td>
<td>3.83±0.66***</td>
</tr>
<tr>
<td>tau +/−, GSK inh.</td>
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<td>9.49±1.04</td>
</tr>
<tr>
<td>tau +/−, AβOs + GSK inh.</td>
<td>19.74±1.18###</td>
<td>12.92±0.98###</td>
<td>6.82±0.73###</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Run length (µm)</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>7.95±0.56</td>
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<td>7.54±0.40</td>
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<td>6.34±0.24</td>
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<td>tau +/−, vehicle</td>
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<td>7.38±0.38</td>
<td>7.66±0.39</td>
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<tr>
<td>tau +/−, AβOs</td>
<td>6.61±0.40</td>
<td>7.21±0.64</td>
<td>6.19±0.55#</td>
</tr>
<tr>
<td>tau +/−, GSK inh.</td>
<td>7.13±0.45</td>
<td>7.29±0.59</td>
<td>6.89±0.46</td>
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<tr>
<td>tau +/−, AβOs + GSK inh.</td>
<td>7.66±0.38</td>
<td>8.15±0.51</td>
<td>7.03±0.36</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Velocity (µm/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tau +/+, vehicle</td>
<td>1.78±0.11</td>
<td>1.90±0.13</td>
<td>1.67±0.11</td>
</tr>
<tr>
<td>tau +/+, AβOs</td>
<td>1.45±0.09*</td>
<td>1.40±0.10**</td>
<td>1.45±0.10</td>
</tr>
<tr>
<td>tau +/+, GSK inh.</td>
<td>1.78±0.09</td>
<td>1.81±0.11</td>
<td>1.70±0.08</td>
</tr>
<tr>
<td>tau +/+, AβOs + GSK inh.</td>
<td>1.63±0.10</td>
<td>1.72±0.13</td>
<td>1.56±0.08</td>
</tr>
<tr>
<td>tau +/−, vehicle</td>
<td>2.06±0.08</td>
<td>2.10±0.11</td>
<td>1.93±0.10</td>
</tr>
<tr>
<td>tau +/−, AβOs</td>
<td>1.42±0.08###</td>
<td>1.41±0.09###</td>
<td>1.45±0.10###</td>
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<tr>
<td>tau +/−, GSK inh.</td>
<td>1.90±0.10</td>
<td>1.94±0.12</td>
<td>1.80±0.10</td>
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<tr>
<td>tau +/−, AβOs + GSK inh.</td>
<td>2.09±0.06###</td>
<td>2.23±0.06###</td>
<td>1.85±0.08###</td>
</tr>
</tbody>
</table>

tau +/+, vehicle n=15 kymographs (15 cells, 951 vesicles), tau +/+, AβOs n=13 kymographs (13 cells, 385 vesicles), tau +/+, GSK inh. n=16 kymographs (16 cells, 1235 vesicles), tau +/+, AβOs + GSK inh. n=13 kymographs (13 cells, 1227 vesicles), tau +/−, vehicle n=14 kymographs (14 cells, 1201 vesicles), tau +/−, AβOs + GSK inh. n=13 kymographs (13 cells, 440 vesicles), tau +/−, GSK inh. n=12 kymographs (12 cells, 861 vesicles), tau +/−, AβOs + GSK inh. n=13 kymographs (17 cells, 1227 vesicles).

*p<0.05, ** p<0.01, *** p<0.001, when compared with tau +/+ vehicle (from each tau +/+ column)
+ p<0.05, ++ p<0.01, +++ p<0.001, when compared with tau +/+ AβOs (from each tau +/+ column)
# p<0.05, ## p<0.01, ### p<0.001, when compared with tau +/− vehicle (from each tau +/− column)
§ p<0.05, §§ p<0.01, §§§ p<0.001, when compared with tau +/− AβOs (from each tau +/− column)
Figure 3.9. Kymographs of Tideglusib rescues DCV transport defects.

Cultures were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then Tideglusib (or vehicle) was applied. Representative kymographs comparing DCV transport effects of AβOs, Tideglusib, and AβOs + Tideglusib in tau<sup>+/+</sup> and tau<sup>-/-</sup> neurons compared to vehicle control.
Figure 3.10. Flux, velocity, and run length comparisons of DCV transport defects rescued by Tideglusib in tau++/− neurons.

Effects of treatments on DCV flux, velocity, and run length. Cultures of tau++/− neurons were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then Tideglusib (or vehicle) was applied. A) AβO treatment decreased bidirectional flux compared to vehicle control; Tideglusib applied to AβOs treated cells reverted bidirectional flux to control levels. Tideglusib also significantly increased total and anterograde flux values over vehicle control. B) AβO cells with Tideglusib had greater total and retrograde average velocity compared to AβO + vehicle control. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, *** p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
Figure 3.11. Flux, velocity, and run length comparisons of DCV transport defects rescued by Tideguslib in tau\textsuperscript{−/−} neurons.

Effects of treatments on DCV flux, velocity, and run length. Cultures of tau\textsuperscript{−/−} neurons were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then Tideguslib (or vehicle) was applied. A) AβO treatment decreased bidirectional flux compared to vehicle control; Tideguslib application to AβOs treated cells reverted bidirectional flux to control levels. Tideguslib also significantly increased total and anterograde flux values over vehicle control. B) AβO cells with Tideguslib had greater average velocity compared to AβO + vehicle control. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, ***p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
Table 3.4. **Quantitative Analysis: DCV Transport Defects Rescued by Tideglusib**

<table>
<thead>
<tr>
<th>Flux (min⁻¹)</th>
<th>Traffic values</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All events</td>
<td>Anterograde</td>
</tr>
<tr>
<td>tau +/+, vehicle</td>
<td>18.52±1.59</td>
<td>9.62±1.09</td>
</tr>
<tr>
<td>tau +/+, AβOs</td>
<td>9.95±0.86***</td>
<td>4.35±0.72**</td>
</tr>
<tr>
<td>tau +/+, Tideglusib</td>
<td>26.35±2.34*</td>
<td>15.41±1.82*</td>
</tr>
<tr>
<td>tau +/+, AβOs + Tideglusib</td>
<td>23.32±1.5***</td>
<td>11.4±1.15***</td>
</tr>
<tr>
<td>tau -/-, vehicle</td>
<td>23.64±2.91</td>
<td>14.27±1.98</td>
</tr>
<tr>
<td>tau -/-, AβOs</td>
<td>10.73±1.4###</td>
<td>5.01±0.86***</td>
</tr>
<tr>
<td>tau -/-, Tideglusib</td>
<td>25.23±2.52</td>
<td>15.61±1.63</td>
</tr>
<tr>
<td>tau -/-, AβOs + Tideglusib</td>
<td>24.11±2.24###</td>
<td>12.36±1.47###</td>
</tr>
</tbody>
</table>

**Velocity (µm/s)**

<table>
<thead>
<tr>
<th></th>
<th>Traffic values</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau +/+, vehicle</td>
<td>1.52±0.1</td>
<td>1.57±0.11</td>
</tr>
<tr>
<td>tau +/+, AβOs</td>
<td>1.36±0.08</td>
<td>1.38±0.09</td>
</tr>
<tr>
<td>tau +/+, Tideglusib</td>
<td>1.81±0.1</td>
<td>1.86±0.13</td>
</tr>
<tr>
<td>tau +/+, AβOs + Tideglusib</td>
<td>1.68±0.08*</td>
<td>1.7±0.13</td>
</tr>
<tr>
<td>tau -/-, vehicle</td>
<td>1.88±0.08</td>
<td>1.95±0.08</td>
</tr>
<tr>
<td>tau -/-, AβOs</td>
<td>1.62±0.09*</td>
<td>1.56±0.09</td>
</tr>
<tr>
<td>tau -/-, Tideglusib</td>
<td>2.08±0.13</td>
<td>2.17±0.13</td>
</tr>
<tr>
<td>tau -/-, AβOs + Tideglusib</td>
<td>1.92±0.07§</td>
<td>2.04±0.11##</td>
</tr>
</tbody>
</table>

**Run length (µm)**

<table>
<thead>
<tr>
<th></th>
<th>Traffic values</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau +/+, vehicle</td>
<td>8.15±0.49</td>
<td>8.83±0.69</td>
</tr>
<tr>
<td>tau +/+, AβOs</td>
<td>7.51±0.79</td>
<td>7.18±1.01</td>
</tr>
<tr>
<td>tau +/+, Tideglusib</td>
<td>8.51±0.67</td>
<td>8.95±0.61</td>
</tr>
<tr>
<td>tau +/+, AβOs + Tideglusib</td>
<td>8.5±0.6</td>
<td>9.28±1.12</td>
</tr>
<tr>
<td>tau -/-, vehicle</td>
<td>7.98±0.48</td>
<td>8.76±0.74</td>
</tr>
<tr>
<td>tau -/-, AβOs</td>
<td>7.48±0.65</td>
<td>7.56±0.67</td>
</tr>
<tr>
<td>tau -/-, Tideglusib</td>
<td>9.04±0.55</td>
<td>9.57±0.74</td>
</tr>
<tr>
<td>tau -/-, AβOs + Tideglusib</td>
<td>8.57±0.6</td>
<td>8.62±0.79</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, *** p<0.001, when compared with tau +/+ vehicle (from each tau +/+ column)
+ p<0.05, ++ p<0.01, +++ p<0.001, when compared with tau +/+ AβOs (from each tau +/+ column)
# p<0.05, ## p<0.01, ### p<0.001, when compared with tau -/- vehicle (from each tau -/- column)
§ p<0.05, §§ p<0.01, §§§ p<0.001, when compared with tau -/- AβOs (from each tau -/- column)
3.4. GLP-1 receptor stimulation rescues AβO-induced transport defects

Internalization of IRs caused by AβOs prevents direct IR stimulation from being most effective in AD progression, thus I wanted to test if the glucagon-like peptide-1 receptor (GLP-1r) pathway was a sufficient alternate intervention strategy for rescuing BDNF transport defects. The GLP-1 agonist, Exendin-4 (Ex-4), stimulates the GLP-1 pathway and activates the PI3K/AKT pathway, independent of the insulin receptor. This activation of AKT could inhibit GSK-3β, thus leading to similar rescuing of BDNF transport defects as seen with the GSK-3β Inhibitor VIII and Tideglusib experiments.

Indeed, the Exendin-4 experiments showed that total axonal flux was significantly restored (145% in Tau+/+, 141% in tau−/−) when AβO treated cells had Exendin-4 applied for 2-5 hours compared to the AβO + vehicle cells (Fig. 3.14, 3.15A, 3.16A and Table 3.5). Additionally, average velocity for anterograde and retrograde transport significantly increased when Exendin-4 was added to AβO treated cells compared to AβOs + vehicle in tau+/+ neurons (Fig. 3.15B and Table 3.5), while there was no significance found in the tau−/− neurons (Fig. 3.16B and Table 3.5). Collectively, these results show that the insulin-signaling cascade is able to regulate transport through a tau-independent manner in the presence of AβOs.
Figure 3.12. Kymographs of Exendin-4 rescues DCV transport defects.

Cultures were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500nM AβOs (or vehicle), then Exendin-4 (or vehicle) was applied. Representative kymographs comparing DCV transport effects of AβOs, Exendin-4, and AβOs + Exendin-4 in tau+/+ and tau−/− neurons compared to vehicle control.
Figure 3.13. *Flux, velocity, and run length comparisons of DCV transport defects rescued by Exendin-4 in tau+/+ neurons.*

Effects of treatments on DCV flux, velocity, and run length. Cultures of tau+/+ neurons were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500 nM AβOs (or vehicle), then Exendin-4 (or vehicle) was applied. A) AβO treatment decreased bidirectional flux compared to vehicle control; Exendin-4 applied to AβOs treated cells reverted bidirectional flux to control levels. B) AβO cells with Exendin-4 had greater total and retrograde average velocity compared to AβO + vehicle control. A minimum of 12 cells from 3 different cultures were analyzed per condition. *p<0.05, **p<0.01, ***p<0.001, relative to vehicle. +p<0.05, ++p<0.01, +++p<0.001, relative to AβO.
Figure 3.14. Flux, velocity, and run length comparisons of DCV transport defects rescued by Exendin-4 in tau−/− neurons.

Effects of treatments on DCV flux, velocity, and run length. Cultures of tau+/− neurons were transfected with BDNF-mRFP, after several hours treated for 18 hours with 500nM AβOs (or vehicle), then Exendin-4 (or vehicle) was applied. A) AβO treatment decreased bidirectional flux compared to vehicle control; Exendin-4 applied to AβOs treated cells reverted bidirectional flux to control levels. No changes in average velocity and run length were found among the treatments. A minimum of 12 cells from 3 different cultures were analyzed per condition. * p<0.05, ** p<0.01, ***p<0.001, relative to vehicle. + p<0.05, ++ p<0.01, +++ p<0.001, relative to AβO.
### Table 3.5. Quantitative Analysis: DCV Transport Defects Rescued by Exendin-4

<table>
<thead>
<tr>
<th>Flux (min⁻¹)</th>
<th>Traffic values</th>
<th>%</th>
<th>All events</th>
<th>Anterograde</th>
<th>Retrograde</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau +/-, vehicle</td>
<td>16.92±2.01</td>
<td>10.09±1.75</td>
<td>6.83±0.92</td>
<td>100±11.87</td>
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<tr>
<td>tau +/-, AβOs</td>
<td>7.67±1.14***</td>
<td>3.15±10***</td>
<td>4.53±0.82*</td>
<td>45.36±6.74***</td>
<td></td>
</tr>
<tr>
<td>tau +/-, Exendin-4</td>
<td>18.9±1.78</td>
<td>11.4±1.3</td>
<td>7.50±0.8</td>
<td>111.74±10.49</td>
<td></td>
</tr>
<tr>
<td>tau +/-, AβOs + Exendin-4</td>
<td>18.73±2.15***</td>
<td>10.17±1.74**</td>
<td>8.56±0.76***</td>
<td>110.72±12.7***</td>
<td></td>
</tr>
<tr>
<td>tau +/-, vehicle</td>
<td>21.21±1.86</td>
<td>13.07±1.33</td>
<td>8.14±1.44</td>
<td>100±8.74</td>
<td></td>
</tr>
<tr>
<td>tau +/-, AβOs</td>
<td>8.19±1.66###</td>
<td>4.11±0.81###</td>
<td>4.08±1.04#</td>
<td>38.61±7.8###</td>
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<tr>
<td>tau +/-, Exendin-4</td>
<td>21.67±1.97</td>
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<td>102.17±9.25</td>
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<td>19.8±1.13###</td>
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<td>8.48±0.69###</td>
<td>93.35±5.29###</td>
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</table>

<table>
<thead>
<tr>
<th>Velocity (µm/s)</th>
<th>All events</th>
<th>Anterograde</th>
<th>Retrograde</th>
<th>%</th>
</tr>
</thead>
<tbody>
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<td>1.43±0.11</td>
<td>100±7.93</td>
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<td>1.30±0.13</td>
<td>88.55±6.74</td>
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<td>tau +/-, Exendin-4</td>
<td>1.60±0.09</td>
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<td>1.43±0.07</td>
<td>108.15±5.51</td>
</tr>
<tr>
<td>tau +/-, AβOs + Exendin-4</td>
<td>1.84±0.12***</td>
<td>1.87±0.15**</td>
<td>1.80±0.09**</td>
<td>124.53±7.55***</td>
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<td>100±6.29</td>
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<tr>
<td>tau +/-, AβOs</td>
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<tr>
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<td>1.96±0.18</td>
<td>101±8.94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Run length (µm)</th>
<th>All events</th>
<th>Anterograde</th>
<th>Retrograde</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau +/-, vehicle</td>
<td>8.12±0.74</td>
<td>8.93±1.27</td>
<td>7.11±0.45</td>
<td>100±9.07</td>
</tr>
<tr>
<td>tau +/-, AβOs</td>
<td>8.55±1.43</td>
<td>9.49±2.77</td>
<td>7.92±1.37</td>
<td>105.42±17.56</td>
</tr>
<tr>
<td>tau +/-, Exendin-4</td>
<td>8.17±0.47</td>
<td>9.02±0.61</td>
<td>7.16±0.36</td>
<td>100.71±5.79</td>
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<tr>
<td>tau +/-, AβOs + Exendin-4</td>
<td>7.77±0.55</td>
<td>7.56±0.81</td>
<td>7.81±0.59</td>
<td>95.78±6.67</td>
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<td>tau +/-, vehicle</td>
<td>7.76±0.42</td>
<td>7.87±0.50</td>
<td>7.78±0.60</td>
<td>100±5.35</td>
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<td>tau +/-, AβOs</td>
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<td>6.81±1.06</td>
<td>6.08±0.70</td>
<td>82.77±8.93</td>
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<td>tau +/-, Exendin-4</td>
<td>7.09±0.58</td>
<td>7.48±0.63</td>
<td>6.72±0.68</td>
<td>91.46±7.38</td>
</tr>
<tr>
<td>tau +/-, AβOs + Exendin-4</td>
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<td>8.43±1.29</td>
<td>7.36±1.10</td>
<td>103.19±15.11</td>
</tr>
</tbody>
</table>

tau +/-, vehicle n=14 kymographs (14 cells, 944 vesicles), tau +/-, AβOs n=12 kymographs (12 cells, 419 vesicles), tau +/-, Ex-4 n=15 kymographs (15 cells, 1017 vesicles), tau +/-, AβOs + Ex-4 n=13 kymographs (13 cells, 801 vesicles), tau +/-, vehicle n=12 kymographs (12 cells, 918 vesicles), tau +/-, AβOs n=12 kymographs (12 cells, 488 vesicles), tau +/-, Ex-4 n=13 kymographs (13 cells, 989 vesicles), tau +/-, AβOs + Ex-4 n=15 kymographs (15 cells, 1169 vesicles).

* p<0.05, ** p<0.01, *** p<0.001, when compared with tau +/- vehicle (from each tau +/- column)
+ p<0.05, ++ p<0.01, +++ p<0.001, when compared with tau +/- AβOs (from each tau +/- column)
# p<0.05, ## p<0.01, ### p<0.001, when compared with tau +/- vehicle (from each tau +/- column)
§ p<0.05, §§ p<0.01, §§§ p<0.001, when compared with tau +/- AβOs (from each tau +/- column)
4. **Discussion**

4.1. **Summary**

The development, function, and survival of a neuron are dependent on an efficient axonal transport system. Several neurodegenerative diseases have transport defects, notably, in early stages of AD\(^{235}\). AβOs, the primary neurotoxin implicated in AD, induce a variety of cellular insults, among them dysregulation of intracellular signaling cascades, tau hyperphosphorylation\(^{38}\), disruption of fast axonal transport\(^{212}\), and ultimately neuronal death. The mechanisms by which signaling cascades can regulate axonal transport in healthy and diseased neurons remain largely unknown.

Recent studies have increasingly shown brain insulin resistance and associated insulin signaling dysfunction to be a common feature of AD\(^{236}\). However, it remains largely unknown if the axonal transport defects are signaling dependent, caused by abnormal activation of kinases in the early stages of AD progression. I sought to prevent GSK3-β overactivation by stimulating the insulin-signaling pathway in hopes that doing so would prevent or even perhaps rescue axonal transport disruption caused by AβOs.

My data show that insulin receptor stimulation prior to AβO application can protect axonal transport defects. In addition, AKT activation, an insulin receptor signaling intermediate, yielded similar results. Furthermore, once AβO-induced transport defects were established, I was able to rescue these defects through the use of two different GSK3-β inhibitors and the anti-diabetes drug, Exendin-4. In addition, performing all of the experiments in both tau\(^{+/+}\) and tau\(^{-/-}\) neurons with similar results, I was able to show that tau is not required for inducing transport defects with AβOs. Furthermore, the insulin-signaling cascade’s ability to prevent and rescue transport defects were via a tau-independent mechanism. My thesis further elucidates the importance of maintaining insulin signaling for the physiology of healthy neurons.
**Figure 4.1.** Graphical summary and proposed mechanism for insulin signaling preventing and rescuing AβO-induced axonal transport defect.

AβOs bind to various membrane receptors causing signaling dysregulation ending in activation of GSK-3β. Aims 1-4 were performed in order to modulate transport by presumably inactivating GSK-3β, thus preventing motor protein phosphorylation. GSK-3β may exert direct motor regulation through phosphorylation (1) and/or disrupt the attachment of cargoes to their motors (2), and impede BDNF transport in the presence of AβOs.

4.2. Insulin prevents AβO-induced DCV transport defects in a tau-independent manner

Previous work suggested that tau has a phosphatase activating domain (PAD) within the N-terminus that when exposed it activates an aberrant GSK3-β cascade, phosphorylating of kinesin light chain (KLC), and inhibiting kinesin-1 based anterograde axonal transport\(^\text{224, 225}\). In addition, AβOs induce kinase activity changes that have been reported to have an affect on axonal transport, but whether this mechanism is tau-dependent is largely unknown\(^\text{237, 238}\). My data demonstrate that insulin pre-treatment prevents AβO-induced axonal transport defects in tau\(^\text{−}\) neurons. There are several AβO binding sites on the membrane, e.g. NMDA receptors, that trigger changes that lead to signaling dysregulation\(^\text{44}\). These results imply that insulin receptor stimulation is sufficient to protect against the negative effects resulting from AβO binding (Fig. 3.2-3.4 and Table 3.1). Furthermore, these results support the hypothesis that the signaling mechanism could be tau-independent for early stage AD transport disruption because
the absence of tau eliminates the possibility that tau hyperphosphorylation would be the sole cause for the aberrant signaling propagation\textsuperscript{224, 239}.

My results demonstrate that AβOs impair bidirectional BDNF transport, and that GSK-3β inhibition prevents reductions in anterograde and retrograde flux. It is possible that GSK-3β largely governs anterograde transport; however, several recent studies have elucidated regulatory mechanisms that are coordinated through opposing motors, whereby disrupting one motor impairs bidirectional transport\textsuperscript{231-234} This bidirectional impairment could be through inhibition of either motor, thus describing a mechanism whereby flux, but not other measures are reduced. Indeed, past studies have show that these bidirectional transport disruptions exist in Alzheimer’s disease models, such as mitochondria\textsuperscript{240, 241}, amyloid precursor protein\textsuperscript{242}, and organelles contained in squid axoplasm\textsuperscript{243} and primary hippocampal neurons\textsuperscript{244}.

The role of tau in transport disruption is contentious, yet may be resolved when considering the specifics of motor regulation and experimental paradigms. Recently, AβO-induced transport defects of mitochondria and the nerve growth factor receptor, TrkA\textsuperscript{241}, were prevented by tau reduction. As I observed tau-independent transport defects of BDNF my results may be different because of the various mechanisms by which motor proteins can be regulated. Primarily, KIF1A is the motor protein for BDNF and other DCV cargoes\textsuperscript{245}. However, mitochondria and TrkA is transported by KIF5. Additionally, another important difference between the studies was that they imaged in the first hour after AβO treatment. It could be possible that tau plays a significant role in transport disruption within that time frame as opposed to the 18 hour point in my experiments. Furthermore, I used nanomolar concentrations of AβOs versus previous experiments reporting micromolar amounts. Their higher amounts of AβOs may induce a strong tau hyperphosphorylation response, initiate tau’s detachment from microtubules, or expose a phosphatase activating domain that induced GSK-3β activation, thereby disrupting transport\textsuperscript{224}. The AβO concentration used in my thesis may allow observation of GSK-3β mediated transport disruption, with little contribution from pathogenic forms of tau.
4.3. Regulation of transport by kinase activity

To further explore mechanisms of insulin signaling protection I sought to modulate the insulin cascade downstream of the receptor as the insulin pre-treatment prevented AβO-induced IR internalization that could serve as a possible protective mechanism\(^4^4\). In AD progression, presumably once AβOs cause IR internalization, insulin can no longer activate the IR to induce a protective response. In order to better understand the signaling mechanisms that regulate transport, I transfected ca-AKT, an intermediate of the insulin pathway, in the presence of AβOs. Even with AβOs binding to the IR, the insulin-signaling pathway was able to overcome the negative signaling cascades caused by AβOs, thereby preventing axonal transport defects. There was no difference between the prevention of transport defects in tau\(^{-/-}\) and tau\(^{+/+}\) neurons (Fig. 3.4-3.7 and Table 3.2), further evidencing that tau may not be required in AβO action or for modulating transport via insulin signaling. There have been conflicting reports on the level of AKT overactivation in AD brains due to the results reflecting perimortem oxidative stress\(^2^4^6\). Some work has shown AβOs in cell culture have increased AKT-pser473 phosphorylation thereby desensitizing the insulin signaling pathway through feedback inhibition similar to diabetes\(^2^4^7\), however AKT can inhibit GSK-3β activation by direct phosphorylation\(^2^3^0\).

There are several mechanisms by which AβOs may disrupt axonal BDNF transport independently of tau. Generally, transport regulation is thought to occur via direct motor regulation and/or disrupting the attachment of cargoes to their motors. Both of these mechanisms are likely controlled through phosphorylation events. One way that motor protein activity could be inhibited is through GSK-3β-dependent phosphorylation of motor proteins. GSK-3β is implicated in many aspects of AD pathogenesis, with studies showing that active GSK-3β disrupted amyloid precursor protein (APP) axonal transport in Drosophila\(^2^4^2\). The study concluded that one of the ways APP transport could be regulated is by reducing the number of kinesin-1 (KIF5) motors that are bound to the microtubule. Another mechanism could be the disruption of the binding domain of motor proteins that attach cargoes. Vagnoni et al. showed that phosphorylation of KLC1 at ser460 would influence the ability of KIF5 to bind calsyntenin-1, a membrane protein that mediates APP transport\(^2^1^2\). KLC1 can also be phosphorylated by GSK-3β signaling.
and casein kinase 2 (CK2) thus causing KIF5 to dissociate from cargoes in squid axoplasm \(^{248, 249}\). Thus, it is possible that the activation of the insulin signaling cascade could prevent these GSK-3\(\beta\) mediated impairments by inhibiting GSK-3\(\beta\) overactivation. To further explore this hypothesis I used specific inhibitors of GSK-3\(\beta\) to induce changes that might provide protection from transport defects. If these cell-signaling mechanisms are truly responsible for the changes in transport, it should be possible to reverse the transport defects. I successfully used the reversible GSK-3\(\beta\) inhibitor, Inhibitor VIII, and the irreversible inhibitor, Tideglusib, to rescue FAT (Fig. 3.8-3.10, Table 3.3; and Fig. 3.11-3.13, Table 3.4, respectively). My work with both GSK-3\(\beta\) Inhibitor VIII and Tideglusib in tau\(^{+/+}\) and tau\(^{-/-}\), further supports the hypothesis that signaling cascades have the ability to modulate GSK-3\(\beta\) cascades (i.e. through the insulin signaling pathway) that may have a central role in the early stages of AD pathology.

### 4.4. Alternatives to the IR stimulated transport protection

In search of an insulin receptor-independent therapeutic approach to activating the insulin signaling pathway, I investigated an approved drug for the treatment of type-2 diabetes, Exendin-4 (Ex-4). This peptide is a selective long-lasting glucagon-like peptide-1 receptor agonist that stimulates the insulin signaling pathway. Ex-4’s insulinotropic properties have been well characterized in peripheral tissues \(^{250}\). In the central nervous system, stimulation of the GLP-1 receptor is thought to play a role in processes of synaptic plasticity and neuronal survival \(^{175}\). Ex-4 has the ability to readily cross the blood-brain barrier, and demonstrates a neuro-protective effect in animal models for Parkinson’s disease and Huntington’s disease \(^{251, 252}\). Ex-4 was also able to prevent the inhibition of IRS-1 that was caused by A\(\beta\)Os \(^{149}\). Furthermore, neuronal binding of A\(\beta\)Os was not inhibited by treatment with Ex-4, suggesting that protection by Ex-4 involves intracellular signaling pathways that block cell damage caused by A\(\beta\)Os, such as insulin signaling cascade stimulation. Axonal transport defects, caused by A\(\beta\)Os, were rescued with Ex-4 application in both tau\(^{+/+}\) and tau\(^{-/-}\) neurons (Fig. 3.14-3.16 and Table 3.5). The rescue was presumably occurred via GLP-1 pathway stimulation, activating the PI3K/AKT pathway, which would inhibit GSK-3\(\beta\) activity by phosphorylating Ser 9, thus relieving the phosphorylation on motor protein and allowing the motor to resume physiological transport.
4.5. Conclusions and Future Perspectives

My body of work further establishes intracellular links between insulin signaling and AβO action, by better understanding that insulin signaling can closely regulate BDNF transport. Not only was insulin signaling able to prevent AβOs from inducing axonal transport defects, but once the defects were present, insulin signaling intermediates were able to return transport to normal physiological levels.

Future experiments should focus on elucidating the mechanisms for direct regulation of KIF1A by GSK-3β in order to better understand the mechanisms involved in AD, and axonal transport more generally. There has been no study that has elucidated the mechanism by which KIF1A is regulated in DCV transport. Understanding the mechanism of motor protein regulation could offer insight into designing new therapies for AD to stave off further cellular insults that result from transport disruption. Additionally, future experiments should measure the kinase activity of only the axonal compartment, rather than whole-cell lysates\textsuperscript{117}, to better understand the regulation mechanism of axonal transport. Understanding the direct signaling environment that regulates transport not only furthers the understanding of how motor proteins function, but how the insults of different neurodegenerative diseases affect axonal transport. The exciting possibility thus emerges that restoring neuroprotective function of CNS insulin signaling as well as reverting axonal transport defects from AβO-induced cellular insults may allow for the development of novel therapies for AD.
References


