The Interaction of Lingo-1 and Amyloid Precursor Protein

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

Rian de Laat
University of Washington
2012
Reading Committee:
Mark Bothwell, Chair
Edwin Rubel
Stanley Froehner

Program Authorized to Offer Degree:
Department of Physiology and Biophysics
1 Abstract

Proteolytic cleavage of amyloid precursor protein (APP) generates the amyloid β peptide (Aβ), the main component of cortical and subcortical plaques in Alzheimer’s disease (AD). APP can be processed at the cell surface or within endosomes after endocytosis, and via an amyloidogenic or non-amyloidogenic pathway. Along the amyloidogenic pathway, APP is first cleaved by β-secretase followed by γ-secretase to produce Aβ. The non-amyloidogenic pathway involves cleavage by α-secretase and then γ-secretase. Aβ generation is thought to occur in a variety of organelles where APP, β- and γ-secretase reside. Proteins that regulate endocytosis and trafficking can thus control the qualitative proteolysis of APP, and consequently may be associated with pathophysiology of AD. One such protein is Lingo-1, which promotes APP trafficking to the lysosome and concomitant degradation, independent of the secretory pathway. In this manner, Lingo-1 may function as a control mechanism for APP levels.
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2 Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by the slow loss of cognitive function over time. The first case of AD was described in 1906 by the German neuropathologist Alois Alzheimer. Despite years of research, the cause and progression of AD are not well understood. The loss of mental faculties is tragic for both patients and family, as there is no cure for AD, and cognitive decline continues to worsen as the disease progresses and disease eventually leading to death. With an increasingly large senior population the incidence of AD continues to rise and the expected cost of patient care for 2012 is $200 Billion.

My research has been dedicated to studying a protein that may play a role in AD onset and decline. To better explain the complexity associated with AD and its potential points of regulation, I will review the trafficking of proteins in the cell, the relevance of lysosome dysfunction in neurodegenerative disease, how sorting proteins determine protein trafficking, and finally discuss a new family of sorting receptors, and in particular Lingo-1, that has been the focus of my research.

2.1 APP and Alzheimer’s disease

The development of amyloid protein deposits in the cerebral cortex, called plaques, and neurofibrillary tangles inside nerve cell bodies suggests that Alzheimer’s disease results from protein aggregation followed by generalized neuroinflammation. Plaques are composed of aggregated proteins, mainly the proteolysis product of a membrane protein, the synaptic amyloid precursor protein (APP), while tangles are composed of a cytoskeletal protein, tau. APP is synthesized in the endoplasmic reticulum (ER) and transported to the cell surface via the secretory pathway. The native biological function of APP also remains unknown, although research suggests it might function in synaptic regulation [1], axonal transport [2], and cell adhesion [1, 3].

Both normal and pathological forms of APP undergo sequential proteolysis by alpha, beta, and gamma (α, β, and γ) secretases yielding peptide fragments of varying sizes. Non-pathogenic fragments are generated by alpha/gamma secretase mediated cleavage within the trans-Golgi network (TGN) and at the cell surface. α/γ secretase generates the large soluble N-terminal APPα and a non-amyloidogenic C-terminal fragment. Pathogenic fragments are generated when endocytosis of intact APP triggers cleavage by β-secretase, generating CTFβ, and then cleavage by γ-secretase, generating Aβ [4-7]. The resulting Aβ peptide fragments vary in size from 36–43 amino acids. Aβ42 is the most fibrillogenic, leading to its preferential inclusion in amyloid plaques [18]. What causes accumulations of Aβ42 in plaques or what factors determine whether APP undergoes pathogenic or non-pathogenic processing is a subject of active investigation.

The amyloid hypothesis of Alzheimer's disease postulates that Aβ, particularly the Aβ42 isoform, initiates a neurotoxic cascade that leads to amyloid deposition, neurodegeneration and cognitive decline [8]. At nanomolar concentrations, soluble amyloid-beta oligomers block
hippocampal long-term potentiation, cause dendritic spine retraction from pyramidal cells and impair rodent spatial memory [19]. The accumulation of peptides forms oligomers, induces microglial and astrocytic activity, pro-inflammatory response, oxidative injury, and altered kinase/phosphatase activity. This is followed by fibril formation and accumulation in senile plaques and neurofibrillary tangles [9-12]. Eventually loss of neuronal dendritic complexity and synapses causes brain atrophy. The amyloid hypothesis is supported by genetic differences that cause early onset or familial forms of AD. Mutations in APP, presenilin 1 (PS1) or presenilin 2 (PS2), [13] lead to increased Aβ deposition and are responsible for most cases of early-onset Alzheimer’s and lead to elevated levels of pathogenic amyloid [14]. Presenilins are the catalytic subunit of the γ-secretase protein complex that releases Aβ peptides from its APP; consequently, mutations in either the cleaved or the cleaving proteins which up-regulate amyloid biogenesis are coupled with AD pathology [15]. Overexpression of APP in humans through duplication of its gene or trisomy of chromosome 21, which causes Down syndrome, results in early-onset AD [16, 17]. Moreover, increased APP gene expression caused by altered regulatory sequences in the APP promoter region, leads to early-onset disease and may be a risk factor for late-onset AD, with levels of APP expression correlating inversely with age of disease onset [18]. Amyloid deposition precedes tangle formation [19] in disease progression, further supporting the primacy of the amyloid hypothesis.

While alterations in APP metabolism are believed to play a central role in Alzheimer disease pathogenesis [15] there are several mechanistic failures that could lead to Aβ accumulation. Increased proteolysis of APP, altered trafficking of full length APP or its proteolysis products, or a decrease in lysosomal protein degradation could all cause an imbalance between Aβ production and Aβ clearance [5, 20]. Current evidence suggests that the processing of APP is regulated by numerous sorting mechanisms which localize interactors to lipidic microenvironments like endosomes, lysosomes, and lipid rafts. The processes of sorting and trafficking APP to different compartments are possible points of regulation and may determine whether APP is proteolytically processed via two pathways; one benign and the other pathogenic. Since nonamyloidogenic production and amyloidogenic APP proteolysis occur in different subcellular locations [20], a possible means for regulation of Aβ formation is the sorting and intracellular transportation of APP in endocytic vesicles to the recycling and degradative pathways [21]. Proteins in control of vesicular trafficking are potential regulators of APP proteolysis, and possibly plaque formation.

2.2 Endocytic pathways and trafficking

My research reveals a novel mechanism for sorting of APP that may determine whether APP is cleaved in a pathogenic or benign manner, therefore I wish to review previous work on the subject.

Many neurodegenerative disorders have been linked to mutations in the genes encoding components of the vesicular transport machinery responsible for normal protein trafficking. Disruption of these processes results in protein mislocalisation, misfolding and aggregation. Cellular functions depend on the correct delivery of proteins to specific intracellular destinations and a variety of proteins determine that sorting, recycling, sequestration, metabolism, and trafficking occur correctly [17].
In the biosynthetic pathway newly synthesized proteins are folded and packaged in the endoplasmic reticulum, then sent to the Golgi for post-translational modification. From the Golgi proteins can be shuttled through the trans-Golgi network and directly transported to the plasma membrane or to degradative lysosomes [21].

Extracellular materials and membrane proteins are internalized by receptor-mediated (clathrin) endocytosis or bulk-phase endocytosis (pinocytosis) into early endosomes/sorting-endosomes (Rab5 positive vesicles). After sorting in early endosomes, some materials are sent back to the plasma membrane via recycling endosomes, some go to the trans-Golgi network for further packaging and trafficking, while others reach late endosomes (LE)/multivesicular bodies (MVB, Rab7 positive), which contain hydrolase vesicles delivered from TGN by shuttle vesicles. The MVB/late endosomes fuse with lysosomes where proteins are degraded.

Lysosomes are acidic vacuolar compartments containing numerous hydrolytic enzymes that degrade proteins. Lysosomes receive proteins directly from the biosynthetic pathway, from the cell surface or extracellular space via receptor-mediated endocytosis, pinocytosis, phagocytosis, and from autophagy [22]. In the process of trafficking to the lysosome, hybrid organelles are formed by fusion. The cargo from hybrid organelles can contain material from various vesicles that originated from the cell surface, or the secretory pathway. In this manner intracellular proteins come into contact with cell surface receptors.

2.3 Lysosomes and AD (Dysfunction)

Dysfunction of the endolysosomal system is one of the first pathological features of AD [23], and increasing evidence links AD with aberrant endocytic trafficking and abnormal lysosomal degradation [24-26]. All the proteins involved in the production of Aβ peptide are membrane associated and hence, membrane trafficking and cellular compartmentalization play important roles [27]. APP is enriched in the lysosome [28], and to generate Aβ, beta and gamma secretases, which are generally present in several compartments of the cell, have to be transported to the endosomal environment [29, 30]. Inhibitors of endocytosis, endosome fusion, and lysosomal processing inhibit the intracellular proteolysis of the amyloid precursor protein [31], and in AD neurons have more endocytic organelles. Autophagosomes and early or late autophagic vacuoles [32] and lysosomes [33] are increased, as well as lysosomal hydrolases [34]. Genome-wide association studies have identified allelic variants of PICALM, which is essential for clathrin/AP-2 dependent endocytosis, as a risk factor for Alzheimer’s disease [74]. Abnormalities in the lysosomal pathway occur early in AD pathogenesis before the appearance of neurofibrillary tangles or senile plaques. Abnormal endosomes associated with earliest elevations of Aβ levels has been found in both AD and Down syndrome (DS) [35]. Aβ has also been detected in enlarged endosomes that are immunopositive for the early endosomal marker rab5 [17]. The E4 isoform of the apolipoprotein E gene is the strongest genetic risk factor for late onset AD, and one or two copies of this allele accelerates and exacerbates endosome dysfunction in early AD [36]. Autophagic vacuoles in Alzheimer's disease have distinct morphologies and accumulate in dystrophic neurites [37, 38], and in neurons expressing mutant APP Aβ-induced abnormalities in MVB endocytic sorting [39-41]. MVBs/late endosomes are relatively rich in APP and APP secretases, and those in AD brain and mouse model of AD contain Aβ peptide [42, 43].
2.4 Sorting proteins

Several membrane proteins control vesicular trafficking of APP and other membrane proteins. The proteins, called “sorting receptors,” function primarily in the trans-Golgi network, interacting with a wide range of ligands comprising other transmembrane receptors as well as soluble proteins from neurotrophic factors to enzymes targeted for lysosomes. Sorting receptors regulate intracellular trafficking and proteolytic processing of APP into amyloidogenic and nonamyloidogenic products [44]. The term “sorting receptor” designates the 5 mammalian homologs of the yeast protein VPS10p - SORLA (SORL1), sortilin, SorCS1, SorCS2 and SorCS3 [45]. Overexpression of SORLA reduces amyloid precursor protein processing and amyloid β peptide formation in cells [46-48]. APP-binding sorting receptors such as SORL1 determine whether APP in endosomes recycles to the cell surface or enters late endosomes, influencing the production of Aβ. Indeed, mutations in SORL1 are genetically linked to Alzheimer’s disease [49, 50].

SORLA shares homology with the LDL receptor gene family, which concentrates in coated pits and is internalized when the pit becomes a coated vesicle. The members of this family, LDLR, VLDLR, apoER2, LRP1, LRP1b, LRP2/megalin, LRP4/MEGF7, LRP5 and LRP6, are referred to as endocytic receptors as they promote clathrin-dependent endocytosis [51, 52]. Sorting and endocytic receptors are functionally similar, as they control the trafficking of target proteins between membrane compartments. Sorting and endocytic receptors share a similar structural organization. Their large extracellular domains (ECDs) consist of multiple repeats of subdomains specialized for protein-protein interaction, allowing them to form specific interactions with structurally diverse partners. Their abbreviated intracellular domains contain sequence motifs that bind cytoplasmic adapter proteins controlling vesicular trafficking.

The ability of sorting receptors to bind diverse partner proteins allows them to be multifunctional. For example, LRP2/megalin binds and internalizes dozens of different ligands, including hormones, growth factors, vitamin/binding protein complexes [53], and LRP1 forms functionally important complexes with over 40 proteins. LRP1, LRP1b [54], ApoER2 [5] and SorLA [48] all bind APP and modulate APP trafficking and processing. ApoER2 promotes endocytosis of cell surface APP [3]. SORLA has been reported to retain APP in the Golgi [48], to promote APP traffic from the Golgi to endosomes [55] or to direct APP into recycling endosomes and away from late endosomes [50]. The relevance of SORLA interaction with APP is demonstrated by the linkage of inherited variants of SORLA with late-onset AD [50].

2.5 LIG family members - New sorting receptors

APP subcellular localization and trafficking is critical to how it is proteolytically processed. SORLA and other membrane proteins that similarly regulate vesicular trafficking in secretory and endocytic pathways have been shown to play a role in AD. Another potential family of sorting receptors is the LIG family. LIG proteins are both implicated in subcellular trafficking and shown to directly bind APP. Consequently, my research has focused on how LIG proteins and particularly Lingo-1 may impact APP processing.

The mammalian LIG family consists of Tropomyosin-related kinase neurotrophin receptors (TrkA, B & C), NGLs (NGL1, 2, & 3), ISLR1 & 2, Amphotericin-Induced gene and open
reading frames (AMIGO1, 2 & 3), Leucine-rich repeat Ig proteins (LRIG1, 2 & 3) and Leucine-rich repeat Ig-domain containing Nogo receptor interacting proteins (Lingo-1, 2, 3 & 4) [56, 57]. LIG family members are enriched in neural tissue and appear to influence neuronal survival, cellular differentiation, neurite outgrowth and synapse formation [58].

LIG proteins are characterized by being single pass transmembrane proteins that contain leucine rich repeats, one or more IgC2 domains proximal to the transmembrane domain and N and C-terminal caps. Leucine-rich repeats (LRR) and Ig-C2 domains mediate protein-protein interactions. LIG proteins, by virtue of possessing many such domains, form specific complexes with structurally diverse partner proteins, as endocytic and sorting receptors do. LIG proteins also typically possess short intracellular domains, as sorting and endocytic receptors do. The intracellular domains of LIG proteins contain regions with sequences that are invariant over mammalian evolution, suggesting that they represent binding sites for cytoplasmic adapter proteins controlling vesicular trafficking.

The most extensively studied LIG is LRIG1, which binds and mediates the endocytosis and lysosomal degradation of receptor tyrosine kinases (RTKs), including ErbB1, ErbB2, ErbB3, ErbB4 [59, 60], c-MET (an HGF receptor) [61] and c-RET, a subunit of GDNF receptors [62].

My data suggests that different LIG subfamilies may each control trafficking of target membrane proteins in the endolysosomal pathway. Different LIG family members promote or inhibit receptor tyrosine kinase signaling. For example LINX promotes signaling by TrkA and c-RET, while Lingo-1 promotes degradation and inhibits signaling of those receptors [56]. Our unpublished evidence indicates that LIG proteins modulate receptor tyrosine signaling by acting as sorting receptors that govern trafficking of receptor tyrosine kinase-bearing endosomes.

2.6 Lingo-1 and its regulatory function

My studies have focused on Lingo subfamily of LIG proteins. Lingo-1 is a membrane protein, the structure of which is illustrated in Figure 1. Lingo-1 mRNA is expressed almost exclusively in the CNS throughout embryonic and postnatal stages, with regional differences [63-68]. Lingo-1 protein is present prominently in neocortex, hippocampus, thalamus, and amygdala, with lower levels in cerebellum and basal nuclei in the adult brain [63]. The LRR domains of proteins have been implicated to have a role in protein–protein interaction and their unique structure may help in cell signaling and/or cell adhesion in specific regions of the brain [63, 64, 69, 70].

Lingo was first identified as a functional component of the Nogo receptor signaling complex ((p75/Troy)/Nogo/Lingo-1) responsible for RhoA activation in the context of inhibition of axonal regeneration by myelin-associated factors [64]. Lingo-1 inhibits axonal growth, and also apparently regulates oligodendrocyte differentiation, as Lingo-1 knockout mice show premature myelination [64]. Interestingly, Lingo-1 is expressed in brain regions and subcellular compartments that do not overlap with other components of the Nogo receptor complex. This expression suggests Lingo-1 may participate in other activities in developing neurons separate from oligodendrocyte maturation or axon extension [63, 71].
2.7 Lingo in PD and ET

More recently there have been a number of papers proposing Lingo involvement in a variety of diseases. Lingo-1 and Lingo-2 variants were identified as risk factors for essential tremor (ET) in a genome-wide association study and several follow-up linkage studies [72-76].

ET is associated with other forms of neurological dysfunction including ataxia, cognitive deficits, and dementia [77], and is associated with increased risk for both PD and Alzheimer’s disease (AD) [78]. Given the clinical overlap of ET with Parkinson’s disease (PD), it has been suggested that there might be genetic overlap between these diseases. Farrer, et al. expanded their study of Lingo variants involved in ET to include two PD patient-control series from the US and Europe [79] and identified Lingo as the first risk gene to genetically link essential tremor and Parkinson’s disease [72]. Lingo-1 and 2 variants altered age of onset of ET and a Lingo-1 variant altered age of onset of PD [80], specifically those presenting the non-rigid-akinetic phenotypes.

Lingo-1 inhibition has been shown to improve dopaminergic neuron activity in a model of Parkinson’s disease [81] and Lingo-1 expression is elevated in the substantia nigra of Parkinson's disease patients compared with age-matched controls [81]. In Lingo-1 knockout mice dopaminergic neuron survival is higher [81] associated with increased p-Akt. Blocking Lingo-1 enhances neurite growth of midbrain neurons. In some populations Lingo variants seem to be linked to essential tremor, while in others Lingo is not a factor. It has been suggested that ethnic differences must be considered in future studies of Lingo-1[82].

In addition, Lingo has been shown to biochemically bind APP. In an effort to characterize the APP interactome, Bai, et al. used chemical cross-linking in vivo to identify proteins that bind APP. While this study showed linkage with many known interactors of APP the study also found 30 new proteins that associate with APP in this experiment. One of these new proteins was Lingo-1. Bai, et al, found that not only did Lingo-1 binds with APP it appeared to modulate its processing. Over-expressed Lingo-1 modestly increased production of Aβ from APP by increasing the production of CTFβ while decreasing the production of CTFα [83].

My lab has studied APP and Lingo independently and was intrigued by the published reports of association of Lingo-1 with APP [68, 84]. My original hypothesis was that Lingo-1 might target other LIG proteins and/or APP to the endosomal compartment but my data supports a model in which Lingo-1 directs sorting of these proteins to the lysosomal sub compartments of the endocytic pathway, potentially providing a key mechanism of control over specific protein levels, including APP. I demonstrate that Lingo-1 regulates APP proteolysis by promoting degradation of APP in a manner similar to Lingo-1 mediated down-regulation of neurotrophin receptors (unpublished results from our lab, Mandai et al. 2010, Meabon et al, in prep). Lingo-1 and APP interact in the endosome/lysosome pathway [56] where Lingo-1 controls APP turnover and regulates the amount of APP available for amyloidogenic processing. Understanding the function of Lingo-1 in the trafficking of APP may provide insights into the regulation of APP
processing in AD pathogenesis. My work suggests that Lingo-1 regulates lysosomal degradation of a variety of membrane proteins in the endocytic pathway.

I have found that Lingos play a role in down-regulation of neurotrophin receptors (Mandai et al. 2010, Meabon et al, in prep). Lingo-1 inhibits receptor signaling by down-regulating the receptors that it binds and speeding endocytic delivery of receptors to lysosomes. Taken together, these phenomena suggest Lingo-1 functions as a negative regulator of RTKs and APP. Lingo promotes lysosomal degradation of endosomally trafficked membrane proteins by preventing those cargo proteins from returning to the cell surface via recycling endosomes.

**Figure 1 Lingo Structure**

![Figure 1 Lingo Structure](image)

**Figure 2 Lingo-1 forms Tetramers**

**Figure 1 Lingo Structure.** LIG proteins are characterized by being single pass transmembrane proteins that contain leucine rich repeats, one or more IgC2 domains proximal to the transmembrane domain, and N and C-terminal caps.
3 Results

3.1 APP/Lingo-1 Binding and Localization

Based on the reported association between Lingo-1 and APP [83] we attempted to replicate these results by immunoprecipitating (IP) Lingo-1 directly from transiently transfected HEK 293 cells using an hemaglutinin (HA) tagged APP. We observed that HA-APP was present in Lingo-1 immunoprecipitates (not shown), and Lingo-1 was present in HA-APP immunoprecipitates (Figure 3), supporting previous reports of a Lingo-1-APP association. Confirming that these proteins co-immunoprecipitate suggests they do associate, but a limitation for experiments employing co-immunoprecipitation to assess whether proteins associates is that these experiments will not distinguish between interactions that are direct and interactions that are indirect (representing bridging complexes with other proteins). Either direct or indirect interactions might mediate functional regulation. It is possible that Lingo-1 binds an as yet unidentified putative adaptor protein potentially containing a C-terminal PDZ binding domain. No PDZ protein has been shown to bind a protein with the ECEV sequence in the C-terminus. However, nearly 260 genes encoding PDZ proteins have been found in the human genome and many of them still lack established binding partners [19]. Another possibility is other LIG family members like the Trks. Trks have also been demonstrated to associate with APP [85].

Confirming that these proteins co-immunoprecipitate suggests they do associate, but in order for direct APP/Lingo-1 interactions to be broadly relevant in brain the two proteins must be expressed in the same cells, and in the same subcellular compartments.
Figure 3 Immunoprecipitation of APP results in co-immunoprecipitated Lingo
In order to assess Lingo-1 and APP colocalization in neurons dissociated mouse cortical neuronal cultures were plated on slides and immunostained for Lingo-1 and APP. I found that...
Lingo-1 and APP are expressed in similar populations of neurons in the brain and colocalize in intracellular membranes of neurons and vesicles (Figure 4).

**Figure 4 Lingo and APP are coexpressed in Neurons**

![Confocal microscopy of Lingo-1 and APP in cultured neonatal mouse cortical neurons. DAPI blue nuclear stain. Red label from antibody against Lingo-ICD and green against APP. Overlapping staining appears orange, indicating colocalization on intracellular vesicles.](image)

Data from the Allen brain institute also reveals strong regional correlations of APP and Lingo. *In situ* hybridization patterns of Lingo and APP mRNA transcription in the developing mouse brain also show coexpression of APP and Lingo (Figure 5).

**Figure 5 Lingo and APP coexpress in the brain**
3.2 Lingo-1 down-regulation of APP

While conducting my immunoprecipitation experiments I found that Lingo-1 Over-expression dramatically reduced levels of APP. When HEK 293 cells were transiently transfected with a uniform amount of APP plasmid (1 µg) and a titration of Lingo-1 plasmid from 0.25 to 1.25 µg, balanced with pcDNA empty expression vector to maintain a uniform amount of total expression plasmid. Lingo-1 down regulated APP in a monotonic fashion (Figure 6). Lingo and APP coexpress in the brain, and Lingo stoichiometrically down regulates APP at roughly equivalent levels suggesting this phenomenon may have physiological relevance.

Figure 5 Lingo and APP co-express in the brain. Lingo and APP expression patterns in the developing mouse brain. Sagittal sections stained by in situ hybridization reveal strong regional correlations of APP and Lingo mRNA transcription. Images courtesy of the Allen Brain Atlas.

Figure 6 APP is down-regulated by Lingo-1
3.3 shRNA knock-down of Lingo-1 expression rescues APP down-regulation

Plasmid overexpression can have a variety of non-specific effects. To determine if APP down-regulation is specific to Lingo-1 expression and not an effect of the expression plasmid itself I tested whether knocking down Lingo-1 expression with shRNAs would rescue APP down-regulation. I transfected a uniform amount of APP expression plasmid DNA, with or without Lingo, and both Lingo, APP, and Lingo shRNA expression plasmids in both N2a, and HEK 293 cells, while maintaining the total protein content using an empty pcDNA expression vector. Lingo-1 shRNA reduced expression of transfected Lingo-1 in N2a, and HEK 293 cells. Reduced Lingo-1 expression rescued APP down-regulation (Figure 7 and 8) in both cell lines.
Figure 7 shRNA rescues APP down-regulation in N2A cells

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Figure 7 shRNA rescues APP down-regulation in N2A cells. Immuno blots from Neuro 2A transfected with APP alone, or co-transfected with Lingo-1 showed down regulation of APP. APP with twice as much Lingo-1 showed further down regulation. Wells that were transfected with APP, Lingo-1 and with shLingo did not show down-regulation of APP. Blot probed with 22C11 (anti-APP).
3.4 Lingo-1 down-regulation of APP in neuronal cultures

Having demonstrated that Lingo-1 down regulates APP in artificial non-neuronal cell lines I wished to determine whether Lingo-1 would down regulate APP endogenously expressed in neurons. Dissociated mouse hippocampal neuronal cultures were transduced with Lingo-1 lentivirus and a GFP lentivirus control. The neuronal cultures were harvested, immunoblotted, and protein expression bands densiometrically quantified. The wells that were GFP transduced and untransduced had greater expression of APP than wells that were transduced with Lingo-1 lentivirus, indicating that Lingo-1 over-expression in neuronal cultures robustly down regulates endogenous APP, just like in cultured cell lines. This suggests that even in primary neurons, Lingo-1 has consistent effect upon APP protein levels. As my previous staining demonstrated high levels of colocalization in neuronal vesicles, we suspect that Lingo-1 may regulate neuronal APP protein levels by modulating vesicular trafficking (Figure 9).
Figure 9 Lingo-1 over-expression down regulates APP in neurons

Transduced LINGO lentiviruses down regulate APP expression in mouse primary neuronal cortical cultures. Cortical cultures were transduced with Lingo-1 and GFP control viruses at 3 MOI and grown for 5 days. Cells were harvested and normalized for protein concentration and immunoblotted. Lingo-1 band density was calculated for untransduced, Lingo-1 virus transduced, and control GFP virus transduced cultures.

3.5 The ability of Lingo Paralogs to down-regulate APP

The Lingo family is comprised of three proteins: Lingo-1 through Lingo-4. The other three paralogs retain high sequence similarity (44-60%) to Lingo-1 and the same domain organization, so they may also affect APP expression. Using the same co-transfection experiment shown in Figure 6, demonstrates that Lingo-1 and Lingo-3 greatly reduce APP expression (Figure 10), and Lingo-2 exhibits moderate reduction in APP (Figure 10). Lingo-4 was not tested because the Upstate Lingo antibody does not recognize Lingo-4 and could not be used to assess level of Lingo-4 expression achieved.
3.6 Does Lingo-1 down-regulation of APP employ a secretase dependent mechanism?

There are two potential mechanisms for Lingo down-regulation of APP. Lingo could cause degradation by targeting APP to the lysosome, thus decreasing all proteolytic products, or Lingo can increase APP cleavage by α- and γ- or β- and γ-secretases causing an increase in APP AICD fragments in the cytoplasm. The APP intracellular domain (AICD) contains the highly conserved YENPTY and NPXY sequence that interacts with FE65 adaptor protein, and functions as a transcriptional factor for target gene activation [86, 87]. To determine whether stimulated APP cleavage is responsible for APP down-regulation I co-transfected Lingo-1 and APP plasmid DNA in Hek 293 cells and treated the cells with a γ-secretase inhibitor DAPT, or the α-secretase inhibitor GM6001. My results show that in addition to down-regulating APP, Lingo-1 also down-regulates CTFα/CTFβ fragments. This indicates Lingo-1 down-regulation of APP is not mediated by increased processing by α or β secretase (Figure 11).

There are two possibilities. Option one: Lingo-1 regulates APP by altering trafficking and driving APP into secretase rich compartments for degradation. Option two: Lingo-1 does an end-run around the secretase rich compartments, and targets APP for degradation by other mechanisms. This suggests strongly that while DAPT does promote a buildup of APP-CTFs, Lingo does not enhance it—in fact it eliminates all traces of APP holoprotein and the CTFs. This supports a model in which Lingo-1 drives APP into an alternative degradatory pathway.

Figure 11 Lingo down-regulates full length APP as well as CTFα/CTFβ fragments
3.7 Does Lingo-1 down-regulation increase APP-AICD

In order to assess what affects Lingo-1 may have on APP nuclear signaling, we used the APPGV16/Gal4-luciferase assay. The APPGV16/Gal4-luciferase assay quantifies γ-secretase mediated cleavage of APP. A construct encoding APP C-terminally tagged with an artificial transcription factor that drives luciferase gene expression when the C-terminal fragment is released by γ-secretase mediated cleavage (Figure 12). Luciferase counts can be quantified using a luminometer.
We cotransfected Lingo-1 and its paralogs with the luciferase transcriptional reporter construct in both HEK 293 and N2a cells. Luciferase activity in lysates of transfected cells were assayed by luminometry. We found that cells co-transfected with Lingo-1 had drastically reduced luciferase counts. The effects of other Lingo-1 family members and of mutated forms of Lingo-1 differed in proportion to their ability to down-regulate APP in comparison to expression of APPGV16/Gal4luc alone as a control. Lingo-2 does not reduce measured production of APP AICD, and Lingo-3 has only a modest effect on APP AICD production (Figure 13). This result is supported by my immunoblot data that indicates Lingo-2 has less of an ability to down regulate APP than either Lingo-1 or Lingo-3. The results for Lingo-2 in the luciferase assay is weaker than its observed ability to down-regulate Lingo as observed by immunoblot, as shown in Figure 10. Generally effects observed by the luciferase assay are weaker than observed biochemically, which probably means two things — first, this is not a secretase dependent mechanism as Lingo-1 and Lingo-3 potently down-regulate AICD-GV16 liberation. Second, there may be some escape of the AICD fragment following Lingo mediated degradation. The luciferase assay data supports my immunoblot results of DAPT treatment of transfected cells. Both indicate that Lingo down-regulates full length APP as well as CTFα/CTFβ fragments.

Figure 13 Lingo down-regulates full length APP and AICD
The same experimental procedure was used to test AICD production when cells were cotransfected with Lingo expression constructs lacking an intracellular domain. To test whether the intracellular domain of Lingo-1 is the portion that interacts with APP we cloned an expression construct lacking the intracellular domain (Lingo-1-ΔICD). We transfected Hek 293 and N2a cells with APGV16 and either Lingo-1 or Lingo-1-ΔICD construct plasmid. Unlike full length Lingo-1, deletion of 40 residues from the intracellular domain of Lingo-1 yields a form that is ineffective in down regulating APP. This suggests that Lingo-1s intracellular localization is important for its interaction with APP (Figure 14).

Figure 13 Lingo-1 down-regulates full length APP and AICD. Hek 293 and N2a cells were cotransfected with Lingo-1 paralogs and a luciferase transcriptional reporter (APGV16) construct. Measured production of APP AICD is reduced by expression of Lingo-1 or Lingo-2, but not by Lingo-3.

Figure 14 Truncated Lingo-1 does not down regulate APP
secretase activity amyloidogenic APP fragments accumulate in lysosomes

Figure 14 Truncated Lingo-1 does not down regulate APP. HeK 293 and N2a cells were cotransfected with full length Lingo-1 and a Lingo-1 mutant missing its intracellular domain (Lingo-1-AICD), as well as a luciferase transcriptional reporter (APGV16) construct. Measured production of APP AICD is not reduced by expression of the Lingo-1 mutant missing its intracellular domain.

3.8 How does Lingo-1 down-regulate APP?

Lorenzen, et al. used expression constructs containing APP fused to a C-terminal fluorescent protein in order to examine the lysosomal distribution and trafficking of APP in cultured cells. They found cell surface APP is trafficked to the lysosome in a rapid and specific manner. They suggested that regulation of lysosomal traffic could regulate APP processing and that the lysosome could play a central role in the pathophysiology of Alzheimer's disease [28]. Because Lingo down-regulation of APP is not caused by increased processing by the alpha (α), beta (β), or gamma (γ) secretase pathway the other possible fate of APP is degradation. Proteins are degraded through trafficking to the proteasome or the lysosome. To test whether Lingo-1 targets APP to lysosomes or proteasomes for degradation I treated neonatal mouse cortical cultures transduced with Lingo-1 virus, or controlled untransduced neurons with a lysosome inhibitor bafilomycin, the proteasome inhibitor epoxomicin, or a DMSO vehicle loading control. Epoxomicin had no effect, but bafilomycin blocks the ability of Lingo-1 to down-regulate APP. This indicates that Lingo-1 targets APP to lysosomes (Figure 15). In presenilin-1 knockout cells lacking γ-secretase activity amyloidogenic APP fragments accumulate in lysosomes [88], but Lingo appears to send full length APP to the lysosome.

Figure 15 Bafilomycin rescues APP expression
My results suggest that an important locus of Lingo-1 function is within the endocytic pathway, specifically lysosomes, but published studies focus on functions of Lingo-1 at the cell surface.

### 3.9 Lingo-1 and TLRs

I tested a variety of Lingo-1 siRNAs but found that both control and Lingo-1 targeted siRNAs caused an increase in Lingo-1 expression. siRNA transfection of 1 nm to 100nm all caused a corresponding increase in Lingo-1 expression (Figure 16) suggesting that off target effects of siRNA cause an increase in Lingo-1. Antisense and siRNA agents have been shown to exert non-target-related biological effects including activation of Toll-like receptors (TLRs), a family of pathogen-associated, molecular pattern recognition receptors. TLRs signal through pathways such as the NF-kappaB pathway which can influence expression of many genes.

Unfortunately using siRNAs did not allow us to conclude whether endogenous Lingo-1 suppresses APP expression. This is a crucial experiment. It is possible transduction of lentiviral shRNA may succeed where siRNAs have failed.
3.10 Lingo-1 localization in the brain

The potential role of Lingo-1 playing a role in AD pathology begs the questions of where in the human brain does expression occur. All reports describe Lingo-1 as a cell surface protein but my results indicate that Lingo-1 targets APP for degradation in the lysosome. To address the question of where Lingo-1 is localized I used confocal immunofluorescence microscopy. My results in the adult human brain show Lingo-1 localized exclusively to neurons and most abundantly in pyramidal neurons of all neocortical layers and hippocampus. Additionally, Lingo-1 is mainly present on intracellular vesicles and not on the cell surface (Figure 17). This result is consistent with the distribution of Lingo-1 mRNA shown by in situ hybridization (Allen Atlas of the Brain).

Figure 177 Lingo expression in adult human brain
Lingo-1 localizes exclusively to neurons and most abundantly in pyramidal neurons of all neocortical layers and hippocampus.

To ensure that the Lingo localization observed with the intracellular domain antibody were specific to Lingo (and not other related family members), I tested a second Lingo specific antibody. Upstate Lingo-1 antibody raised to the intracellular domain of Lingo-1 localized to the same intracellular vesicles as the Lingo antibody from R&D systems raised to the extracellular domain.

The Upstate Lingo-1 ICD antibody raised to the intracellular domain of Lingo-1 recognizes Lingo-1, Lingo-2 and Lingo-3, while the ECD antibody (R&D) is specific for Lingo-1. I costained mouse neonatal cortical cultures and human brain slices with both antibodies, the ECD stained in red and ICD in green, while orange indicates overlapping stain from both antibodies. For both cortical cultures and brain slices the results obtained with the two antibodies were identical and showed overlap of both Lingo stains. This demonstrates that Lingo-1 localization is specific to intracellular vesicles and Lingo-1 is probably the most predominant isoform in neurons (Figure 18).

Figure 17 Lingo expression in adult human brain. Confocal microscopy of Lingo in adult human brain. DAPI blue nuclear stain. Red label from antibody against Lingo-1 ECD and green label from antibody against Lingo-1 ICD overlaps as orange. Lingo-1 localizes exclusively to neurons and most abundantly in pyramidal neurons of all neocortical layers and hippocampus.

Figure 18 Lingo ICD and ECD antibodies co-immunostain
3.11 Lingo localization to intracellular vesicles

Lingo-1 was originally described as a protein that interacted functionally with NgR and p75 in mediating growth cone collapse [64] at the cell surface. However, I find that the majority of Lingo-1 expression is in intracellular vesicles in membranes of the biosynthetic secretory pathway and the endocytic system of mouse neonatal cortical cultures (Figure 18) and cerebellar granule neurons (Figure 19). Cerebellar granule neurons were biotinylated and either stained with a streptavidin dye to label cell surface proteins and immunostained for Lingo-1, or subjected to streptavidin pull-down of biotinylated cell surface proteins.

Labeling of cell surface proteins using a red streptavidin tagged dye clearly outlines the cell, while immunostaining to Lingo-1 shows cytoplasmic Lingo punctae (Figure 19). Streptavidin pull down of cell surface proteins followed by immunoblotting and probing for pyruvate kinase, Lingo, and pan Trk showed Lingo expression in the whole cell lysates, but none in the streptavidin pull down lane. Trks, which are expressed at the cell surface, pulled down with streptavidin. We could not detect any Lingo immunoreactivity localized with the plasma membrane. If Lingo is associated with the cell surface it is only transiently.

Figure 19 There is no Lingo on the cell surface
Figure 20 Biotinylation of plasma membrane proteins does not pull down Lingo

3.12 Lingo costaining of markers of various vesicular subcompartments

Endogenous APP is extensively colocalized with markers of the early endosomal (Rab5), late endosomal (Rab9) and lysosomal (LAMP1) compartments in dissociated mouse cortical
neurons. I used these and other markers to determine the localization of Lingo-1 in mouse neuronal cortical cultures. Since Lingo-1 staining indicates it is expressed in endocytic vesicles throughout the cell I chose multiple secretory and endocytic markers to establish the identity of the intracellular vesicles containing Lingo-1. I fluorescently co-immunostained Lingo-1 (Red) and various endocytic markers (Green) in endogenously expressing dissociated mouse neonatal cortical culture neurons. Rab5 is a marker of early endosomes, EEA1- early endosome antigen marks the early endosome, GM130 stains the cis-Golgi, β-cop is a marker for late endosomal/multivesicular body, Rab 11 stains recycling endosomes, Rab 7 stains late endosomes, mannose 6-phosphate receptor (M6PR) is a marker for the late endosome, and Lamp 2, a lysosomal marker.

There was very little costaining of Lingo with markers of the endoplasmic reticulum and trans-Golgi network, but Lingo-1 co-localized strongly with β-cop, Rab5, LAMP2, and M6PR indicating Lingo is located in the endocytic pathway, with an enrichment in endosomes and lysosomes, but less so in the secretory pathway (Figure 21).

The confocal microscopy immunofluorescence data indicates that ISLR2 is localized at the plasma membrane, whereas Lingo-1 is localized to late endosomes and lysosomes, and Lingo-1 has been detected as a component of late endosomes [89]. This data further confirms my hypothesis that the two LIG family members encounter the proteins they interact with at different locations and through different mechanisms.

Figure 201 Lingo co-localizes with various endocytic markers
Lingo-1 appears to be expressed throughout the secretory and endocytic pathway. To better visualize co-localization I labeled points of staining overlap, and split the red and green channels. Labels that showed yellow when merged were easier to visualize when split (Figure 22).

![Figure 21 Lingo co-localizes with various endocytic markers. Fluorescent co-immunostaining of Lingo-1 (Red) and various endocytic markers (Green) in endogenously expressing dissociated mouse cortical neurons. Blue is DAPI nuclear counter stain. EEA1- early endosome antigen 1, Rab 5 - marker of early endosomes, GM130 - cis-Golgi, Rab 11 - recycling endosomes, Beta cop – late endosome, Rab 7 - late endosomes, M6PR - late endosome/multi-vesicular body, Lamp 2 - lysosomal marker.](image1)

**Figure 212** Fluorescent co-immunostaining of Lingo-1 and markers of various vesicular subcompartments
Additionally, I chose vesicles that appeared to have expression of both Lingo-1 and markers of vesicular subcompartments and generated the orthogonal perspectives by taking a Z-stack of images and projecting them in the respective directions (Figure 23) to illustrate that the Lingo-1
and marker expression occurred in the same vesicles instead of different vesicles that are oriented one above the other, indicating actual colocalization in the same vesicle. The split views and the orthogonal perspectives supported my original conclusions from Figure 21 and 22, Lingo targets to endocytic and lysosomal compartments, consistent with my previous data showing a lysosome dependent effect on APP protein levels.

Figure 223 Fluorescent co-immunostaining of Lingo-1 and markers of various vesicular subcompartments
Figure 23 Fluorescent co-immunostaining of Lingo-1 and markers of various vesicular subcompartments. The captured orthogonal slice view from a point of orange overlap between the two stains show co-stained vesicles in a depth view.
3.13 APP/Lingo-1 in Alzheimer’s diseased brain

My results indicating that Lingo-1 down-regulates APP by a non-amyloidogenic pathway suggest that the level of Lingo-1 expression in brain might be an important determinant of risk to develop AD. For this reason, I performed experiments to assess whether Lingo-1 expression in AD brains differs from controls. Astoundingly, I found that in all AD brain samples tested, Lingo levels were dramatically down-regulated.

To further verify my results I repeated this experiment with 10 more diseased brain homogenates and 10 more control brain homogenates. The new samples were immunoblotted and quantified for Lingo-1 and β tubulin. The level of Lingo-1 expression was normalized by β tubulin levels to test for loading consistency. The amount of Lingo-1 expressed in AD brain homogenates was half that expressed in age matched controls with a p value of 0.0362 (Figure 24).

Figure 234 Lingo expression in AD brain is greatly reduced

![Image of Lingo and Tubulin expression in AD and Control brain samples](image)

**Figure 24 Lingo expression in AD brain is greatly reduced.** Expression of Lingo in AD brain is greatly reduced. Three control brain homogenates and three diseased brain cortical grey matter homogenates were normalized for protein concentration and analyzed by Immunoblot. All three of the control samples had strong expression of Lingo while diseased samples had almost no measurable expression. The Immunoblots were stripped and re-probed with beta tubulin to test loading consistency. The results were repeated with five AD patients and 5 controls. Immunoblot bands for Lingo and β tubulin were densitometrically measured using Image J and Lingo levels were normalized by β tubulin levels.
The experiment was repeated with a greater number of brain samples to improve the statistical reliability. Grey matter samples from three different cortical regions were obtained from 15 AD patients and 14 age matched controls. The samples were homogenized, normalized for protein quantity, and immunoblotted. The immunoblots for Lingo-1 expression were quantified, as was the β tubulin expression. Both Lingo-1 levels normalized by β tubulin expression and unnormalized Lingo-1 expression showed Lingo-1 levels were significantly reduced in AD cortical samples relative to age matched controls (see Figure 22). This effect was observed in all three cortical regions examined - parietal, frontal and temporal. I observed that Lingo-1 levels, normalized against total protein or against β-tubulin levels, were reduced by almost half in AD in frontal cortex (p = 0.0002), parietal cortex (p = 0.0007) and temporal cortex (p = 0.02). These results are consistent with the hypothesis that neurons in AD brains have defects in late endosomal function, reducing degradation of βAPP by the lysosomal pathway and promoting βAPP degradation by the amyloidogenic pathway.
One hallmark of AD is global changes in neuronal protein content in brains undergoing degeneration. Advanced AD patients exhibit brain atrophy and neuron loss. In an effort to address whether decreased Lingo expression is a result of total neuron loss, the data was normalized by adding equivalent amounts of total protein to each gel lane and additionally normalized in some cases by normalizing versus beta tubulin content. I also stripped and reprobed the immunoblots with actin, β-tubulin, and 22C11 for APP expression. All of these proteins concentrations remained uniform in both sets of samples. AD causes loss of neurons in specific brain regions (e.g., of pyramidal cells in lamina II of the entorhinal cortex and in the
CA1 region of the hippocampus) but much of the overall loss of brain volume appears to be due to the shrinkage and loss of neuronal processes [90]. Loss of synapses and dendritic spines correlates better with cognitive decline in AD than loss of neurons [91].

Because it was not possible to match age of death perfectly for AD and control samples, I tested whether Lingo-1 levels varied depending on age, for both AD and controls. Lingo-1 expression levels graphed as a function of age for patient and controls showed that Lingo-1 did not vary with age at death for either AD or control samples (Figure 26).

**Figure 256 Decreased Lingo is not a function of age**

![Graph showing Lingo VS Age Control and Lingo VS Age AD](image)

**Figure 26 Decreased Lingo is not a function of age.** Graphing L.ingo-1 concentration as a function of age for control or AD samples indicates that decreased Lingo-1 is not a result of aging.

4 Discussion

4.1 Lingo-1 down-regulates APP in the endocytic pathway

I have shown that Lingo-1 controls APP turnover by enhancing degradation through interaction in the endolysosomal pathway. I examined the likelihood that Lingo-1’s down-regulation of APP reduces the amount of APP available for proteolysis, thereby reducing amyloidogenic peptide production.

I have confirmed that APP and Lingo-1 co-express in the brain, co-localize in neurons, co-immunoprecipitate, and Lingo-1 stoichiometrically down-regulates APP at physiologically relevant concentrations in cell lines and neonatal mouse cortical cultures. All these factors suggest the interactions between Lingo-1 and APP have physiological relevance.
I have found that Lingo paralogs down-regulate APP with varying degrees of efficacy. Other Lingo family members appear to have functional redundancy; however, as Lingo-1 appears to be the predominant isoform, I expect that compensation for Lingo-1 deficiency by other Lingo paralogs may be only partial.

My results suggest that deletion of the intracellular domain of Lingo-1 abrogates Lingo down-regulation of APP. The finding that such a mutant form of Lingo-1, which lacks normal restriction to endosomal compartments, fails to affect APP processing suggests that endosomal localization of Lingo-1 is essential for its functional interaction with APP. Stein and Walmsley used mutagenesis to identify regions of Lingo-1 that are involved in homo- and heterotypic interactions [84]. They found that the N-terminal region containing the leucine-rich repeats along with the transmembrane and cytoplasmic domains of Lingo-1 are not required for self-interaction or interaction with APP. Regions of the ectodomain outside of the N-terminal region containing the LRRs are wholly sufficient for the interaction with APP.

4.2 Non Aβ pathogenesis

For many years the amyloid hypothesis has had the largest number of supporters and been the focus of AD research. Recently numerous groups have pointed to “non-amyloid” based disease mechanisms for AD pathogenesis. There is evidence from several labs that excess βCTF, not Aβ, is the culprit in Alzheimer-related endocytic dysfunction [92, 93]. The intracellular domain of APP is hypothesized to have potential signaling capacity and AICD has been shown to contribute to the pathologic effects of APP processing [94].

Lingo-1, by eliminating all these potentially toxic products of APP, should suppress AD no matter which mechanism prevails. Lingo-1’s role as a potential “gain control mechanism” is ideally situated to provide an extra level protein level regulation.

4.3 Lingo is not a cell surface protein

All of my results indicate that Lingo is not a cell surface protein, but resides in intracellular membranes of the endocytic pathway. This discovery contradicts the body of Lingo literature and will be discussed in the section Caveats, controversies and contradictions.

4.4 Lingo diverts APP from the amyloidogenic pathway

I have demonstrated that Lingo down-regulates full length APP as well as CTFα/CTFβ fragments. Over-expression of Lingo-1 almost entirely eliminates processing of APP through the γ-secretase mediated pathway that produces Aβ. Lingo diverts APP from amyloidogenic pathway into lysosomal degradative pathway. Importantly, the ability of Lingo-1 to down-regulate APP is blocked by the lysosomal inhibitor, bafilomycin, indicating that Lingo-1 targets APP to lysosomes.

My results contradict those of Bai et al. literature and will be discussed in the section Caveats, controversies and contradictions.
4.5 Lingo-1 is down-regulated in AD

AD predominantly affects the cerebral cortex and certain subcortical regions like the hippocampus. Atrophy affects the temporal lobe, the parietal lobe, frontal cortex, and the cingulate gyrus [95]. Intriguingly, I observed a substantial decrease in Lingo expression across these pathogenically impacted regions in AD patient brains. While Lingo-1 down-regulates APP, Lingo itself was found to be reduced in AD patients. Lingo-1 is down-regulated in AD brain in a manner that suggests that down-regulation of Lingo-1 might increase amyloidogenic APP processing and contribute to the disease process. Lingo-1 levels were found to be significantly reduced in AD cortical samples relative to controls in all three cortical regions examined - parietal, frontal and temporal, and Lingo-1 levels did not vary with age at death for either AD or controls.

While Lingo has been reported to interact with APP [68, 84] this is the first report involving AD brain. All my data support the physiological importance of Lingo and APP interactions. Linking Lingo and AD is important and will be explored further under the section heading of Disease Implications.

4.5.1 Possible mechanism for reduced Lingo in AD brain

I have investigated Lingo-1 down-regulation at the protein level. It remains to be determined whether the Lingo is down-regulated at the transcriptional level, or increased Lingo-1 turnover.

Microarray Analysis of Alzheimer’s Disease tangle-bearing hippocampal neurons from the CA1 region indicates that tangle-bearing neurons in AD brains have approximately three times more down-regulated genes (18%) compared with up-regulated transcripts (6%) [96]. Histone deacetylase 2 (HDAC2) may contribute to such transcriptional changes. Aging and AD are associated with epigenetic dysregulation at various levels, including abnormal DNA methylation and histone modifications [97]. HDAC2 accumulates in the brain early in the course of Alzheimer's disease in mouse models and in people with the disease [98] and blocks the expression of genes needed for brain plasticity. Reversing the build-up of HDAC2 by short-hairpin-RNA-mediated knockdown suppresses the levels of APP and stem cell markers, reinstates structural and synaptic plasticity, and abolishes neurodegeneration-associated memory impairments [98]. Inversely, promotion of histone acetylation improved learning and memory in a mouse model of AD and increased learning-related gene expression in aged wild-type mice [99, 100]. Further, HDAC inhibitors appear to be effective in ameliorating some of the primary phenotypes associated with AD [101-103]. Sodium butyrate and phenyl butyric acid are HDAC inhibitors being researched as potential AD drugs. Also, recent screening work done in collaboration with Dr. Wiley demonstrates that HDAC inhibitors promote APP degradation.

Another possibility is that down-regulation occurs at the level of Lingo-1 turnover, as a result of alterations in endolysosomal function known to occur in AD. Alternatively, diminished neurotrophin/Trk signaling in AD might transcriptionally down-regulate Lingo-1, and the resulting Lingo-1 down-regulation might enhance amyloidogenic processing of APP. Lingo-1 is transcriptionally up-regulated by NGF-dependent activation of TrkA in sensory neurons[67] and BDNF-dependent activation of TrkB in hippocampal neurons [104] while over expression of APP reduces retrograde transport of NGF in cholinergic neurons [93], leading to cell death.
Bulbarelli, et al. show that administration of (pro-apoptotic) Aβ25-35 or oligomeric Aβ1-42 to primary cultures of hippocampal neurons produces a temporary and marked elevation of NGF release, TrkA protein, NGF RNA and TrkA RNA, and of TrkA, Akt, and GSK3β phosphorylation; the observed TrkA activation is largely NGF-induced and partly independent of NGF. The investigation raised the question whether NGF and TrkA up-regulation might be a defense mechanism against increased Aβ or part of a pro-apoptotic response to Aβ.

Matrone, et al. found in NGF-dependent cultures of hippocampal neurons that NGF withdrawal causes an increase in Aβ that leads to pro-apoptotic signaling of TrkA. Their results also indicate a direct interaction of TrkA, p75, and Aβ and a role of multiprotein TrkA-p75 complexes in this apoptosis. According to their interpretation, NGF withdrawal causes an imbalance of beta and gamma-secretase activities that induces apoptosis by increased levels of Aβ and of p75 fragments and by Aβ- and p75-mediated TrkA signaling.

4.6 How do Lingo and APP encounter each other?

Neurodegenerative disorders represent some of the most common and debilitating disorders in aging. Dysfunction of the endocytic system is an early event in a wild range of neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease, ALS, frontotemporal dementia (FTD) and ALS. My results suggest that dysfunction of Lingo-1 actions in the endocytic pathway may contribute to Alzheimer’s disease.

My data suggests that Lingo-1 resides primarily in late endosomes, where it encounters Trks and APP following their endocytosis. The fact that Lingo-1 down-regulates the proteins it encounters, while Lingo’s fellow LIG family member ISLR2 up-regulates the proteins it encounters, suggests there is a different underlying mechanism of association between the different LIG family members and the proteins they regulate. To address this mechanism the Bothwell lab immunoprecipitated Lingo-1 from detergent extracts of mouse brain and ran the samples on SDS page gels. The gels were silver stained and the appropriately sized bands were cut from the SDS gel for analysis by tandem mass spectrometry. Identical studies were performed for ISLR2 in the laboratory of my collaborator, David Ginty [105].

The ISLR2 immunoprecipitate contained multiple subunits of members of the AP1/AP2/AP3 clathrin adapter gene family, whereas none of these proteins were present in the Lingo-1 immunoprecipitate [105]. Instead the Lingo-1 immunoprecipitate contained several proteins known to influence vesicular trafficking, including GIT1 and CHIP. GIT1 regulates ARF GTPases, key regulators of vesicular trafficking [66] and CHIP is a ubiquitin ligase that ubiquitinates APP and regulates its processing [67]. The Lingo-1 immunoprecipitate also contained multiple subunits of the ESCORT III complex including CHMP-1b1, 1b2, 2a,4b,5 and 6, as well as EXCORT complex accessory proteins including Annexin A2 [68], ARP2/3 [69], LIP5, and ALG-2 [71], and ALG-2 [71]. These protein associations suggest that Lingo-1 controls delivery of cargo to late endosomes and lysosomes while ISLR2 regulates clathrin-dependent vesicular trafficking.
APP is internalized from the plasma membrane via a mixture of clathrin and raft-dependent endocytosis [16, 72], and ARF6 controls APP processing by mediating the endosomal sorting of BACE1 [73], suggesting subcellular trafficking as a regulatory mechanism for this proteolytic processing step. Lingo-1 either doesn’t traffic to the cell surface or is present there only very transiently. Lingo promotes lysosomal degradation of endosomally trafficked membrane proteins by preventing those cargo proteins from returning to the cell surface via recycling endosomes. Lingo and APP encounter each other in hybrid organelles or directly from the trans-Golgi network where Lingo targets APP for degradation.

### 4.7 Caveats, controversies and contradictions

Current literature indicates that Lingo is a cell surface protein. However, as my data shows in section 3.11, Lingo may actually reside in the intracellular membrane instead. The discrepancy in my data and previously published work likely has to do with the precise nature of the reagents employed in the experiments. Many of the experiments done regarding Lingo function used an expression construct of Lingo-1 with a C-terminal FLAG epitope tag. The C-terminal 8 residues of Lingo-1 are absolutely conserved from zebrafish to man. I have observed that any modification of these C-terminal residues, either by deletion or addition of C-terminal epitope tags, alters the subcellular distribution of Lingo-1 (data not shown). Lingo-1 in neurons is normally expressed exclusively on endosomal vesicles whereas Lingo-1 with a modified C-terminus resides mainly on the plasma membrane. My results indicate that Lingo-1 with a modified C-terminus does not effectively down-regulate APP. Bai, et al.[68] used this C-terminally tagged Lingo-1 in their 2008 paper that reported an interaction of Lingo-1 with APP that led to modulation of APP proteolysis and subsequent Aβ generation. However, altering the C-terminal domain likely alters the intracellular trafficking signals leading to altered function of the over-expressed Lingo.

The use of a C-terminal FLAG epitope tagged Lingo construct may also have influenced Bai, et al.’s findings that over-expressed Lingo-1 modestly increased production of Aβ from APP by increasing the production of CTFβ while decreasing the production of CTFα [83]. As discussed in section 3.6, we found that Lingo-1 down-regulation of APP is not mediated by increased processing by α or β secretase. If Lingo expression is changed to the cell surface it will not encounter APP in the same location and will not have the chance to down-regulate APP before it encounters the various secretases. Additionally Bai, et al. used a form of APP found in a Swedish family that has a double mutation (Swedish APP) which causes early onset AD for their study of Lingo function. Swedish APP is cleaved by BACE in more proximal compartments that are probably not identical, at least on a quantitative stoichiometric basis, to those where wild-type APP is cleaved by BACE [106-109]. Since the Swedish mutant APP is processed in a Golgi compartment where Lingo1 does not function, it is not surprising that they failed to see the dramatic effects of Lingo-1 on APP processing that I observed.

### 4.8 Experimental knock down of Lingo expression

shRNA knock-down of Lingo rescues APP down-regulation in cell lines, but showing Lingo down-regulation by siRNA transfection in neonatal cortical cultures increases endogenous APP is a key piece of evidence I have been unable to obtain. siRNA transfection of Lingo or control siRNA causes an increased Lingo expression instead of the expected knockdown.
Despite the number of commercially available Lingo siRNAs, knockdown of Lingos in primary cultures have not been reported in current literature. Bai, et al. transfected siRNA Lingo in a HEK 293 cell line that stably overexpressed APP harboring the Swedish double mutation (HEK 293 APP<sub>sw</sub>), but any immunoblot data they showed was a knockdown of transfected Flag tagged Lingo-1, not Lingo endogenous to Hek 293 cells. They used Lingo-1-specific quantitative RT-PCR to establish endogenous expression of Lingo-1 and identify a pool of siRNAs that mediated efficient knockdown of endogenous Lingo-1. Using RT-PCR and a pool of siRNAs have their own complications. RT-PCR measures RNA transcript levels in a quantitative fashion, but RNA transcript levels do not always correlate to protein levels and my studies are at the protein level. Additionally, using a pool of siRNAs increases the chance of off-target effects.

Many papers cite using the anti-Lingo-1 antibody or the antagonist Lingo-1-Fc to inhibit Lingo, but my subcellular localization data shows that Lingo targets to several intracellular degradation pathways and is only minimally present at the cell surface. However, FC fusion proteins and antibodies can be endocytosed and potentially have their effects intracellularly. It might be possible to knock-down Lingo if the endosome containing the endocytosed FC fusion protein then fuses with a Lingo containing endosome. Mi, et al. reported using Lingo-1 RNAi lentivirus to infect oligodendrocytes and knock-down endogenous Lingo-1. Like Bai, et al., they used RT-PCR to measure endogenous expression [110]. It is possible that Lingo-1 RNAi lentivirus may succeed in knocking down Lingo expression where siRNA only caused an increase in Lingo expression. On the other hand, transcriptional upregulation of Lingo1 by siRNA may be cell type specific, if so oligodendrocytes may respond differently than cortical cultures.

5 Conclusions

My results are important for several reasons. First, consistently altered expression of an endosomal protein in multiple cortical regions in AD supports the popular hypothesis that there is widespread endocytic dysfunction in AD. Second, since Lingo-1 down-regulates APP, the reduced expression of Lingo-1 in AD might increase APP expression. Studies have demonstrated that a variety of types of mutations that modestly increase expression of APP increase the risk of AD [111]. Multiple studies have linked Lingo-1 to essential tremor (reviewed in [112]), while essential tremor is linked to neurodegenerative disease associated with an increased risk of AD [78]. Finally, if Lingo-1 down-regulation is an effect of APP-related pathology, rather than a cause of that pathology, resulting perturbation of Lingo-1-mediated regulation of receptor tyrosine kinases such as neurotrophin receptors may have positive pathological consequences.

Alzheimer’s disease is the most common form of dementia and the subject of intense study for years. Despite the years of research, no therapeutics are available that slow disease progression [113]. Multiple proteins have been targeted as possible drug targets with varying degrees of efficacy. AD is neurodegenerative disorder that is probably caused by interactions among multiple genetic, epigenetic, and environmental pathways that will require drugs with a combinative, complimentary approach that has more than one target.
Attempting to treat AD is further complicated by the late onset of disease. Ideally we would be able to prevent AD instead of attempting to halt or reverse the disease once cognitive impairments have emerged in a fragile aging population. The ability to determine pivotal factors that start the degenerative process could be critical in catching and preventing a disease that is usually a result of degeneration that has progressed silently for many years [114].

Changes in diagnostic ability have made it easier to identify patients earlier in the course of their disease. Biomarkers such as MRI scans, PIB scans, and measurements of Aβ and tau in the cerebrospinal fluid have allowed for earlier identification of people at risk for developing the disease [115]. The ability to identify more sensitive and specific biomarkers might allow for the earlier identification of potential genetic risk factors [116].

Many of the therapeutics in trial for AD have focused on preventing or altering cleavage of APP [117-119]. Changing cleavage of APP affects cleavage of other proteins like Notch and voltage-gated sodium channel subunits [114]. Lingo regulation of APP does not increase cleavage and does not pose a risk for notch cleavage. Lingo has been suggested as a potential molecular target for neuroprotective drug trials for essential tremor [120], but as yet so little is known about Lingo’s mechanism in either ET, PD or AD. It is possible Lingo-1 function influences onset of AD. Perhaps Lingo down-regulation combines with environmental factors and genetic variables in AD brain.

If Lingo-1 shifts APP processing from amyloidogenic to degradative its mechanism will have to be explored. It is tempting to hope that a protein that functions as a “gain control” mechanism for APP levels will lead to a compelling drug target, both for mitigation and prevention of Alzheimer’s disease. Lingo itself is a questionable target, as tampering with Lingo function will cause alternative vesicular trafficking and mis-sorting of proteins other than APP. It is possible that even if Lingo is not a possible drug target, there may be a related sorting protein in the same pathway that is more compelling.

Understanding the function of Lingo-1 in the trafficking of APP may provide insights into the regulation of APP processing in AD pathogenesis. If Lingo-1 down-regulation is an effect of APP-related pathology, rather than a cause of that pathology, resulting perturbation of Lingo-1-mediated regulation of receptor tyrosine kinases such as neurotrophin receptors may have pathological consequences. With the wide variety of receptors Lingo-1 modulates, it would be unsurprising if Lingo-1 is involved in other protein sorting and trafficking based diseases.

I have shown that AD brain expresses substantially reduced amounts of Lingo compared to age-matched controls, and Lingo down-regulates APP using cultured cells and primary neurons. Lingo interactions determine whether APP in endosomes recycles to the cell surface or enters late endosomes and the lysosome for degradation. I suggest that this down-regulation reduces the quantity of APP available for amyloidogenic proteolytic processing and may provide insights into Alzheimer’s disease pathology.
6 Exploration of additional Lingo functions

6.1 Lingo modulation of other receptors

Because Lingo-1 stems from a common LIG origin, has an ECD consisting of LRRs and Ig domains (which are commonly implicated in protein-protein interactions), is structurally similar to Trks (which are known to dimerize) and physically associates with a Trk-interacting transmembrane protein (p75\textsuperscript{NTR}) I hypothesized Lingo-1 may directly modulate Trk RTK activity similar to the inhibition of EGFR activity and expression by LRIG. LRIG is described to effect cell surface RTKs that have been internalized in response to binding ligand. As a first step, I tested Lingo-1 regulation of cell surface receptor levels. Towards that end I chose to evaluate the first Lingo-1 report which described the modulation of a cell surface complex consisting of the LRR molecule NgR and a known TrkA interacting protein, p75\textsuperscript{NTR}.

My lab has also demonstrated that Lingo-1 regulates p75 and TrkB. I co-transfected Hek 293 cells with a uniform amount of p75 (176 pg) plasmid DNA and a titration of Lingo-1 Lingo-1 plasmid DNA increasing from 0.25 the amount of p75 (44 pg) to 16 times the amount of p75 (2.8 µg) plasmid DNA. Increasing Lingo-1 caused a corresponding decrease in p75 (Figure 27). Similarly when this experiment was repeated with first TrkB alone (Figure 28), and then with TrkA, TrkB, and TrkC (Figure 29) all were down regulated like APP. My results suggest that Lingo/Trk association may impede trafficking of Trk to the cell surface, leading to eventual degradation of Trks.
Like APP, down-regulation of Trks occurs through degradation in the lysosome. Inhibition of lysosomal function by bafilomycin treatment rescued Trks from Lingo-1-mediated down-regulation. In contrast, the proteasome inhibitor epoxomicin had no effect. These results indicate that Lingo-1 down-regulates Trks by promoting delivery of Trks to lysosomes.
TrkA and TrkB were expressed with Lingo-1 and treated with the proteasome inhibitor epoximicin and the lysosomal inhibitor bafilomycin. As with APP i found that Lingo-1 mediated down-regulation of TrkA was prevented by the lysosome inhibitor bafilomycin whereas the proteasome inhibitor was ineffective. (Figures 29)
Figure 289 Lingo down regulates all Trks

![Image of Lingo Trk regulation](image)

Figure 29 Lingo down-regulation is rescued by bafilomycin

![Image of Lingo Trk regulation with bafilomycin](image)

Lingo-1 down-regulation of p75 is also ameliorated by treatment with bafilomycin.
7 Closing remarks

This body of work gives new understanding of Lingo localization, associations, and functions. It raises the exciting possibility that Lingo is a general controller of vesicular trafficking. Lingo promotes lysosomal degradation of endosomally trafficked membrane proteins by preventing those cargo proteins from returning to the cell surface via recycling endosomes. Regulation of proteins by Lingo-1 extends far beyond its interactions with APP. More and more evidence points to Lingo-1 modulating whole families of receptors down regulating APP, Trk receptors, and p75. The wide variety of proteins modulated by Lingo-1 concentration suggests these interactions may not be specific, but rather that Lingo-1 is a general controller of vesicular trafficking. This further leads to the possibility that Lingo-1 is involved in other protein sorting diseases.

8 Future work

To complete the narrative of my current research there are a few further experiments that could be completed by the Bothwell lab. Preliminary results suggest that Lingo-1 expression correlates with Braak score, or CERAD score for individuals who were not clinically diagnosed with AD. Using the three cortical regions originally immunoblotted for Lingo-1, the same
samples would be immunoblotted and probed for APP to determine if APP expression is higher in AD patients versus age matched controls.

There are a few results still needed to establish correlation of Lingo-1 expression with age and severity of diagnosis. First I would like to strip and re-probe the 87 immunoblot samples taken from 3 cortical regions to determine if my preliminary result showing APP expression is higher in AD patients versus age matched controls is validated with a higher number of samples. Preliminary results suggest that Lingo-1 expression levels correlate with Braak score, or CERAD score for individuals not clinically diagnosed with AD. Data mining the existing three cortical region immunoblots measuring Lingo-1 levels should give us a better understanding of how Lingo-1 levels correlate to severity of AD diagnosis, and whether controls with low Lingo-1 levels may be patients with some level of dementia.

8.1 Immunoblot studies

I would also like to extend immunoblot studies to include more individuals, as well as other regions of the brain. It would also be interesting to examine mouse AD models and Lingo-1 knockout mice to test the hypothesis that Lingo-1 influences APP turnover and capacity to undergo amyloidogenic processing.

8.2 Do SNP variations associated with ET and PD change Lingo down-regulation?

It would be valuable to determine whether SNP variations linked with essential tremor and Parkinson’s disease have any effect on Lingo down-regulation of APP. I would like to look at APP and Lingo expression in essential tremor and Parkinson’s brains. People with ET have increased risk of AD might suggest that the ET linked Lingo SNPs might affect APP in AD, but APP is increased in PD and ET. The main SNP is in an intron, so it might affect Lingo transcriptional regulation. The transcriptional effect could be opposite in different neurons.

8.3 Mouse models

I would expect that Lingo-1 knockout mice would have higher levels of APP compared to age matched controls. It would also be possible to examine Lingo-1 and APP levels at a variety of ages. In Down syndrome early endosomes are enlarged in some pyramidal neurons as early as 28 weeks of gestation. That endosome enlargement occurs decades before classical AD neuropathology develops. This suggests that changes in Lingo-1 expression levels probably occur fairly early in disease development. Mouse AD models and Lingo-1 nulls would allow comparisons of brain tissue from a variety of ages.

I would also like to breed a transgenic mouse model of AD (APPswe/PS1dE9 mice, available from colleague Warren Ladiges) into a Lingo-1 null genetic background, and repeat these studies, additionally using ELISA to determine whether levels of ɑβ peptide are increased, and assessing whether amyloid accumulation in brain is increased compared to APPswe/PS1dE9 expressing Lingo-1.
8.4 ELISA

I would like to do further studies transducing mouse neonatal cortical cultures. I would like to compare Aβ40 and Aβ42 levels in transduced and non-transduced neurons. Aβ40 and Aβ42 levels will be assessed by ELISA.

8.5 Pulse Chase experiments

My hypothesis predicts that APP is down-regulated when Lingo-1 is over-expressed (and up-regulated when Lingo-1 is absent or inhibited) because Lingo-1 promotes lysosomal degradation of APP. To test this prediction directly, the lab will employ metabolic pulse-chase experiments examining APP synthesis and turnover in the HEK 293 cell system. For these experiments, cells will be exposed for 30 minutes to $^{14}$C-methionine/cysteine, followed by an excess of unlabeled methionine/cysteine, detergent lysates will be prepared at intervals of 30 minutes thereafter, and the labeled APP products will be detected on gels by autoradiography. I predict that newly synthesized APP will turn over with a shorter half-life when Lingo-1 is over-expressed and that this effect will be countered by the exposure of cells to the lysosome inhibitor bafilomycin.

8.6 NF-kappaB activation

siRNA results suggest that even low levels (1nM) of siRNA transfection cause an increase in Lingo-1 expression regardless of the siRNA sequence used (control or Lingo-1) leads us to believe that off-target effects cause an increase in Lingo-1. My lab has several tools to study NF-kappaB activation or inhibition. I would like to try siRNA treatment of cells transfected with an IκB expressing plasmid.

9 Materials and Methods

9.1 Plasmid constructs

A eukaryotic expression plasmid for full length human Lingo-1 [Lingo-1, hL1], IMAGE: 4214343, was purchased from Thermo Scientific Open Biosystems. A preprotrypsin-FLAG amino tagged human Lingo-1 lacking the native secretion signal [Flag-Lingo-1] was created employing standard molecular biology techniques with the oligonucleotides 5’-CACAAGCTTTGCCCCGCCCG-3’ and 5’-CACTCTAGATCATATCATCTTCATGGTGAACCTTGCGG-3’ and pFLAG-CMV-1 from Sigma. A V5-6His carboxyl epitope fusion to human Lingo-1 [Lingo-1-V5] and Lingo-1 missing the prospective intracellular domain [Lingo-1-ΔICD-V5] were created according to manufacturer’s instructions with PCR generated DNA fragments using the forward oligonucleotide primer 5’-CACCATGCAGGTGAGCAAGG-3’ and the reverse primers 5’-TATCATCTTCATGGTGAACCTTGCG-3’ and 5’-GTTGCCCTTGCCCCGGCTCCA-3’, respectively, and the pcDNA3.1 Directional TOPO expression kit (Invitrogen, #K4900-01). The sequence for the transmembrane and intracellular domain of Lingo-1 was amplified via 5’-CAGCCTTCCAAGACCCTCATCATCGCCACC-3’ and 5’-CACTCTAGACTCATATCATCTTCATGGTGAACCTTGCGG-3’ oligonucleotides and was spliced into and expressed from pSecTagB (BamH1-XbaI). The kinase incompetent pCMV5 rat TrkA D671A [TrkA-KD] and pCMV5 rat TrkB D693A [TrkB-KD] have been described previously
[121]. Empty vector control is pcDNA3.1/Zeo(-) and was purchased from Invitrogen (Carlsbad, CA, USA). A carboxyl myc tagged version of mouse LINX [LINX-myc] is in the vector pcDNA3.1 myc/His(-)A (Invitrogen) and described previously [56]. A plasmid vector driving the expression of an amino myc tagged version of human Lingo-1 with a deleted extracellular domain, a non-native secretion signal and IRES mediated DsRed expression [DsRed ECDdelta-hL1] was created in a two-step process using the three plasmids: IMAGE clone 4214343 (Thermo Scientific Open Biosystems, Huntsville, AL, USA), pSecTagB (Invitrogen) and pIRES2-DsRed2 (Clontech, Mountain View, CA, USA). First, a DNA fragment encoding myc-ECDdelta-hL1 was created using standard Taq based PCR, the IMAGE clone and the oligonucleotide primers 5'-CACGGATCCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGAATGAAAAGACCCCATCATCGCCACC-3' and 5'-CACGAATTCCTCATATCATCTTCATGTTGAACCTTGCAGG-3'. This fragment is then digested with the endonucleases BamH1 and EcoR1 and ligated into a similarly digested pSecTagB using T4 DNA ligase. The resulting 371 base pair CDS is then removed via Nhe1 and EcoR1 and inserted into an identically opened pIRES2-DsRed2. Plasmids generating short hairpin RNAs against Lingo-1 [shLingo-1; (Open Biosystems, #RMM4534-NM_181074, Constructs TRCN0000174355 and TRCN0000175194)] encode antisense sequences 5'-ATTGAATTATTGACTCTGCCGT-3' and 5'-ATTGAAACCCAAGTTACAGATT-3'. Rat TrkA and rat TrkB constructs were gifts from Moses Chao and NgR-Flag was a gift from Z.-g. He. All oligonucleotides were purchased from Integrated DNA Technologies.

9.2 Cell culture, Transfection and Treatments

For transient transfections, HEK 293 cells were plated at 70–80% confluency on 35 mm plates and transfected with Lipofectamine 2000 (Invitrogen). HEK 293 and N2A cells were cultured in pH 7.2 DMEM (MediaTech, #50-003-PB) containing 10% fetal bovine serum (Hyclone, #SH30396.03) at 37 °C, 5% CO2. For experiments, unless indicated otherwise, cells were plated at 30-50% confluency in 6-well plates 20-24 hr prior to transient transfection and transfected using Lipofectamine 2000 (2.5 µl/µg LIPO:DNA; Invitrogen, Cat No. 11668-019) and 1 µg (unless otherwise indicated) of plasmid DNA (per construct transfected) in DMEM+10% fetal bovine serum without antibiotics. Cells were harvested 24hr (unless otherwise indicated) post transfection.

9.3 Dissociated Mouse Hippocampal Neuronal Culture

Primary cortical neurons were cultured from C57BL/6 postnatal day zero mouse pup brains. Pup brains were removed in Hanks' medium without Ca2+ and Mg2+, and then further dissected in dissection media (Neurobasal-A™ (Invitrogen, #10888) medium supplemented with 10 mm HEPES, and 500 µM L glut). Post dissection the cortices were digested with trypsin for 25 minutes at 37 °C. The trypsin digestion was halted with trypsin inhibitors and the neurons were dissociated by multiple rounds of pipetting. The neurons were spun at 800 rpm for 3 minutes and resuspend in dissection media. This process was repeated three times to wash the neurons. On the final wash the neurons were resuspended in plating media (Neurobasal-A™/2% B27 supplement 500 µM L glut and 0.25% Pen/strep(MediaTech 30-002-CI,100X)). The neurons were diluted to the appropriate density and transduced with virus. The neurons were plated at 1.0 x10^5 cells/ml on poly-D-lysine (Sigma, #P7405-5MG, 0.1mg/ml in dH2O) coated 12 well plates,
or wells containing glass cover slips that were washed before being coated with poly-d-lysine. The neurons were cultured for 1 week without changing the media.

9.4 Virus Transduction

Dissociated Mouse Hippocampal Neuronal Cultures were plated at the desired confluency (between $1.5 \times 10^5$ cells and $5 \times 10^4$) in plating media (Neurobasal-A™ with 2% B27 supplement and 500 µM L glut and 0.25% Pen/strep) and the requisite volume of virus. Any disparity in volume was compensated for with additions of the appropriate amount of B27 supplement, L glut and Pen/strep. The media was changed after twelve hours and the neurons cultured for an additional four days without changing the media before harvesting.

9.5 Brain tissue sample preparation

-80°C frozen tissue was obtained from the University of Washington Alzheimer Disease Research Center (ADRC). Tissue was harvested using Grant #P50 AG05136. A small chunk of brain was sliced off the frozen sample, weighed, and added to a microcentrifuge tube. A matching volume of lysis buffer (20mM Tris, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 250 mM sucrose, and protease inhibitor mixture (Sigma)) was added to each tube and the sample gently squished against the bottom of the tube using a microcentrifuge tube pestle. The samples were triturated using 20 gauge needles to homogenize, and then sonicated for 10 pulses at 20% duty/output #2. The homogenized tissue was spun at 14,000 g for 20 minutes in the microcentrifuge. The supernatant was removed and the protein concentrations were quantified by Bradford assay for use in SDS-PAGE. SDS loading buffer (62 mM Tris pH 6.8, 2% SDS, 10% Glycerol, 2% BME, 0.02% BPB) was added to the lysates and they were boiled for five minutes.

9.6 siRNA

Dissociated Mouse Hippocampal Neuronal Cultures were plated at the desired confluency (between $1.5 \times 10^5$ cells and $5 \times 10^4$) in plating media (Neurobasal-A™ with B27 supplement and L glut and Pen/strep) and grown for 2-3 days. The cells were rinsed with PBS and changed to antibiotic free media. The neurons were either transfected using Lipofectamine 2000 or HiPerfect. For Lipofectamine 2000 transfection 2 µl Lipofectamine 2000 was diluted in 100 µl OptiMEM I per well. The siRNAs were diluted in 100 µl OptiMEM I and both solutions incubated at room temperature for 5 min. The two solutions were mixed and incubated at room temperature for 20 min. 200 µl OptiMEM I containing DNA/Lipofectamine 2000 transfection complexes was added to each well. The plates were swirled gently to ensure uniform distribution of the transfection complexes. The media was changed 2-6 hours post transfection. Neurons were harvested 2-4 days post transfection.

For HiPerfect transfection siRNAs were diluted in 100 µl OptiMEM I per well. 3 µl of HiPerfect was added and the solution vortexed and incubated at room temperature for 5 minutes. 100 µl of the siRNA/HiPerfect/ OptiMEM I solution was added to each well and the plate swirled gently to ensure uniform distribution of the transfection complexes. Neurons were
harvested 2-4 days post transfection. For both HiPerfect and Lipofectamine 2000 transfected a variety of siRNAs and concentrations were used. Concentrations ranged from 0.5 nM to 150 nm.

9.7 Immunoprecipitation

Immunoprecipitation from whole p0 mouse brain was conducted using the following protocol: p0 mouse brain was dissected and snap frozen in liquid nitrogen. A pre-chilled mortar and pestle was used to grind the brains into a fine dust. Low salt homogenization buffer (10 mM Tris pH8, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100 plus protease inhibitors) was added to the brain dust which was further homogenized by trituration with decreasing diameters of needles from 18 to 25 gauge. After the 25 gauge needle allowed for easy (if slow) uptake into the needle the slurry was spun at high speeds, the pellet brought up in hypotonic buffer, and the solution incubated nutating for one hour at 4°C. After being ultracentrifuged for 1 hour at 100,000 x g at 4°C the supernatant was brought to a final NaCl concentration of 0.15M. The lysate was pre cleared by rotating for an hour at 4°C with Protein A Sepharose CL4B beads (GE 17-0780-01) before being aliquoted into different tubes for the various antibodies for immunoprecipitation.

9.8 Immunocytochemistry

Cover slips were rinsed with PBS (pH 7.4) then fixed with 4% formaldehyde (4% w/v PFA in PBS) for 30 minutes. Cover slips were then rinsed with PBS and permeabilized with 0.25% v/v Triton-X100 in PBS for 15 minutes. Permeabilization was followed by another PBS rinse. Cover slips were then blocked in 10% w/v BSA in PBS (BSA/PBS) for 15 minutes then incubated overnight at 4°C in primary antibody mixtures. Primary antibodies and dilutions used follow: rabbit anti-Lingo-1 (Upstate, #07-678, 1:1000), mouse anti-Flag (Sigma, #F 1804, 1:1000), mouse anti-V5 (Invitrogen, #R960-25, 1:1000), mouse anti-c-myc (previously described in Kanning et al., 2003; 1:500), mouse anti-Rab5(BD Transduction,#610724, 1:500), mouse anti-mannose 6-phosphate receptor (Abcam, #ab2733, 1:1000), mouse anti-β-COP (Sigma, #G6160, 1:2000), goat anti-LAMP2 (Santa Cruz Biotech., #sc-8100, 1:200). At the conclusion of incubation, primary antibodies were removed and cover slips were rinsed three times with PBS (one quick, two for 10 minutes each). Cover slips were then incubated for 3 hrs at 4°C in appropriate secondary reagents (AF568 donkey anti-goat IgG (Molecular Probes, #A11057, 1:1000), AF555 donkey anti-rabbit IgG (Molecular Probes, #A31572, 1:1000), AF568 donkey antimouse (Molecular Probes, #A10037, 1:1000), AF488 donkey anti-rabbit (Molecular Probes, #A21206, 1:1000) and or streptavidin-conjugated AF546 (Molecular Probes, #S-11225, 1:2000). Cover slips were then washed six times with cold PBS (three quick, three for 10 minutes each), incubated with Hoechst 33258 (1:20000) in dH2O for 7 minutes, post-fixed with 4%PFA (10min), rinsed with PBS and then washed twice for 10 minutes in dH2O. Cover slips were mounted onto slides with 1:1 glycerol/dH2O (plus thimerosal, 0.01% final concentration). All primary and secondary antibody mixtures were in 2.5% BSA/PBS.

Paraffin imbedded slides were de paraffinized by two three minute washes in xylene followed by three minutes washes in xylene 1:1 with 100% ethanol, 100% ethanol, 95% ethanol, 70 % ethanol, 50 % ethanol, and a final rinse under cold running tap water. Antigen retrieval was heat induced by thirty minute incubation in 10 mM Sodium citrate, 0.05% Tween 20, pH 6.0 at a constant 98°C. The slides were allowed to cool to room temperature and then rinsed under cold.
running tap water. The slides were then stained using the immunohistochemical staining protocol listed above without any permeabilization step.

9.9 Confocal Microscopy

Fixed cells (HEK 293, N2A, or mouse neuronal cover slip cultures) were imaged using a Zeiss 510 META confocal microscope. Excitation lasers used were 405nm Diode, 488nm Argon and 543 HeNe lasers. Images were routinely acquired at a depth yielding maximum fluorophor signal throughout the highest amount of neuritic arborization resolvable. Only healthy cells with normal morphology and non-pyknotic nuclei were imaged. For transfected cells, cells expressing low levels of construct were routinely observed to minimize over expression effects. Images were obtained at 40x or 63x (oil immersion) in multi-track, multi-frame mode with line averaging (set to 4). For co-localization studies, signal contrast was optimized post image acquisition to determine for coincident localization.

9.10 Luciferase Assay

Two constructs were employed to measure \( \gamma \)-secretase activity in a variety of cell types. The APP-Gal4-VP16 and Gal4-Luc-GFP constructs have been previously described [2] and were subcloned into the pSL6 lentiviral shuttle vector [31]. Cells were plated at a density of 50,000 cells/well in a 24 well plate the day prior to transfection. The cells were then transfected using Lipofectamine 2000 (described above), the two constructs listed, plus vector DNA. The transfection mix was incubated on the cells for 2–4 h, prior to a replacement of the medium of fresh growth medium. The cells were grown for 72 hours and then rinsed twice with cold PBS and lysed in 100 \( \mu \)l of cold lysis buffer (0.1% Triton X-100/1 mM dithiothreitol/6 mM MgSO4/4 mM ATP/100 mM potassium phosphate, pH 7.8) and the plate frozen at -80°C. For the assay the plates are defrosted and banged together to help the melting lysates finish lysing. Each well of a 96-well microtiter white bottomed plate is filled with 200 \( \mu \)l (100 mM potassium phosphate, 6 mM MgSO4/4 mM ATP, pH 7.8) and 25 \( \mu \)l lysates added to each well. The plate is then assayed for luciferase activity using the luminometer as described [122, 123]. Galactosidase assays were performed essentially as described in the same paper. Luciferase activity was divided by \( \beta \) galactosidase activity to normalize for differences in transfection efficiency.

The \( \gamma \)-secretase inhibitors used DAPT was used at 10 \( \mu \)m. The cells were treated with DAPT \( \gamma \)-secretase inhibitors starting approximately 24 h post-transfection and remained in the inhibitors for 16–20 h prior to lysis.

9.11 Western Blots

Cells were washed twice with cold PBS on ice, lysed in 20mM Tris, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 250 mM sucrose, and protease inhibitor mixture (Sigma), and centrifuged 15 min at 16,000 x g; supernatant protein concentrations were quantified by Bradford assay for use in SDS-PAGE. SDS loading buffer (62 mM Tris pH 6.8, 2% SDS, 10% Glycerol, 2% BME, 0.02% BPB) was added to the lysates and they were boiled for five minutes. The boiled lysates and a molecular weight marker were run on pre-poured 4-20% Tris- HCl acrylamide gels, (Bio-Rad, #345-0033) using the recommended conditions, and SDS PAGE and Western Blotting was performed as described previously [124]) and transferred to
polyvinylidene difluoride membranes (Millipore) using a semidry transfer system ([124]) and transferred to polyvinylidene difluoride membranes (Millipore) using a semidry transfer system (Invitrogen). The membranes were blocked with 0.01% Tween 20, phosphate-buffered saline (PBS-T) containing 5% nonfat dried milk at 4 °C overnight and then probed with a primary antibody overnight. After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000; Vector), or anti-mouse IgG (1:5,000; Vector) in 5% milk, PBS-T for 1 h at room temperature. After washing the membranes were treated with enhanced chemiluminescence (Upstate), and the membranes were visualized by autoradiography.

9.12 Antibodies

The following antibodies, diluted from the supplied source, were used as specified: rabbit anti-Lingo-1 (Upstate, #07-678, 1:1000 IP, WB; 1:500 ICC), rabbit anti-Trk (Santa Cruz Biotech., #sc-139, 1:1000 IP & WB), goat anti-phospho TrkB (A gift from Dr. Moses Chao, 0.3 µg/ml), goat anti-TrkB (R&D, #AF397, 0.1µg/ml), mouse anti-myc (The monoclonal anti-myc antibody 9E10 was purified from hybridoma culture supernatant using protein G-Sepharose), mouse anti-Flag (Sigma, #F 1804, 1:1000), mouse anti-V5 (Invitrogen, #R960-25, 1:1000), mouse anti-Rab5(BD Transduction,#610724, 1:500), mouse anti-mannose 6-phosphate receptor (Abcam, #ab2733, 1:1000), mouse anti-β-COP (Sigma, #G6160, 1:200), goat anti-LAMP2 (Santa Cruz Biotech., #sc-8100, 1:200), goat anti-pyruvate kinase (Rockland, #100-1178, 1:2000), mouse anti-V5 (Invitrogen, #R960-25, 1:1000), rabbit anti-GFAP (AbCam ab7260 1 to 1000), mouse anti-APP 22C11 (Millipore # MAB348SP 1 to 1000), mouse anti-GM130 (AbCam ab12991 to 500), Rabbit polyclonal antisera 9992 (A gift from Dr. Moses Chao), mouse anti-β-actin (Sigma A5316 1 to 1000), mouse anti-Acetylated Tubulin (Sigma T6793 1 to 500), rabbit anti-Rab 7 (Sigma R4779 1 to 100), and rabbit anti-Rab 11 (Sigma SAB2500848 1 to 500).

9.13 Secondary Reagents and Treatments

For mitochondrial imaging, MitoTracker Red CM-H2XRos (2.5µM, 30 min; Molecular Probes, #M7513) was used to label actively respiring mitochondria; the signal was similarly acquired using 543nm excitation. MitoTracker Red was applied as per manufacturer’s protocol.

(AF568 donkey anti-goat IgG (Molecular Probes, #A11057, 1:1000), AF555 donkey anti-rbt IgG (Molecular Probes, #A31572, 1:1000), AF568 donkey anti-mouse (Molecular Probes, #A10037, 1:1000), AF488 donkey anti-rabbit (Molecular Probes, #A21206, 1:1000) and or streptavidin-conjugated AF546 (Molecular Probes, #S-11225, 1:2000)) . Cover slips were then washed six times with cold PBS (three quick, three for 10 minutes each), incubated with Hoechst 33258 (1:20000) in dH2O for 7 minutes, post-fixed with 4%PFA (10min), rinsed with PBS and then washed twice for 10 minutes in dH2O. Cover slips were mounted onto slides with 1:1 glycerol/dH2O (plus thimerosal, 0.01% final concentration). All primary and secondary antibody mixtures were in 2.5% BSA/PBS.

To study the possible involvement of local APP processing in Aβ accumulation within lysosomes, a γ- secretase inhibitor DAPT (500 nM) was administrated.
9.14 Densitometry and Statistics

Using ImageJ (http://rsbweb.nih.gov/ij/download.html), densitometry of subject bands (minus background) were quantified from Western blot images. Subsequent analysis was conducted using Microsoft Excel and GraphPad Prism 4. Normalization of conditions was calculated as treatment conditions divided by baseline conditions/control treatments. Analysis of samples was conducted using Student’s t-test. Data are given as mean (SEM) and values with $p<0.05$ were considered significant.

9.15 Abbreviations

Aβ  β-amyloid;
AD Alzheimer’s disease
AICD APP intracellular domain
APOE apolipoprotein E
APP amyloid precursor protein
AVs autophagic vacuoles
BACE β-site APP cleaving enzyme
CNS central nervous system
CTF C-terminal fragment
CTFα C-terminal α fragment
CTFβ C-terminal β fragment
CysC cystatin C
C83 CTFα
C99 CTFβ
DAPI 4’ 6-diamidino-2-phenylindole
DS  Down syndrome
ECD extracellular domain
EE early endosomes
ELISA enzyme-linked immunosorbent assay
ET essential tremor
ER endoplasmic reticulum
FAD Familial AD
GFP green fluorescent protein
GSK-3β glycogen synthase kinase 3β
HEK 293 human embryonic kidney cells
IB Immunoblot
IHC immunocytochemistry
LAMP lysosome-associated membrane protein
LE late endosomes
Lingo-1 leucine-rich repeat and Ig domain-containing
MPRs mannose 6-phosphate receptors
MVB multivesicular body
M6P mannose-6-phosphate
NGF nerve growth factor
NgR Nogo-66 receptor
NFT neurofibrillary tangles
NT nontransfected
PBS phosphate-buffered saline
PD Parkinson’s disease
PS presentin
SAD sporadic AD
sAPP secreted APP
sAPPα soluble α-APP fragments
sAPPβ soluble β-APP fragments
shRNA short hairpin RNA
siRNA small interfering RNA.
SP senile plaques
TGN trans-Golgi network
TM transmembrane
Trks receptor tyrosine kinases
Wt wild type
YFP yellow fluorescent protein

10 Bibliography


105. Ginty, D., *personal communication of Mandai and Ginty*.


