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Confirmation and Characterization of a Functional APP/GAP-43 Protein Interaction

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Confirmation and Characterization of a Functional APP/GAP-43 Protein Interaction

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Table of Contents

List of Tables and Figures

Abstract

Alzheimer's disease (AD) is the leading form of dementia worldwide. It is associated with advanced age and involves a progressive cognitive decline. Pathologically, AD is characterized by two defining features: neurofibrillary tangles and amyloid plaques. The neurofibrillary tangles are composed of cleaved and hyperphosphorylated forms of the protein tau. The amyloid plaques are aggregates of the amyloid β (Aβ) peptide. Aβ is derived from a larger precursor protein, the amyloid precursor protein (APP), through sequential proteolytic cleavages. APP can first be enzymatically cleaved by either α- or β-secretase. If first cleaved by α-secretase, it is cleaved within its Aβ domain; therefore, the formation of Aβ is precluded. If first cleaved by β-secretase, subsequent cleavage by γ-secretase results in the production of differentially sized Aβ peptides of 40-42 amino acids that can accumulate to form the characteristic plaques of the disorder. Since genetic evidence indicates that Aβ accumulation is the primary causative agent of AD, much emphasis has been placed on elucidating factors that influence its formation. My thesis will discuss the impact of one such influential factor: the growth-associated protein-43 (GAP-43). I found that overexpression of GAP-43 and APP in HEK293 cells, a human embryonic kidney cell line, results in a profound alteration of APP processing as evidenced by a significant reduction in both α- and β-CTF fragments and that this effect is dependent on the palmitoylation of cysteines 3 and 4 of GAP-43. To our knowledge, this paper is the first known report to describe a functional interaction between APP and GAP-43. By shedding light on factors that play a crucial role in determining the processing fate of APP, we gain a better understanding of the pathology of the disease and consequently, bring ourselves closer in the search for an effective therapy for the treatment of AD.

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia worldwide, afflicting approximately 5.3 million people in the United States alone (alz.org, 2017). Due to the aging "baby boomer" population, this number is expected to climb drastically within the next 20 years. Currently, it is the sixth leading cause of death in the United States and is the only leading cause that cannot be prevented, cured, or slowed (alz.org, 2017).

AD is associated with advanced age, generally manifesting in the 6th decade and beyond, and involves a progressive cognitive decline. It is commonly divided into the early, mid, and late stages, or alternatively, into seven clinical stages (Sheehan, 2012). In the early stages, individuals with Alzheimer's begin to experience memory lapses (McKhann *et al.*, 1984). They will occasionally misplace commonly used items and will begin to misuse words. As the disease progresses, the symptoms become progressively more severe until the afflicted individuals can no longer carry out necessary life functions (McKhann *et al.*, 1984). They will no longer be able to control their own bodily functions and will come to completely depend on others. This downward spiral is not only devastating for the individual, but for society, as demonstrated by the $200 billion cost associated with the care of AD patients in both the clinical and private settings in 2014 alone (alz.org, 2014).

Although AD symptomatology has been well characterized for quite some time, it was not until 1907 that the disease was formally characterized by Alois Alzheimer, a Bavarian psychiatrist (Stelzmann *et al.*, 1995). In 1907, Alois Alzheimer performed an autopsy on Auguste Deter, a former patient of his that had experienced what today would be described as AD-like symptoms towards the end of her life. During the autopsy, he noted two hallmark features in her brain: extracellular plaques, which he characterized as "minute miliary foci

caused by deposition of a particular substance in the cortex," and intracellular neurofibrillary tangles (Stelzmann *et al.,* 1995). It was not until the early 1980s that Glenner and Wong determined that the extracellular plaques were aggregates of the beta amyloid peptide (Aβ), which has since been determined to be derived from a series of sequential proteolytic cleavages of a larger precursor protein called the β-amyloid precursor protein (APP) (Glenner & Wong, 1984; Kang *et al.*, 1987). It was not until the mid-1980s that Grundke-Iqbal discovered that the intracellular neurofribrillary tangles were hyperphosphorylated and cleaved forms of the protein tau (Grundke-Iqbal *et al.*, 1986). These seminal findings remain the foundation upon which investigations into the pathological underpinnings of this disease are built.

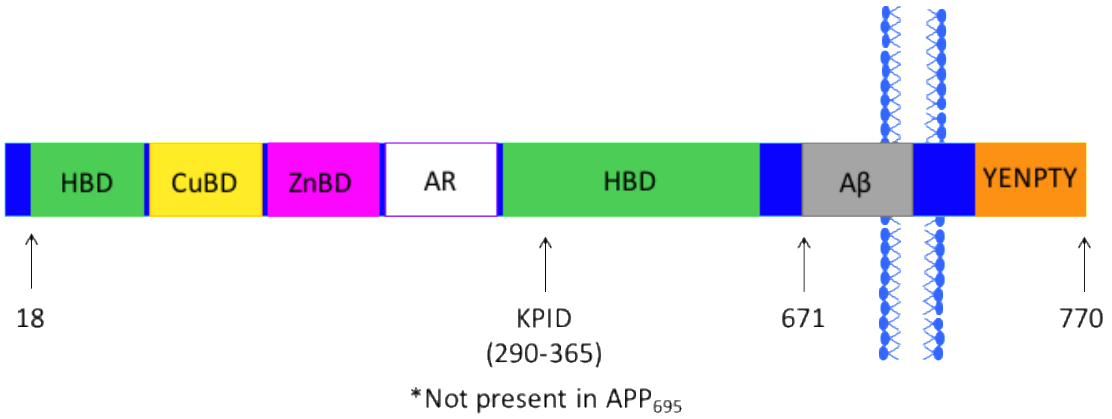
Despite the fact that AD has been symptomatically and pathologically described for over 100 years, there is still no cure nor effective treatments for this disorder. Due to its high prevalence and destructive nature, an effective treatment option is of the utmost importance. Because genetic evidence clearly indicates that the accumulation of Aβ is the primary pathological manifestation in AD, most current treatment options under investigation target the formation and/or the accumulation of Aβ. Since the formation of Aβ depends on the processing fate of its larger precursor protein, APP, understanding factors that influence its processing destiny is crucial. Thus, the primary goal of this study was to confirm a novel protein-protein interaction between APP and the growth-associated protein-43 (GAP-43) as well as to investigate any potential influence of this novel interaction on the processing fate of APP with relevance to AD pathology and therapy.

Literature Review

APP structure

APP belongs to a family of type I transmembrane proteins, which includes the APP like proteins 1 and 2 (APLP1 and APLP2 respectively) (Wasco *et al.*, 1992; Wasco *et al.*, 1993). Members of this family possess a long extracellular domain of approximately 600 amino acids, a transmembrane helix, and a short intracellular domain of approximately 50 amino acids (Wasco *et al.*, 1992; Wasco *et al.*, 1993). While APP shares a high degree of sequence identity with APLP1 and APLP2 and thus, many structural similarities, only APP possesses the sequence that encodes the Aβ domain, while APLP1 and APLP2 are divergent in this region (Wasco *et al.*, 1992; Wasco *et al.*, 1993). In addition to an Aβ domain, APP also possesses several distinct domains within its extracellular region, including: a heparin binding/growth factor like domain, a copper binding domain, a zinc binding domain, an acidic region, and a second heparin binding domain (Lazarov & Demars, 2012) (**Figure 1**). Additionally, APP possesses an intracellular tail that contains sites for adapter protein binding as well as a YENPTY domain (Lazarov & Demars, 2012). While the extracellular domains primarily influence dimerization, stability, and processing, the intracellular YENPTY domain is required for the proper endocytosis and recycling of APP (Small *et al.*, 1994; King *et al.*, 2003; Spoerri *et al.*, 2012; Hoefgen *et al.*, 2014).

Figure 1: APP Structure



Adapted from Lazarov and Demars, 2012

Figure 1 is a schematic representation of the structure and domains of APP. APP is a type I transmembrane protein with several extracellular and one intracellular domain. The extracellular domains include: heparin binding domain/growth factor like domain (HBD), copper binding domain (CuBD), zinc binding domain (ZnBD), kunitz-type protease inhibitor domain (KPID), a second HBD, and an Aβ domain. The intracellular domain consists of the YENPTY domain.

It is important to note that through alternative splicing, eight APP isoforms exist; however, only 3 are commonly expressed: APP_{695}, APP_{751}, and APP_{770}, which contain 695, 751, and 770 amino acids respectively (Kang & Müller-Hill, 1989). Although these 3 isoforms are very similar, some differences do exist. Notably, the APP_{695} isoform is predominantly expressed in neurons, while the other 2 isoforms are predominantly expressed in non-neuronal tissues (Rohan de Silva *et al.*, 1997). Additionally, the longer isoforms of APP, APP_{751} and APP_{770}, possess a kunitz-type protease inhibitor domain in the extracellular region, while the APP_{695} isoform does not (Rohan de Silva *et al.*, 1997). Despite these differences, all APP isoforms are modified and metabolized in a similar fashion.

APP post-translational modifications

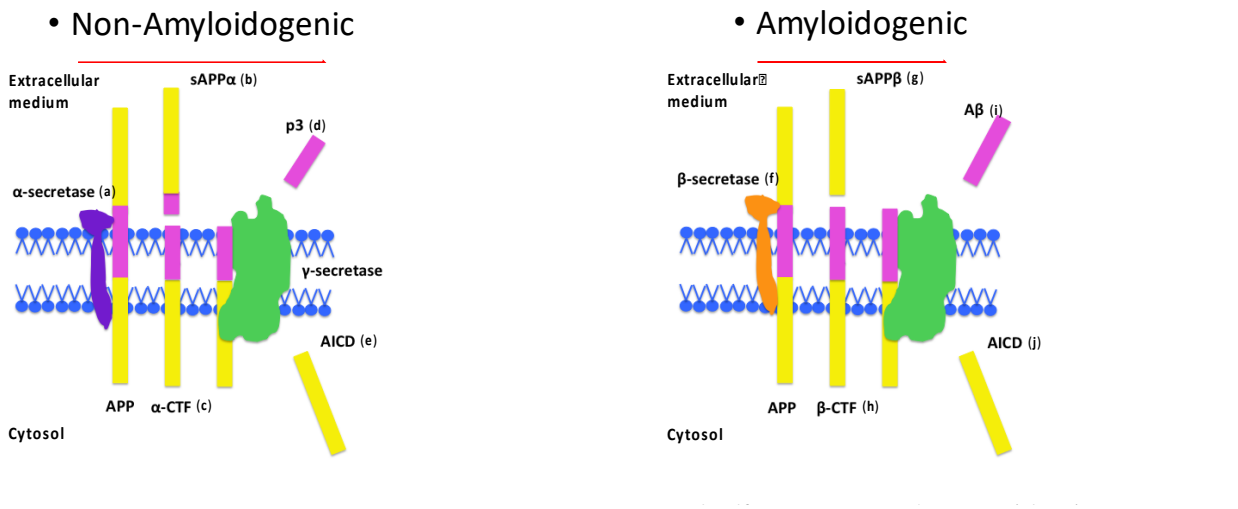
APP contains two potential N-linked glycosylation sites (Asn_{467} and Asn_{496}) as well as a single O-linked glycosylation site (Ser_{124}) (McFarlane *et al.*, 1999). The McFarlane group demonstrated that N-linked glycosylation is required for the proper trafficking of APP to the cell surface by treating cells stably expressing the APP_{695} isoform with mannosidase inhibitors (McFarlane *et al.*, 1999). Compared to the untreated control group, the mannosidase inhibitor-treated group exhibited a higher degree of APP retention in the Golgi as indicated by enhanced perinuclear staining of the APP protein; thus, they concluded that N-linked glycosylation is required for the proper trafficking of APP (McFarlane *et al.*, 1999). Another group independently demonstrated that O-linked glycosylation serves a similar role. Using cells overexpressing an O-linked glycosylation deficient APP mutant in which Ser_{124} was mutated to Cys, Tomita et al. noted a retention of APP in the endoplasmic reticulum by confocal microscropy as well as a decrease in overall APP processing via immunoblot analysis (Tomita *et al.*, 1998). Thus, they concluded that O-linked glycosylation is necessary for the targeting of APP to the plasma membrane as well as for the processing of APP (Tomita *et al.*, 1998).

In addition to N- and O-linked glycosylation sites, APP possesses two sulfation sites (Tyr_{217} and Tyr_{262}) and multiple phosphorylation sites (Ser_{198}, Ser_{206}, Ser_{655}, and Ser_{675}) (Walter & Haass, 2000). Both sulfation and phosphorylation are known to impact trafficking and protein-protein interactions; however, the precise roles of these post-translational modifications at each of the aforementioned sites remain somewhat ambiguous. Nevertheless, it is well established that these critical post-translational modifications influence the processing fate of APP (Tomita *et al.*, 1998; Walter & Haass, 2000; Tamayev *et al.*, 2009).

APP processing and its metabolites

APP_{695} can be processed through one of two mutually exclusive pathways: non-amyloidogenic or amyloidogenic (**Figure 2**), so named because the latter pathway leads to the formation of extracellular amyloid deposits composed of the Aβ peptide, while the former pathway generates different metabolites that do not have the propensity to aggregate (Selkoe, 2011). Thus, since the amyloidogenic pathway is associated with amyloid accumulation, it is also associated with disease onset, while the non-amyloidogenic pathway, which is not associated with amyloid accumulation, is not (Selkoe, 2011). It is important to note that both pathways occur in all tissues that express APP.

Figure 2: APP Processing



Adapted from *Cheng et al., 2007*

Figure 2 is a schematic representation of the processing of APP. APP can be processed through one of two mutually exclusive pathways: non-amyloidogenic or amyloidogenic. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase (**2a**) within its Aβ domain. This results in the release of a soluble fragment, sAPPα (**2b**), that is released extracellularly and a carboxyl-terminal fragment, α-CTF (**2c**), that remains in the membrane. α-CTF is subsequently cleaved by γ-secretase to produce a soluble p3 fragment (**2d**) which is released into the extracellular medium and an AICD fragment (**2e**) that is released into the cytosol. In the amyloidogenic pathway, APP is cleaved first by β-secretase (**2f**) which results in the production of sAPPβ (**2g**) that is released into the extracellular medium and a carboxyl-terminal fragment, β-CTF (**2h**), that remains in the membrane. β-CTF is subsequently cleaved by γ-secretase to produce the neurotoxic Aβ peptide (**2i**) that is released extracellularly and the AICD fragment (**2j**) that is released into the cytosol.

In the non-amyloidogenic pathway, APP is first cleaved by α-secretase, a zinc metalloproteinase activity that is mediated by members of the ADAM (A Disintegrin and Metalloprotease) family of proteins (a family of type I transmembrane enzymes), at residue 612 (APP_{695} numbering will be used throughout this document) (Roberts *et al.*, 1994) (**Figure 2a**). Of the ADAM family of proteins, ADAM10 appears to be the primary effector of α-secretase activity (Lammich *et al.*, 1999). Cleavage of APP by α-secretase precludes the formation of Aβ, since α-secretase cleaves APP within the Aβ domain (Esch *et al.*, 1990). Cleavage of APP by α-secretase results in the formation of a soluble APP fragment, sAPPα, that is released into the extracellular medium (**Figure 2b**) and a carboxyl-terminal fragment, α-CTF, that remains in the

membrane (Esch *et al.*, 1990; Roberts *et al.*, 1994; Lammich *et al.*, 1999) (**Figure 2c**). The α-CTF fragment is subsequently cleaved within its transmembrane domain by γ-secretase, a complex consisting of four components: presenilin (PS, PS1, or PS2), nicastrin (NCT), anterior pharynx-defective-1 (APH-1), and presenilin enhancer 2 (PEN-2) (Kimberly *et al.*, 2003). Presenilin is the catalytic component of the complex, while the other proteins serve to activate the pro-enzyme and regulate activity (Wolfe *et al.*, 1999). Cleavage of α-CTF at residues 636-638 by γ-secretase results in the formation of two products: an extracellular fragment called p3 (**Figure 2d**) and an intracellular fragment called the APP intracellular domain (AICD) (**Figure 2e**).

The products of the non-amyloidogenic pathway are soluble and nontoxic. Although still under debate, some studies have found evidence that the products of the non-amyloidogenic pathway provide neurotrophic benefits. For instance, sAPPα has been implicated in CNS development, neuronal plasticity, and survival (Zhang *et al.*, 2011). In support of its role in neuronal plasticity and survival, Thornton et al. found that intracerebroventricular administration of sAPPα shortly after traumatic brain injury in rats results in improved motor outcome as well as in a reduction in the number of apoptotic axons as compared to the vehicle-treated controls as determined using the rotarod test and immunohistochemical analysis using an antibody against caspase 3 (a marker of apoptosis) respectively (Thornton *et al.*, 2006). Additionally, AICD, a fragment that results from both non-amyloidogenic and amyloidogenic processing, has been implicated in regulating the transcription of several genes (Kinoshita *et al.*, 2002; von Rotz *et al.*, 2004). In support of this function, Zhou et al. found that AICD indirectly regulates gene transcription by inhibiting Wnt signaling, a pathway that is involved in cellular proliferation, cell polarity, and cell fate determinations (Zhou *et al.*, 2012). They found that AICD interacts with

glycogen synthase kinase-3 β (GSK3β) and that this interaction facilitates the formation of the Axin-GSK3β complex and consequently, the phosphorylation and degradation of β-catenin (Zhou *et al.*, 2012). This in turn decreases the transcription of several genes including c-Myc, with the end result being reduced proliferation and enhanced differentiation (Zhou *et al.*, 2012). Additionally, another group found that AICD effectively regulates its own production as the APP gene was identified as one of the transcriptional targets of AICD (von Rotz *et al.*, 2004). Although both sAPPα and AICD are believed to possess neurotrophic properties, the fragment p3 has not been associated with any physiological role as it is rapidly degraded (Zhang *et al.*, 2011). Thus, although the precise roles for these metabolites are still somewhat ambiguous, it is clear that the manner in which APP is metabolized in the non-amyloidogenic pathway results in products that are not associated with the disease state.

In the alternative pathway, APP is first cleaved by β-secretase, a membrane-bound aspartyl protease identified to be BACE1 (β-site APP Cleaving Enzyme 1) (**Figure 2f**) (Vassar, 1999). Cleavage of APP at residue 596 by β-secretase results in the release of a truncated, soluble APP fragment, sAPPβ, that is released extracellularly (**Figure 2g**) and a carboxyl-terminal fragment, β-CTF, that remains in the membrane (**Figure 2h**) (Seubert *et al.*, 1993; Sinha & Lieberburg, 1999). β-CTF is subsequently cleaved by γ-secretase at residues 636-638, which releases an extracellular fragment, Aβ (**Figure 2i**), and an intracellular fragment, AICD (**Figure 2j**) (Seubert *et al.*, 1993; Sinha & Lieberburg, 1999). Since cleavage of β-CTF by γ-secretase can occur at a variety of sites, a spectrum of differentially sized Aβ fragments of approximately 4 kilodaltons (kDa) results, the majority species being $A\beta_{40}$ and the more amyloidogenic being $A\beta_{42}$ (Zhang *et al.*, 2011).

The products of the amyloidogenic pathway are not all soluble and some are even toxic. Evidence supports sAPPβ as a ligand for death receptor 6 and as a regulator of neuronal cell death (Zhang *et al.*, 2011). In support of this, Nikolaev et al. discovered that trophic factor deprivation results in the release of sAPPβ and its binding to death receptor 6 on neuronal cells, which in turn activates caspase 6-dependent apoptosis (Nikolaev *et al.*, 2009). In addition to sAPPβ, the amyloidogenic pathway also produces the neurotoxic Aβ peptide. The toxicity of the Aβ peptide is well established and is discussed in more detail in the following section.

The role of β-amyloid in Alzheimer's pathology

As briefly mentioned above, the neurotoxic properties of Aβ are well established. These toxic properties are dependent upon the oligomerization and accumulation of the peptide into plaques. It is important to note that the Aβ peptide can, once metabolically released from APP, be cleared through physiological clearance mechanisms such as breakdown by insulin degrading enzyme and removal by carrier proteins like transthyretin and apolipoprotein E (Blancas-Mejía & Ramirez-Alvarado, 2013; Baranello *et al.*, 2015). However, if not cleared by one of these mechanisms, Aβ accumulates and forms plaques. Aβ plaque formation in the brain is an obligatory pathological feature of AD. Three primary lines of evidence support this: 1) In familial AD, the gene encoding tau has not been shown to be mutated; rather, tau mutations result in a distinct form of dementia known as frontotemporal dementia that is separate from AD (Selkoe, 2011; Mackenzie & Neumann, 2016). 2) Numerous incidents have been reported in which individuals with late onset AD present with few neurofibrillary tangles (Selkoe, 2011). 3) All familial forms of AD have been shown to stem from mutations in genes that result in the increased production and/or accumulation of Aβ (Selkoe, 2011). This includes APP gene

duplication events and mutations in the APP gene in regions encoding the Aβ peptide as well as in the enzymes responsible for generating Aβ. In addition to these three lines of evidence, a protective mutation has recently been identified in the gene encoding APP near the N-terminal portion of the Aβ region (Jonsson *et al.*, 2012). This mutation has been found to reduce the amount of Aβ formed and is found at a much higher prevalence in the elderly without AD; thus, further supporting the causative role of Aβ in AD pathology (Jonsson *et al.*, 2012; Maloney *et al.*, 2014).

Once formed, the aggregation of Aβ is a crucial event in the toxic gain-of-function change that occurs in the disease state (Lorenzo & Yankner, 1994; Larson & Lesné, 2012). In the aggregated state, Aβ changes from a soluble conformation to an amyloid conformation (Lorenzo & Yankner, 1994). Although the focus of this study is on the production of Aβ from the APP protein, it is important to note that amyloid proteins, defined as having a high beta-sheet content and red-green birefringence when stained with the histological dye Congo-red, can be produced by several different proteins, such as transthyretin, often as a consequence of a misfolding event (Lorenzo & Yankner, 1994). Since amyloid proteins are insoluble in nature and highly resistant to proteolytic clearance, they are known to aggregate and are associated with toxicity in several disease states collectively known as the amyloidoses (Blancas-Mejía & Ramirez-Alvarado, 2013). Furthermore, these diseases are not restricted to the brain; rather, they can impact several tissues such as the heart, the liver, and muscles (Blancas-Mejía & Ramirez-Alvarado, 2013). Within the brain, amyloid is thought to disrupt neuronal function by toxic effects at the synapse that cause synaptic loss and neuronal cell death (Selkoe, 2011). The toxic nature of amyloid proteins further supports the causative role of Aβ in Alzheimer's disease as

does the fact that all genetic links to AD result in the increased production and/or accumulation of the Aβ peptide.

Genetic links to AD

As briefly mentioned, several different mutations have been identified in APP, as well as in proteins that regulate the processing of APP, that result in increased Aβ production and/or accumulation (Selkoe, 2011; Lanoiselée *et al.*, 2017). As of this writing, 52 APP, 238 PSEN1, and 45 PSEN2 mutations have been linked to autosomal dominant forms of AD, while carriers of the Apolipoprotein E (APOE) ε4 allele are associated with sporadic forms of AD (Campion *et al.*, 1999; Liu *et al.*, 2013; Lanoiselée *et al.*, 2017).

Mutations in the APP protein have been identified around the β- and γ-secretase cleavage sites as well as within the Aβ domain. More specifically, two mutations have been identified around the β-secretase cleavage site (Mullan *et al.*, 1992). Located at positions 670 and 671, these two mutations occur simultaneously in what is referred to as the Swedish mutant of APP (APP_{SWE}) and result in an increase in the overall production of all Aβ species (Mullan *et al.*, 1992). While mutations that cluster around the β-secretase cleavage site result in enhanced Aβ production, those that cluster around the γ-secretase cleavage site and those within the Aβ domain itself increase the production of $Aβ_{42,}$ the more amyloidogenic species, and consequently, enhance Aβ aggregation (De Jonghe, 2001; Selkoe, 2011). Additionally, overexpression of wild type APP as the result of gene duplication, such as with trisomy 21, results in the increased production of all Aβ species, similar to the impact β-secretase cleavage site mutations have on Aβ production (Masters *et al.*, 1985; Citron *et al.*, 1992; Mullan *et al.*, 1992; Selkoe, 2011; Potter *et al.*, 2016). Thus, autosomal dominant APP mutations result in the

enhanced formation and accumulation of Aβ plaques, earlier disease onset, and a more rapid progression of the disease.

Mutations in PSEN1 and PSEN2 are also implicated in autosomal dominant forms of AD (Levy-Lahad *et al.*, 1995; Campion *et al.*, 1999; Tanzi & Bertram, 2005b; Lanoiselée *et al.*, 2017). All the identified mutations in PSEN1 and PSEN2 have been found to increase the production of $A\beta_{42}$, which ultimately increases the production and accumulation of Aβ plaques (Levy-Lahad *et al.*, 1995; Rogaeva E, 2001; Tanzi & Bertram, 2005a). As with the APP mutations, these autosomal dominant mutations result in earlier disease onset and a more rapid progression of the disease.

Although not causative of familial AD, individuals carrying the APOE ε4 allele are at a much higher risk (up to 5 times greater) for developing AD as compared to those carrying other APOE variants such as APOE ε2 (Michaelson, 2014). It is well established that APOE ε4 carriers are not able to clear Aβ as efficiently as non-carriers (Schmechel *et al.*, 1993; Michaelson, 2014). It is hypothesized that the ε4 variant, which normally functions to clear cholesterol from the body, is not as effective at clearing lipids, which promotes the aggregation of Aβ oligomers and hence, plaque formation (Schmechel *et al.*, 1993; Selkoe, 2011; Liu *et al.*, 2013; Michaelson, 2014).

It is important to note that all of the aforementioned mutations result in the enhanced production and/or accumulation of Aβ. Since the generation of Aβ is the primary causative agent of AD, and since the formation of Aβ depends upon the processing fate of APP, identifying the subcellular locations in which APP undergoes amyloidogenic versus non-amyloidogenic processing is critical.

APP subcellular localization and trafficking

The processing fate of APP is determined to a large extent by its subcellular location, which in turn is dependent upon its synthesis and trafficking. After synthesis in the rough endoplasmic reticulum, APP is modified in the Golgi complex and then transported down the axon via fast anterograde transport to the cell membrane (**Figure 3**). At the plasma membrane, α-secretase is primarily responsible for the cleavage of APP (Lammich *et al.*, 1999). However, APP can be reinternalized into the endo-lysosomal pathway before cleavage by α-secretase (Koo & Squazzo, 1994). In endosomes and lysosomes, APP is primarily cleaved by β-secretase (Koo & Squazzo, 1994). Alternatively, once internalized from the plasma membrane, APP can be returned to the *trans*-Golgi network (TGN) via retrograde transport where α- and β-secretase compete for the cleavage of APP and where the majority of APP has been found to reside (Choy *et al.*, 2012). While it is now clear that the processing fate of APP is largely dependent upon its subcellular location and consequently its trafficking pattern, the factors that influence the trafficking patterns and subcellular distribution of APP still remain a mystery. One possible factor that may potentially influence the internal trafficking of APP and consequently its processing fate is the focus of this study: growth-associated protein-43 (GAP-43).

Figure 3: APP Trafficking

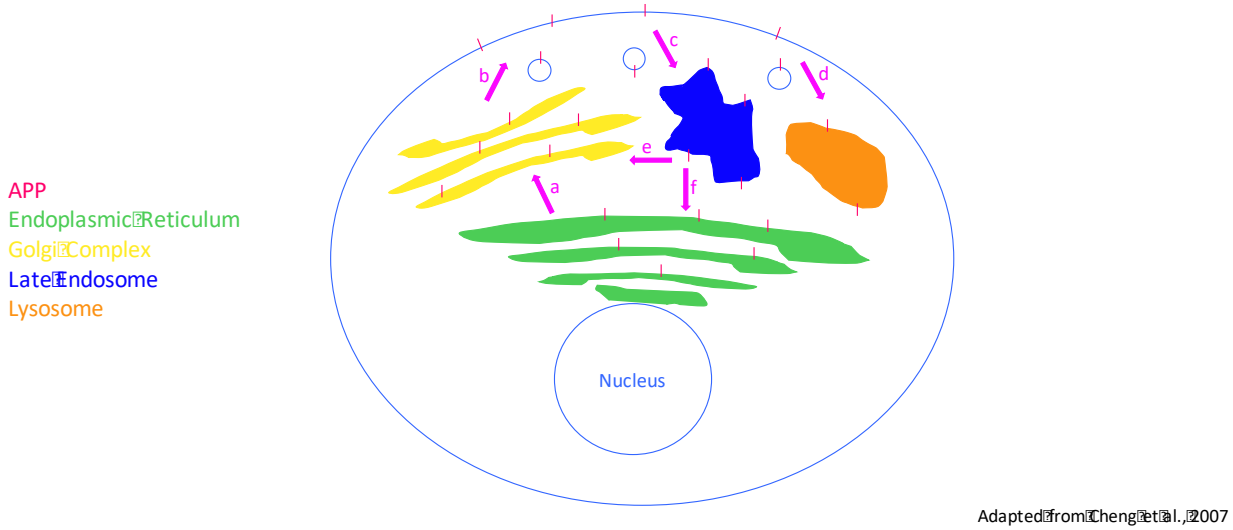


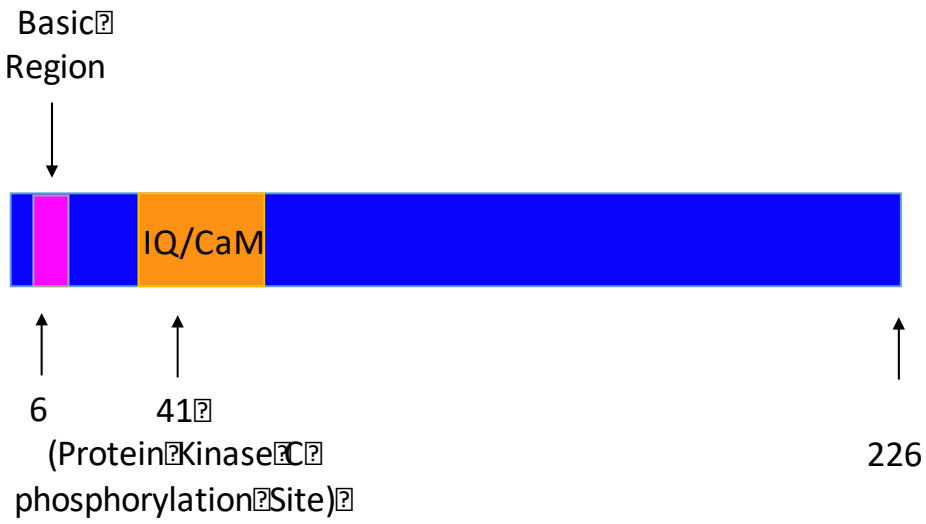
Figure 3 depicts the subcellular trafficking of APP. APP is synthesized in the rough endoplasmic reticulum (**3a**) and then transported down the axon via fast anterograde transport to the Golgi complex (**3b**) for processing before being inserted in the plasma membrane. If not processed by α-secretase at the plasma membrane, APP can be reinternalized into endosomes (**3c**) or degraded by lysosomes (**3d**). In endosomes, if not processed by β-secretase, it can be trafficked to the Golgi complex (**3e**) or shuttled to the rough endoplasmic reticulum (**3f**) via retrograde transport.

GAP-43 structure

GAP-43, which is also known as neuromodulin, B-50, P-57, F1, and pp46, is encoded by three exons located on human chromosome 3 (Grabczyk *et al.*, 1990; Holahan, 2017). The first exon codes for the 5' untranslated region (UTR) and the N-terminal 10 amino acids (Grabczyk *et al.*, 1990). The second exon codes for the majority of the coding region and the third codes for the remainder of the coding region and the 3' UTR (Grabczyk *et al.*, 1990). The end result is a 23.6 kDa, 226 amino acid protein (Rosenthal *et al.*, 1987). Although the calculated molecular mass of GAP-43 is 23.6 kDa, it typically migrates at 40-60 kDa when assayed by protein electrophoresis due to its highly hydrophilic nature (Rosenthal *et al.*, 1987). The high prevalence of charged amino acids also accounts for its primarily linear tertiary structure (**Figure 4**); however, a complete crystal structure has not yet been achieved (Hayashi *et al.*, 1997). Although

GAP-43 is a highly hydrophilic protein, it does contain a small hydrophobic region in its N-terminal domain, which plays an integral role in membrane attachment (Skene JH, 1989). In addition to a membrane binding domain, GAP-43 also possesses an effector or IQ domain that extends from amino acids 38-56 and which contains the protein kinase C phosphorylation site at amino acid 41 (Akers & Routtenberg, 1985; Alexander *et al.*, 1988). Phosphorylation at this site is implicated in actin polymerization, long-term potentiation, and neurotransmitter release (Heemskerk *et al.*, 1990; Benowitz & Routtenberg, 1997; He *et al.*, 1997). Additionally, GAP-43 contains a calmodulin (CaM) binding domain, which plays a role in neurotransmitter release as well as several phosphorylation sites, which play a role in synaptic plasticity and cellular morphology (Biewenga, 1996; Gamby *et al.*, 1996; Holahan, 2017). The various domains of GAP-43 allow for a variety of complex post-translational modifications to occur such as phosphorylation, CaM binding, and palmitoylation.

Figure 4: GAP-43 Structure



Adapted from Cheng et al., 2007

Figure 4 is a schematic representation of the primarily linear structure of GAP-43. GAP-43 contains a small basic region near its N-terminus as well as an IQ/calmodulin (CaM) binding domain. The IQ/CaM binding domain also possesses a protein kinase C phosphorylation site at amino acid 41.

GAP-43 palmitoylation

GAP-43 can be dually acylated by palmitate and/or sterate causing association with the ER-Golgi intermediate compartment, Golgi apparatus, and the plasma membrane (Skene JH, 1989). Although palmitoylation/acylation is required for the initial targeting of GAP-43 to the plasma membrane, Liang et al. observed that only 35% of GAP-43 at steady state is fatty acylated, while nearly 100% is membrane-bound (Liang *et al.*, 2002). Therefore, they concluded that palmitoylation is not necessary for GAP-43 to maintain attachment to the plasma membrane (Liang *et al.*, 2002). Using mass spectrometric methods, Liang and colleagues showed that once associated with the plasma membrane, the basic residues at the N-terminus of GAP-43 are

critical for maintenance of membrane association (Liang *et al.*, 2002). Although not required for maintaining GAP-43 at the plasma membrane, palmitoylation is required for the initial targeting of GAP-43 to the membrane and transcytosis to the axon (El-Husseini *et al.*, 2001; Liang *et al.*, 2002). Liu, Fisher, and Storm confirmed this finding as they demonstrated that GAP-43 palmitoylation-deficient mutants are primarily localized in the cytosol, while their palmitoylated counterparts are membrane-bound (Liu *et al.*, 1994). The capacity of GAP-43 to undergo palmitoylation as well as its other aforementioned post-translational modifications allow it to perform a myriad of physiological roles.

Physiological roles of GAP-43

Several studies have verified the role of GAP-43 during neural development and regeneration, including those conducted by Maier et al. In 1999, Maier and colleagues noted that GAP-43 double knock out mice demonstrated abnormal neural development and lethality shortly after birth; thus, they concluded that GAP-43 is required for neural development (Maier *et al.*, 1999). Due to the pivotal role of GAP-43 during neural development, it is not surprising that GAP-43 expression levels peak during development and decline drastically after birth except in associative areas of the brain and the hippocampus where GAP-43 levels remain relatively high (Benowitz & Routtenberg, 1997). Due to this expression pattern, GAP-43 has also been implicated in learning processes (Benowitz & Routtenberg, 1997). By using a transgenic mouse line overexpressing either wild type GAP-43, a non-phosphorylatable form of GAP-43, or a constitutively phosphorylated form, Routtenburg and others found that overexpression of the constitutively phosphorylatable form results in enhanced long-term potentiation and significantly fewer errors in a Win-Shift delay behavioral assay, thus indicating that phosphorylation of GAP-

43 is required for learning (Routtenberg *et al.*, 2000). In addition to playing a vital role in neural development and learning, GAP-43, by mediating actin dynamics, is also responsible for growth cone formation, filopodia formation, neurite outgrowth, and cell shape (Aigner & Caroni, 1993; Nguyen *et al.*, 2009; Grasselli *et al.*, 2011). Using cultured dorsal root ganglia neurons depleted of GAP-43, Aigner and Caroni demonstrated the necessity of this protein in growth cone formation as well as axon guidance in response to external growth cues (Aigner & Caroni, 1993). Additionally, Nguyen, He, and Meiri found that overexpression of a constitutively phosphorylated form of GAP-43 results in F-actin polymerization and growth cone formation, whereas overexpression of an unphosphorylatable form of GAP-43 in the same cell line induces neurotubulin assembly and neurite outgrowth (Nguyen *et al.*, 2009). In further support of this, Grasselli and others determined that GAP-43 maintains climbing fibre structure; is necessary for axonal sprouting and regenerative potential; and mediates axon length, the number of branching fibres, and the number and density of varicosities (Grasselli *et al.*, 2011). GAP-43 has also been implicated in signal transduction systems. Caprini and colleagues demonstrated that GAP-43 stimulates the release of Ca^{+2} from the endoplasmic reticulum in response to hypotonicity and thus, plays an important role in signal transduction as well as in neurotransmitter release (Caprini *et al.*, 2003). GAP-43 has also been implicated in endocytosis as it has been shown to interact with Rab5 to mediate endocytosis and vesicle recycling (Neve *et al.*, 1998). Although it is clear that GAP-43 is capable of performing a multitude of physiological roles, the expression profile of GAP-43 restricts its functions in both a time- and space-dependent manner.

Expression of GAP-43

As previously mentioned, GAP-43 is ubiquitously expressed throughout the brain during development, but is restricted to certain areas of the adult brain (Benowitz & Routtenberg, 1997). In the adult brain, GAP-43 is predominantly expressed in neurons of the hippocampus, amygdala, cortex, and midbrain, all of which are impacted in AD (Rosenthal *et al.*, 1987). This expression profile is controlled by a variety of extracellular and intracellular factors (Biewenga, 1996).

The extracellular factors that control the expression pattern of GAP-43 include sex hormones and brain injury/trauma (Biewenga, 1996). The expression profile of GAP-43 is differentially regulated by sex hormones in the developing brain, certain areas being stimulated by androgen (bed nuclei of the stria terminalis and the medial preoptic nuclei) and others by estrogen (cortex and ventromedial hypothalamus) (Shughrue & Dorsa, 1994). On the other hand, brain injury/trauma consistently results in the up-regulation of GAP-43 mRNA (Doster *et al.*, 1991; Chong *et al.*, 1992). The intracellular factors that regulate its expression include protein kinase C (PKC) and cyclic AMP (cAMP), both of which enhance GAP-43 mRNA expression (Biewenga, 1996).

It is evident that GAP-43 expression is tightly regulated by a variety of factors. However, this expression pattern is altered in the brains of individuals with AD. Of note, de la Monte, Ng, and Hsu noted that postmortem individuals with AD exhibited a decrease in expression of GAP-43 in neurons and an increase in expression of GAP-43 in white matter in the cerebral cortex as compared to aged controls (de la Monte *et al.*, 1995). What causes this change in expression and its physiological relevance is still unclear. Despite this uncertainty, it is known that once GAP-43 is expressed, it undergoes a variety of post-translational modifications,

including palmitoylation by palmitate and/or sterate at Cys_3 and Cys_4, which plays an essential role in the trafficking of GAP-43 (Skene JH, 1989; Biewenga, 1996).

GAP-43 transport

Although most membrane-bound proteins are translated in the rough ER, GAP-43 mRNA is translated by free ribosomes in the cytosol (Denny, 2006). Shortly after translation, it associates with membranes of the early secretory pathway as the result of palmitoylation (Skene JH, 1989). As GAP-43 traverses this membranous network, it undergoes post-translational modifications (Biewenga, 1996). Once fully mature, GAP-43 is targeted to the growth cones of neuronal cells in vesicles via fast, anterograde transport (Skene & Willard, 1981b; a; Skene & Willard, 1981c). Alternately, GAP-43 mRNA can be transported directly to the growth cones of neurons via unknown means (Smith *et al.*, 2004). Once at the growth cone, GAP-43 mRNA is stabilized by the protein HuD, an RNA-binding protein, and translated by free-floating ribosomes (Smith *et al.*, 2004). The significance of this form of translation is unclear, but it is reasonable to hypothesize that it may play a role in rapid changes in growth cone formation, filopodial formation, and the modulation of actin dynamics (Smith *et al.*, 2004).

Statement of Proposed Research

Since the initial discovery of the pathological hallmarks of the disease by Alois Azheimer nearly 110 years ago, the mechanism of disease onset and progression has become clearer (Stelzmann *et al.*, 1995; Sanabria-Castro *et al.*, 2017; Wang *et al.*, 2017). Evidence strongly implicates Aβ as the primary causative agent (Selkoe, 2011; Jonsson *et al.*, 2012; Maloney *et al.*, 2014; Mackenzie & Neumann, 2016). Additionally, it is well established that Aβ is derived from the amyloidogenic processing of a larger precursor protein called APP (**Figure 2**). However, it is also known that there is an alternative pathway by which APP can be processed, the non-amyloidogenic pathway (**Figure 2**). What we don't know, and what is crucial to determine, are the factors that influence the processing destiny of APP. Several labs have shown that the processing fate of APP is influenced by its interacting partners (Russo *et al.*, 2005; Norstrom *et al.*, 2010; Hao *et al.*, 2011). Therefore, a great need exists to determine the *in vivo* interactome of APP. If we can determine all the players in AD and their individual roles, we can gain a better understanding of the pathology of the disease which is essential for the design of an effective treatment option.

In order to address this pertinent question, Norstrom and colleagues assessed the native APP interactome in brain using mice generated to express human APP_{695} fused to a C-terminal affinity tag (Norstrom *et al.*, 2010). The use of a C-terminal affinity tag circumvented the need for antibodies targeted toward the C-terminus of APP. This is significant as it allowed for the identification of overexpressed APP without interfering with the binding sites of potential APP-interacting proteins. Norstrom and colleagues purified the complexes using rabbit IgG coated magnetic beads and subsequently performed mass spectrometry on the eluates in order to identify novel interacting partners (Norstrom *et al.*, 2010). The validity of their experimental

approach was confirmed by the presence of several well-known APP-interacting proteins in the purified complexes, including Fe65 and APLP1 and 2 (Norstrom *et al.*, 2010). Additionally, several novel proteins were identified as potential APP-interacting proteins (Norstrom *et al.*, 2010).

Norstrom et al. went on to confirm and characterize a novel APP-NEEP21 interaction (Norstrom *et al.*, 2010). However, the validity of the other potential APP-interacting proteins remained to be determined. Of note, GAP-43 stood out as another prime, investigative target. Although GAP-43 could have co-purified with affinity tagged APP_{695} as the result of a non-specific interaction, several other lines of evidence support the idea of a potential APP-GAP-43 interaction.

Further supporting the hypothesis of an APP-GAP-43 interaction, is the fact that GAP-43 possesses several of the same physiological roles as APP (**Table 1**). Proteins possessing similar functions often overlap spatially as well, thus, increasing the likelihood of an *in vivo* interaction. This is indeed the case for APP and GAP-43 as both proteins are known to localize to several of the same subcellular locations (**Figure 5**). More backing for this rationale comes from the collective findings of several independent laboratories that implicate a potential role of GAP-43 in AD pathogenesis. For instance, Masliah and colleagues observed that APP is colocalized with GAP-43 in 57.5 +/- 11% of the aberrant sprouting neurites associated with AD pathology and 3.3 +/- 0.4% of presynaptic terminals (Masliah *et al.*, 1992). Additionally, in a separate study conducted by the same laboratory, they found that in AD brains, GAP-43 protein expression is significantly reduced in the frontal cortex and the hippocampus, areas of the brain profoundly impacted in AD, as compared to non-demented, aged controls (Masliah *et al.*, 2001). Other groups found similar results in which a reduction in GAP-43 protein and/or mRNA levels were

observed in AD brains relative to non-demented, aged controls (Coleman *et al.*, 1992; Masliah *et al.*, 1992; de la Monte *et al.*, 1995; Cheetham *et al.*, 1996; Masliah *et al.*, 2001). Several laboratories have also noted a change in the distribution of GAP-43 protein expression between AD and age-matched control brains. For instance, de la Monte, Ng, and Hsu noted that postmortem individuals with AD exhibited a decrease in expression of GAP-43 in neurons and an increase in expression of GAP-43 in white matter as compared to aged controls (de la Monte *et al.*, 1995). Although this data is correlative in nature, it indicates that GAP-43 may play a pathophysiological role in AD; and importantly, the sum total of these findings strongly indicate that APP and GAP-43 may interact in a bona fide protein complex with functional consequences relevant to AD pathogenesis. Thus, we chose to investigate and characterize a possible APP-GAP-43 neuronal protein interaction.

Table 1: Physiological Roles of APP and GAP-43

Physiological Function	APP	GAP-43	References
Cell adhesion	+	-	Zhang et al., 2011
Neurite outgrowth	+	+	Zhang et al., 2011; Benowitz and Routtenberg, 1997
Synapse formation	+	+	Zhang et al., 2011; Benowitz and Routtenberg, 1997
Axonal pathfinding	+	+	Sosa et al., 2017; Benowitz and Routtenberg, 1997
Cytoskeletal organization	-	+	Benowitz and Routtenberg, 1997
Neurite maintenance	-	+	Grasselli et al., 2011
Long-term potentiation	+	+	Ludewig and Korte, 2017; Benowitz and Routtenberg, 1997
Development	+	+	O'Brien and Wong, 2011; Benowitz and Routtenberg, 1997
Regeneration	-	+	Benowitz and Routtenberg, 1997
Learning and memory	+	+	Zhang et al., 2011Benowitz and Routtenberg, 1997

Table 1 depicts a comparison of the physiological roles of APP to GAP-43 as well as the sources from which the information was derived. A (+) indicates the protein has been implicated in the physiological function and a (–) indicates it has not.

Figure 5: APP and GAP-43 Subcellular Localization

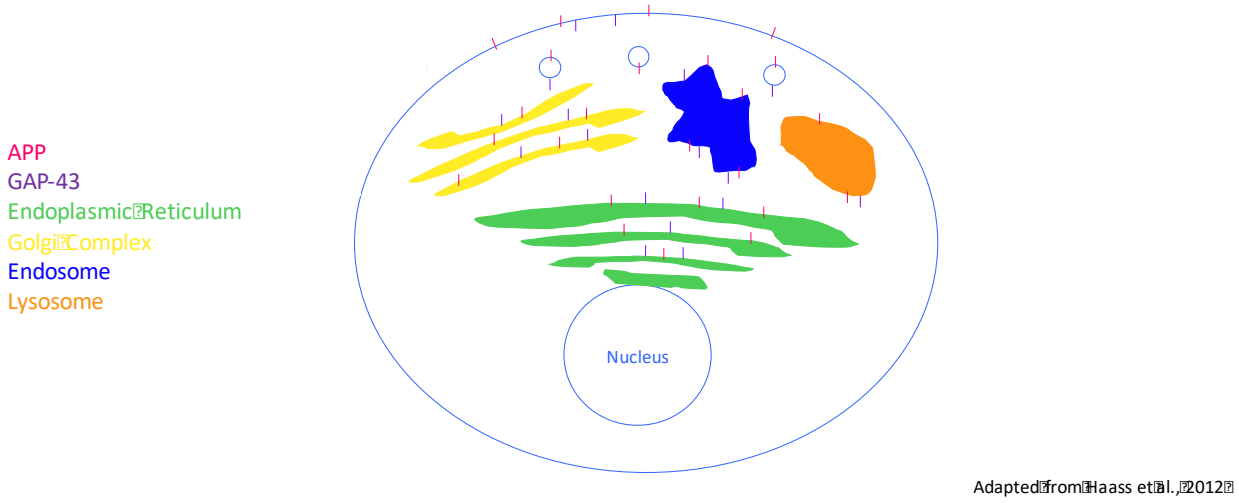


Figure 5 depicts the subcellular locations at which APP and GAP-43 are known to localize.

Hypothesis

I hypothesize that GAP-43 interacts with and influences the processing of APP and may consequently play an important physiological role in modulating the levels of Aβ produced during APP processing.

Aims

1. Determine the effect of GAP-43 expression on the formation of APP metabolites in a relevant cell line.
2. Confirm a potential novel APP-GAP-43 interaction.

Methods

In order to test my hypothesis and aims, four different experimental designs were employed, all of which followed a similar sequence of events. Briefly, cells were transfected to over- or underexpress proteins of interest, expression/silencing was allowed for 40 to 48 hours, cells were lysed, and lysates analyzed via Western blot analysis. A more detailed explanation of the methods employed for these studies is provided below. Where variations on these protocols were used, they will be noted in the text.

Transformation and plasmid purification

To generate plasmid cDNA for transfection, 100ng of plasmid vector containing the cDNA representing the open reading frames of the proteins of interest were combined with DH5α cells in microcentrifuge tubes. The mixture was incubated on ice for 5 minutes and then transferred to a 42°C water bath for 30 seconds after which they were placed back on ice for 2 minutes. Luria Broth (LB) liquid growth medium (1% w/v Peptone, 0.5% w/v yeast extract, 0.5% sodium chloride) was then added and the mixtures were incubated at 37ºC with shaking for one hour. The transformed cells were plated on LB/agar plates supplemented with 50μg/mL ampicillin and then incubated overnight at 37°C. Isolated colonies were selected and each individual colony was incubated in 3mL LB supplemented with 50μg/mL ampicillin at 37°C overnight on a shaker at 250rpm. Cultures were pelleted by centrifugation at 8,000rpm for 2 minutes at room temperature and the pellets purified by alkali lysis combined with pH-based matrix using the GeneJET Plasmid Miniprep Kit (Fermentas) according to the manufacturer's protocol. DNA quantity and purity were analyzed using spectrophotometric analysis at 260 and

280 nanometers absorbance. Plasmid purity was assessed by the ratio of 260:280 and needed to be in the range of 1.8+/- 0.1.

Cell culture

N2a mouse neuroblastoma cells and human embryonic kidney 293 (HEK293) cells were acquired from the American Type Culture Collection (ATCC – Manassas, VA) and were grown in Delbeco's Modified Eagle's Medium (DMEM – Life Technologies) supplemented with 10% fetal bovine serum and 1% penicilin/streptomycin. The cells were incubated at 37°C under 5% CO_2 and passaged 1:10 every 3-4 days.

Transfection

The day before transfection, HEK293 cells were plated at a density of 600,000 cells per 30mm cell culture dish. The following day, the cells were visually inspected to confirm that cell confluency was between 70-90%, and if so, the cells were transiently co-transfected with 1μg of purified plasmid cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Expression was allowed for 40-48 hours at 37°C under 5% CO_2. **Table 2** lists the plasmid cDNA constructs used for transfection. The specific constructs utilized in each experiment are noted in the text.

Table 2: Plasmid cDNA and Expression Vectors

Plasmid cDNA Constructs	Expression Vector
pEYFP	pAG3
GAP-43	pAG3
GAP-43 CCAA	pAG3
APP_{SWE}	pCMV
$APP_{695\text{-}MYC}$	pCB6

Table 2 depicts the plasmid cDNA constructs and expression vectors utilized in the transfection experiments.

Generation of stable cell lines

HEK293 cells were transfected as outlined above with 1µg of pAG3 plasmid containing FLAG-tagged versions of either GFP, GAP-43, or GAP-43 CCAA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Expression was allowed for 40-48 hours at 37°C under 5% CO_2 after which selection of stably expressing cells was performed using Geneticin (Invitrogen) at 400µg/mL. Medium was changed every two days until stably expressing cells were selected. Cells were split into fresh plates and selective pressure was maintained at 80µg/mL Geneticin.

siRNA

Four different siRNA constructs (siRNA1-4) and a control were purchased from QIAGEN. Each construct targeted a different sequence present on Gap-43 mRNA (**Table 3**). 40 picomoles of each siRNA construct was transiently transfected into N2a cells, either alone or in combination, using the transfection protocol previously described.

Table 3: siRNA Constructs and Target Sequences

Construct	Target Sequence (5' – 3')
siRNA1	AAGAACATGCCTGAACTTTAA
siRNA2	CAGGAAAGATCCCAAGTCCAA
siRNA3	CCGGCTCAGCGGAGACAGAAA
siRNA4	TTGGGAAATTCTTGCACTGTA
Control siRNA	AATTCTCCGAACGTGTCACGT

Table 3 depicts the target sequences that correspond to each of the four siRNA constructs and the control.

Cell lysis

After 40-48 hours of expression, cells were lysed on ice according to the following protocol. 200µL of medium were removed from each plate and transferred to microcentrifuge tubes to which the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 2µM. The remaining medium was discarded and the monolayers of cells were washed twice with 1mL of ice-cold phosphate buffered saline (PBS) at 1x without calcium or magnesium. The 1x PBS was removed by vacuum and the cells were then incubated in 200µL of lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% IGEPAL, 0.5% Na:deoxycholate and a protease inhibitor cocktail (Sigma-Aldrich)) for 5 minutes on ice during which time the microcentrifuge tubes containing the medium were centrifuged for 2 minutes at 16,000 x g and the resultant supernatants transferred into new microcentrifuge tubes and the pellets discarded. The cells in the lysis buffer were then transferred to fresh microcentrifuge tubes, vortexed briefly (1-2 seconds), and centrifuged for 1 minute at 16,000 x g to remove nuclei and insoluble cell debris. The resultant supernatants were transferred to new

microcentrifuge tubes and the pellets discarded. The lysis contents were analyzed for protein concentration using the BCA method (Pierce).

Western blot

For analysis of the lysates by Western blot, equal protein concentrations, 20μg, were separated on Tris/glycine/SDS polyacrylamide gels (Bio-Rad) or hand-poured Tris/tricine/SDS gels (Miller *et al.*, 2016). The acrylamide concentration of each gel was determined by the relative sizes of the proteins to be detected and is noted where appropriate. After electrophoretic separation, proteins were transferred onto polyvinylide fluoride membranes (Bio-Rad) in a tris/glycine buffer for 1 hour 15 minutes at 350mA or overnight at 90mA. Upon completion of the transfer, the membranes were washed 3 times with 1x PBST (phosphate buffered saline and 0.1% Tween® 20) for 5 minutes each with rocking at room temperature and then blocked with 5% milk in 1x PBST for 45 minutes at room temperature with rocking. The membranes were then transferred to containers containing the appropriate primary antibody diluted in 1x PBST with 1% milk and then incubated on a rocker at room temperature for 3 hours or at 4°C overnight. After incubation, the membranes were washed 3 times for 5 minutes each with 1x PBST at room temperature with rocking and then incubated with the appropriate secondary antibody in 1x PBST with 1% milk with rocking for 1 hour at room temperature. The membranes were then washed 4 times for 5 minutes each with 1x PBST at room temperature with rocking and briefly rinsed with distilled water before antibody detection by chemiluminesence. Immunoreactivity was visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized using FluorChem HD2 imager or Aplegen OmegaLum G imager.

Antibodies

APP polyclonal antibody CTM1, a kind gift from Dr. Gopal Thinakaran, was raised against the C-terminus of APP with a C-myc epitope tag and provided as serum (Thinakaran *et al.*, 1998). Monoclonal antibody 26D6, also graciously provided by Dr. Gopal Thinakaran, was raised against the N-terminal region of the Aβ peptide; of note, it also detects sAPPα since it contains the same sequence (Thinakaran *et al.*, 1998). Polyclonal antibody raised against recombinant GAP-43 was purchased from EMD Millipore (AB5220). Affinity purified rabbit anti-FLAG antibody detecting the FLAG epitope, DYDDDDK, was purchased from Sigma Aldrich. Mouse anti-actin antibody was purchased from Santa Cruz Biotechnology. All secondary antibodies, goat anti-rabbit HRP and rabbit anti-mouse HRP, were acquired from Pierce-Thermo Scientific. See **Table 4** for antibodies and dilutions.

Table 4: Antibodies and Dilutions

Antibody	Dilution	Secondary Antibody	Dilution
CTM1	1:2,000	anti-rabbit	1:40,000
26D6	1:1,000	anti-mouse	1:10,000
anti-GAP-43	1:1,000	anti-mouse	1:10,000
anti-FLAG	1:10,000	anti-rabbit	1:40,000
anti-Actin	1:10,000	anti-mouse	1:10,000

Table 4 lists the primary antibodies, the corresponding secondary antibodies, and the dilutions utilized.

Mutagenesis

To construct the GAP-43 CCAA double mutant, the template, wild type GAP-43, and the primers, containing the appropriate codon mutations, were ordered from Integrated DNA Technologies and two rounds of PCR performed. The first round consisted of two reactions that

amplified GAP-43 from the area of mutation to the 5' end of the cDNA and the area of mutation to the 3' end of the cDNA. The second round of PCR was performed using only the flanking primers which contained the BamHI and HindIII restriction enzyme sites. The products of the two rounds of PCR as well as the pAG3 expression plasmid were subsequently digested with BamHI and HindIII restriction enzymes in separate reactions containing the 50μL reaction volume and 1μL of each enzyme for 1 hour at 37°C. The reaction products were then run on 0.8% agarose gels at 90V until a separation of bands was evident. The appropriate bands were determined by their migration pattern, excised, and then purified using a QIAGEN gel purification kit. Once purified, the products were ligated into the expression plasmid, pAG3, using T4 DNA ligase with a 3:1 ratio of insert to plasmid and subsequently used to transform DH5α cells as outlined above. Clones containing the insert were submitted to sequencing at the University of Chicago core sequencing facility. Mutations were confirmed before use in experiments.

Cell fractionation

HEK293 cells stably expressing GAP-43 or GAP-43 CCAA were scraped into isotonic lysis buffer (10mM tris pH7.4, 1mM EDTA, 0.5mM PMSF), incubated on ice for 10 minutes, homogenized via 20 strokes through a ball bearing homogenizer with a 12μm clearance, and then centrifuged at 5,000 x g for 5 minutes to remove nuclei. The supernatant from this spin was centrifuged at 20,000 x g for 30 minutes at 4°C. The supernatant was labeled as the cytosolic fraction and the pellet labeled as the membrane fraction.

Co-immunoprecipitation

HEK293 cells were co-transfected with APP_{SWE} and either FLAG-tagged GFP, FLAG-tagged GAP-43, or FLAG-tagged GAP-43 CCAA using the protocol previously described. After 40 hours of co-expression, cells were lysed in a co-immunoprecipitation buffer consisting of 150mM NaCl, HEPES pH 7.0 and 0.5% Igepal. To precipitate protein complexes, 100ng of rabbit anti-FLAG antibody was incubated with 5µl of lysate for 3 hours at 4^{o}C with rocking after which complexes were captured using paramagnetic beads conjugated to anti-rabbit antibodies. After washing with co-immunoprecipitation buffer, complexes were released with citric acid, pH 3.0, and rebuffered by the addition of tris-HCl to pH 6.8. The captured protein complexes were submitted to SDS-PAGE and Western blot analysis.

Densitometric analysis

Quantitation by optical density analysis of Western blot CCD images was performed on three separate experiments using ImageJ software. Average optical density values were compared using the unpaired t-test with GraphPad Prism.

Results

GAP-43 silencing in N2a cells resulted in inconsistent results

Since APP and GAP-43 are known to reside in many of the same subcellular locations (**Figure 5**) and to possess many similar physiological functions (**Table 1**), it is reasonable to hypothesize that the two proteins may have a functional interaction. We predicted that if our hypothesis is true, then the knockdown of GAP-43 in N2a cells would result in an alteration in the level of APP metabolites as compared to untreated and scrambled control-treated groups. To test our hypothesis, N2a cells were transiently transfected with one of four siRNA constructs, either alone or in combination, each targeted against a different sequence within Gap-43 mRNA, or a scrambled control (**Table 3**). Cells were harvested as described in **Methods** and the lysates run on SDS-PAGE and probed with an anti-GAP-43 antibody to determine knockdown efficiency. As evidenced in **Figure 6**, the combination of siRNA1-4, in equal parts, resulted in the most efficient knockdown of GAP-43 expression in N2a cells. Therefore, we utilized this combination in future experiments to determine the impact of GAP-43 knockdown on APP processing.

Although the combination of GAP-43 siRNA1-4 appeared to knockdown GAP-43 expression efficiently in N2a cells, we were not able to collect consistent data between trials. At the time, we were unaware of inconsistent transfection efficiencies in this particular cell line. Importantly, the lack of conclusive data from this particular set of experiments did not rule out the possibility of a functional relationship between APP and GAP-43; therefore, we next sought to determine if overexpression of GAP-43 influences APP processing. For these experiments, we chose the HEK293 human cell line originally derived from human embryonic kidney tissue. Although these cells do not express detectable levels of GAP-43, they are easily transfectable

and express all the components necessary for APP catabolism. Additionally, HEK293 cells are a highly used line for the investigation of APP processing and thus, are a suitable line for the exploration of an APP-GAP-43 interaction through expression protocols.

Figure 6: Efficiency of GAP-43 Silencing

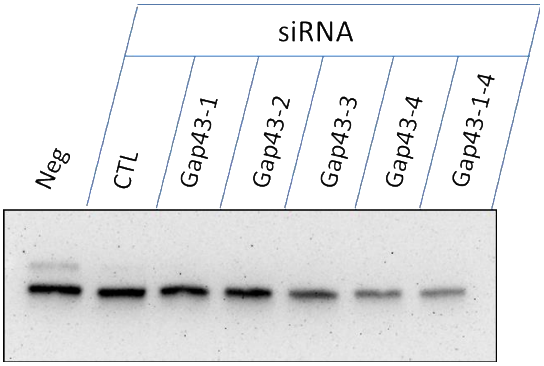


Figure 6 depicts the efficiency of knockdown of each of the GAP-43 siRNA constructs, either singly or in combination (equal parts), as well as that of a scrambled control (CTL) in N2a cells.

GAP-43 overexpression reduces APP C-terminal fragment levels in HEK293 cells

Due to the finding that GAP-43 co-immunoprecipitates with affinity-tagged APP *in vivo* from mouse brain, as well as the fact that APP and GAP-43 have highly overlapping physiological functions **(Table 1)** and are found within similar areas of the neuron (**Figure 5**), we hypothesized that GAP-43 may influence the processing fate of APP (Norstrom *et al.*, 2010). Therefore, if GAP-43 influences APP processing, then we would expect to see changes in either α-CTF fragments, β-CTF fragments, or both with overexpression of GAP-43 in HEK293 cells. In order to test our hypothesis, we transiently transfected APP_{SWE} with either GAP-43 or GFP control into HEK293 cells as described in **Methods**. Cells were harvested approximately 40 hours after transfection and the lysates subjected to Western blot analysis. In order to determine if GAP-43 overexpression impacts APP processing, the lysates were run on a gradient tricine

polyacrylamide gel with a 10-16.5% acrylamide gradient and probed for C-terminal fragments of APP, either α- or β-CTF, using the CTM1 antibody (Miller *et al.*, 2016). **Figure 7** depicts the results of these experiments. As indicated in the figure, we observed a reduction in both α- and β–CTF levels with co-expression of GAP-43 as compared to GFP as indicated by the weaker signals migrating at approximately 10 and 12 kDa relative mass in the GAP-43 co-expressing lane (**Figure 7a – left panel**). Importantly, no changes in full-length APP (migrating at approximately 100 kDa relative mass on the Western blot) were observed in these experiments. This observation indicates that GAP-43 expression does not depress the production of APP metabolites by reducing APP expression. Expression of GAP-43 was confirmed by probing portions of the membrane with an anti-GAP-43 antibody (**Figure 7a - bottom panels**).

Since wild type APP is known to be differentially processed by α- and β–secretases as compared to APP_{SWE}, we wanted to determine if GAP-43 overexpression on APP processing is mutation specific (Mullan *et al.*, 1992). Therefore, we also tested wild type APP under the same paradigm to investigate potential differences in the processing fate of mutant and wild type APP in the presence of GAP-43 overexpression. Since wild type APP does not bear the Swedish mutation and thus, is not a very efficient substrate for β-secretase, we did not expect to observe a reduction in β–CTF as we did with APP_{SWE}; however, we did expect to see a reduction in α-CTF since wild type APP is effectively cleaved by this secretase (Mullan *et al.*, 1992; Lammich *et al.*, 1999). The results for this experiment are shown in the right panel of **Figure 7a**. Here again we observed a reduction in C-terminal fragments without a concomitant reduction in full-length APP; thus, we concluded that the effect of GAP-43 overexpression on APP processing is not dependent on the presence of the Swedish mutation. Overexpression of GAP-43 was confirmed by probing portions of the membrane with an anti-GAP-43 antibody.

In order to determine if GAP-43 overexpression has a statistically significant impact on the processing of APP by both α- and β-secretases, we quantified data from three independent trials in which APP_{SWE} was co-expressed with either GFP or GAP-43. To this end, densitometric analysis was performed using ImageJ software and the values were compared using the unpaired t-test with GraphPad Prism software. The results of this analysis indicated that GAP-43 overexpression has a statistically significant impact on the processing fate of APP by both α- and β-secretases as the p value that resulted was less than 0.05. These results imply that GAP-43 may potentially interact with APP, either directly or indirectly, to reduce APP processing by both α- and β-secretases. We next sought to explore whether membrane tethering was essential for the influence of GAP-43 overexpression on APP processing.

Figure 7: GAP-43 Expression Reduces the Overall Processing of APP

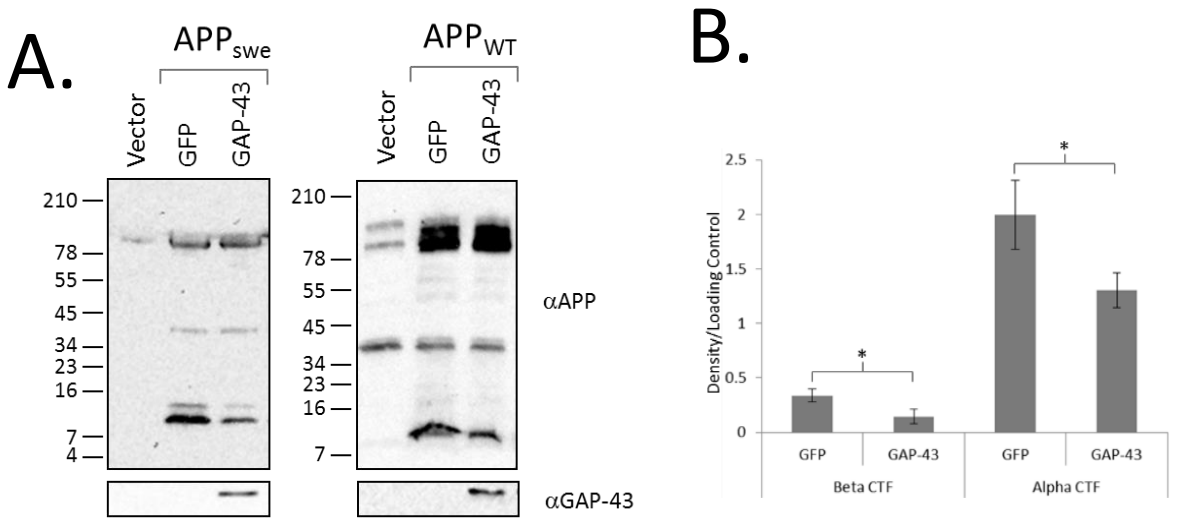


Figure 7 A. Western blot of cell lysates from HEK293 cells co-expressing wild type APP (APP_{WT}) or APP_{SWE} and GAP-43 reveal a reduction in the levels of C-terminal fragments in both cases relative to a control protein (GFP). Both α- and β-CTF were reduced in the presence of GAP-43**. B.** Quantitation of C-terminal fragments by optical density analysis of Western blot CCD images. Quantitation was performed using ImageJ software. Values were compared using the unpaired t-test with GraphPad Prism. * indicates $p < 0.05$. N=3 for each analysis.

Palmitoylation of Cys_3 and Cys_4 of GAP-43 is required for membrane association

Since APP is a type I transmembrane protein with a short cytoplasmic tail, and since GAP-43 is targeted to the inner leaflet of the plasma membrane as a result of the palmitoylation of cysteine residues 3 and 4, then it is logical to hypothesize that GAP-43 may interact with the C-terminal intracellular domain of APP and by doing so, may potentially alter its processing fate (Skene JH, 1989; Wasco *et al.*, 1992; Wasco *et al.*, 1993). If our hypothesis is true, then mutating cysteine residues 3 and 4 to alanines, residues that cannot be palmitoylated, should abolish the effect of GAP-43 overexpression on APP processing; consequently, no reduction in α- or β-CTF levels should be observed with GAP-43 overexpression. To test our hypothesis, point mutagenesis was carried out to mutate Cys_3 and Cys_4 to alanines to create the GAP-43 CCAA double mutant. Once the construct was created, we generated HEK293 cells stably expressing either wild type GAP-43 or GAP-43 CCAA and separated the membrane fraction from the cytosolic fraction through differential centrifugation as described in **Methods**. The two fractions were run on SDS-PAGE and probed for GAP-43 using an anti-GAP-43 antibody to determine distribution differences between wild type GAP-43 and the GAP-43 CCAA double mutant. Since wild type GAP-43 transitions between cytosolic and membrane fractions, we expected to observe wild type GAP-43 in both fractions, while we expected GAP-43 CCAA double mutants only in the cytosolic fraction (Liu *et al.*, 1994; Liang *et al.*, 2002). As shown in **Figure 8**, wild type GAP-43 was indeed found in both fractions, although the majority of the signal was detected in the membrane fraction, suggesting that GAP-43 is primarily bound to the membrane. Conversely, mutation of Cys_3 and Cys_4 to alanines completely abolished the targeting of GAP-43 to the membrane fraction as shown in **Figure 8**. With this mutant, GAP-43 was only detectable in the cytosolic fraction. As a control for the relative purity of each fraction,

we performed the experiment using GFP, a soluble protein with no membrane-association properties (Tsien, 1998). In this case, GFP was found to completely associate with the cytosolic fraction (data not shown). This finding is significant because the change in the subcellular distribution of GAP-43, from a predominantly membrane to a predominantly cytosolic distribution, should consequently also abolish the impact of GAP-43 overexpression on the processing fate of APP if that outcome is based on GAP-43 membrane targeting and a potential APP-GAP-43 interaction. Thus, we next sought to determine if the impact of GAP-43 overexpression on APP processing requires the targeting of GAP-43 to the membrane fraction.

Figure 8: Palmitoylation of GAP-43 is Required for Membrane Association

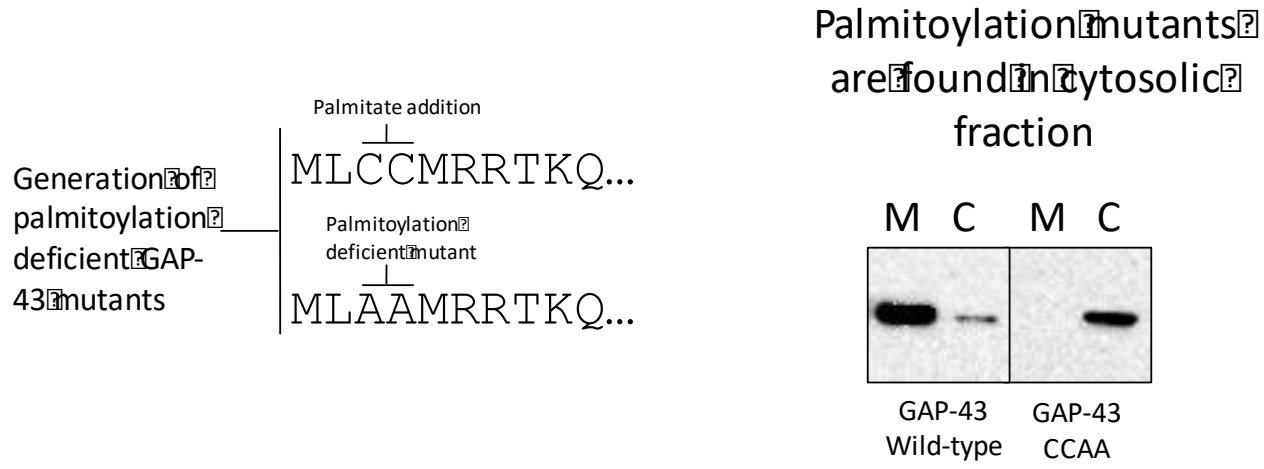


Figure 8 Left. Sequence of the first 10 amino acids of GAP-43. Palmitate is enzymatically added to cysteines 3 and 4. To determine the role of palmitoylation in the GAP-43 mediated alterations in APP metabolism, cysteines 3 and 4 were mutated to alanines, which do not contain a sulfur atom required for the thioester bond created during palmitoylation. **Right.** HEK293 cells expressing GAP-43 or GAP-43 CCAA were fractionated into membrane (M) and cytosolic (C) fractions and assayed for the relative presence of GAP-43 in each by Western blot. Wild type GAP-43 was found in both fractions; conversely, GAP-43 CCAA was only found in the cytosolic fraction.

Palmitoylation of Cys_3 and Cys_4 of GAP-43 is required for GAP-43 to exert its influence on APP processing

Since palmitoylation of Cys_3 and Cys_4 is required for the association of GAP-43 with the membrane fraction, and since APP is a type I transmembrane protein, we hypothesized that the impact of GAP-43 overexpression on APP processing is most likely dependent upon palmitoylation of these residues (Skene JH, 1989; Wasco *et al.*, 1992; Wasco *et al.*, 1993). If our hypothesis is true, then co-expression of APP_{SWE} and GAP-43 CCAA should not result in a reduction in α- and β-CTF levels. To test this hypothesis, we transiently transfected HEK293 cells with APP_{SWE} and either an empty vector control, GFP, wild type GAP-43, or GAP-43 CCAA. After approximately 40 hours of co-expression, the cells were harvested as described in **Methods**. The lysates were then run on SDS-PAGE and probed for full length APP and α- and β-CTF using the CTM1 antibody as well as for sAPPα using the 26D6 antibody to determine the impact of GAP-43 CCAA overexpression on APP processing. The same lysates were also probed for GAP-43 using an anti-GAP-43 antibody as well as for a loading control using an anti-actin antibody. As indicated in **Figure 9**, when APP_{SWE} was co-expressed with the empty vector control or GFP, APP_{SWE} was processed normally, with both α-CTF and β-CTF detectable in both conditions (**Figure 9 – lanes 2 and 3**). Additionally, when APP_{SWE} was co-expressed with wild type GAP-43, we observed a reduction in α- and β-CTF levels as expected **(Figure 9 – lane 4)**. Conversely, when APP_{SWE} was co-expressed with GAP-43 CCAA, the α- and β-CTF levels were comparable to the control conditions and not reduced in level (**Figure 9 – lane 5**). This is strong evidence that an APP-GAP-43 functional interaction is dependent on the membrane targeting of GAP-43 via palmitoylation of Cys_3 and Cys_4. This finding is significant because it is the first known report of a functional interaction between APP and GAP-43 with relevance to AD.

Figure 9: Mutation of Cysteine Residues 3 and 4 to Alanines Inhibits the Effects of GAP-43 Overexpression on the Processing of APP

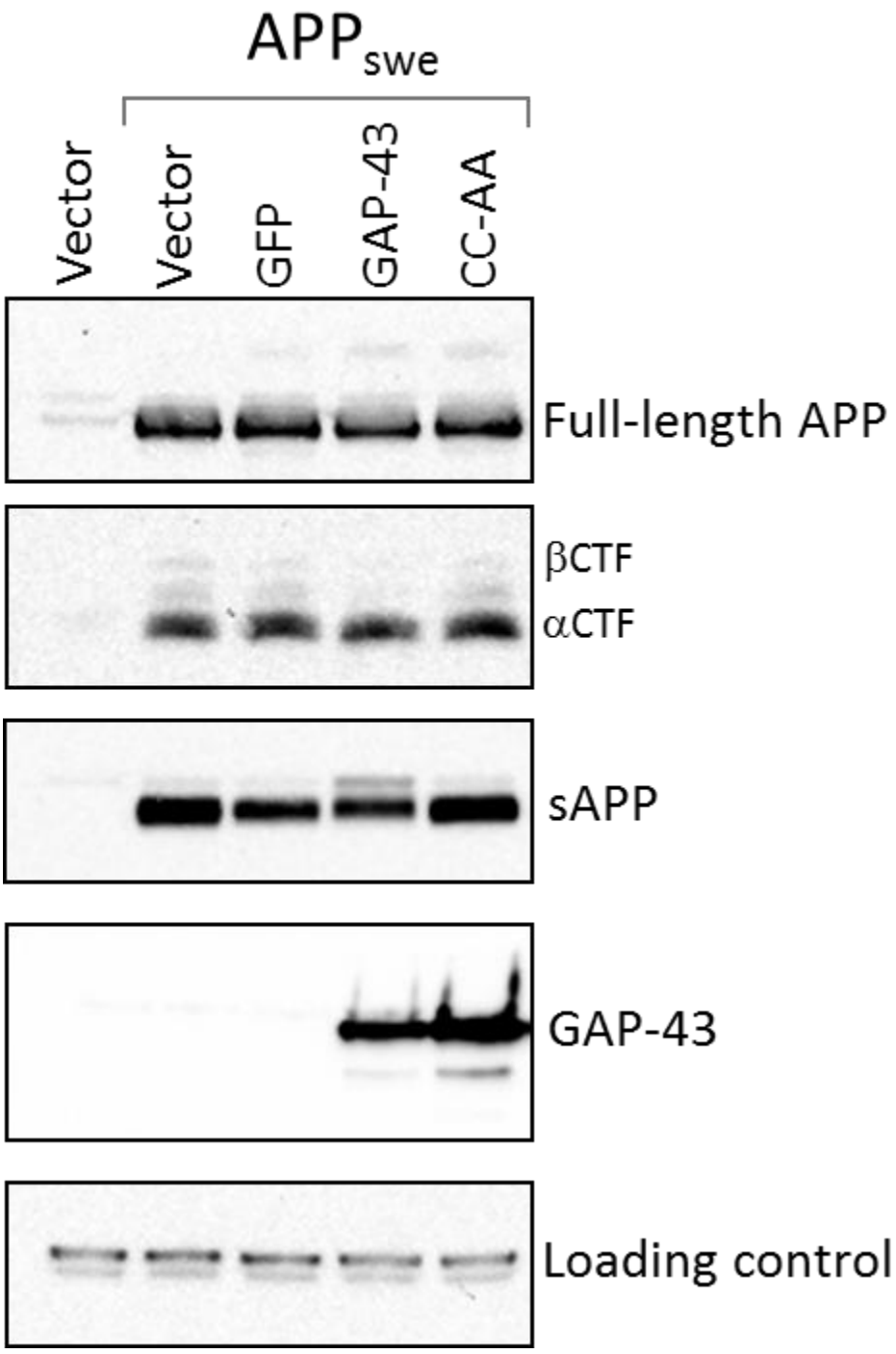


Figure 9: Mutation of cysteines 3 and 4 of GAP-43 to alanines results in a loss of the CTF-reducing effects of GAP-43 overexpression. Co-expression of GAP-43 reduces APP C-terminal fragment levels (lane 4), but this effect is lost when the palmitoylation consensus sequence is mutated (lane 5). Loading control probed with anti-actin antibody indicates equal loading for each lane.

Palmitoylation of Cys_3 and Cys_4 of GAP-43 is required for association with APP

Since we confirmed the necessity of Cys_3 and Cys_4 for the proper targeting of GAP-43 to the membrane, as well as for the influence of GAP-43 overexpression on APP processing, we next wanted to determine if the inability of the GAP-43 CCAA double mutant to influence the processing of APP could be due to a lack of interaction between the two proteins. We hypothesized that palmitoylation of Cys_3 and Cys_4 of GAP-43 is required for an APP-GAP-43 interaction since APP is a type I transmembrane protein and since GAP-43 must be palmitoylated at Cys_3 and Cys_4 in order to associate to the membrane fraction (Skene JH, 1989; Wasco *et al.*, 1992; Wasco *et al.*, 1993). Although previous results indicated that APP and GAP-43 interact *in vivo*, we now predicted that the GAP-43 CCAA double mutant would not immunoprecipitate along with APP *in vitro*, but that wild type GAP-43 would. To test our prediction, HEK293 cells were transiently transfected with APP_{SWE} and either FLAG-tagged GFP, FLAG-tagged GAP-43, or FLAG-tagged GAP-43 CCAA. After 40 hours of co-expression, the cells were lysed and the protein complexes precipitated using a rabbit anti-FLAG antibody as described in **Methods**. The captured protein complexes were submitted to SDS-PAGE and Western blot analysis. To determine whether GFP, GAP-43, and GAP-43 CCAA formed complexes with APP within the cells, membranes were probed with the CTM1 antibody. As indicated in **Figure 10**, APP specifically immunoprecipitated with wild type GAP-43; it did not immunoprecipitate with GFP as expected, nor did it immunoprecipitate with GAP-43 CCAA. In three repeats of the experiment, we did not detect APP in the immunoprecipitate when it was co-expressed with GAP-43 CCAA, indicating that Cys_3 and Cys_4 are required for an APP-GAP-43 interaction. By extension, we concluded that membrane association, mediated at least in part

by palmitoylation of GAP-43 at Cys_3 and Cys_4, is necessary for the functional interaction of GAP-43 with APP.

Figure 10: APP does not Co-immunoprecipitate with GAP-43 CCAA

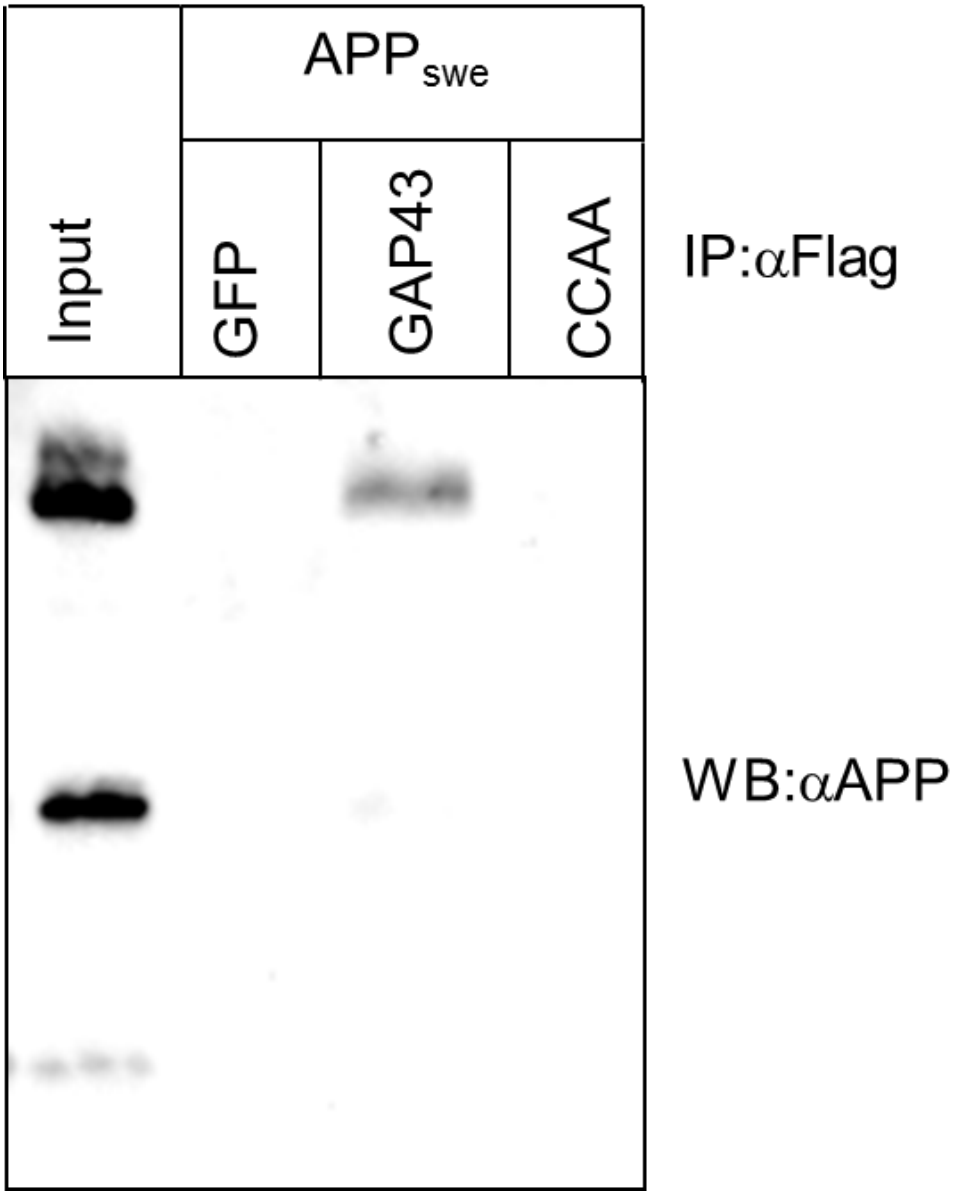


Figure 10: APP_{SWE} was transiently co-expressed with FLAG-tagged versions of either GFP, wild type GAP-43, or GAP-43 CCAA. Immunoprecipitation using an anti-FLAG antibody and Western blot detection of APP revealed the presence of APP in wild type GAP-43 protein complexes, but not mutant GAP-43 or GFP complexes.

Discussion

Importance of studying AD

AD is a devastating disorder that afflicts millions of individuals worldwide (alz.org, 2017). Its ever-increasing prevalence makes the study of this disease more and more relevant (alz.org, 2017). Since no effective treatment options exist, it is important to shed light on the molecular underpinnings of the onset and progression of this disorder. If we can determine why and how Aβ accumulates in afflicted individuals, we can design tailored therapies aimed at preventing just that. Although we do know that Aβ production results from a series of proteolytic cleavages which involves multiple protein-protein interactions, we still do not fully know or understand all of the pertinent protein interactions underlying the onset and progression of AD. In order to design a truly efficacious therapy, more work needs to be done to fully elucidate the complex pathophysiology of this disorder.

Importance of investigating a potential APP-GAP-43 interaction

In an attempt to address a possible factor in the onset and/or progression of AD, we decided to investigate a potential novel protein-protein interaction between APP and GAP-43. More specifically, we wanted to determine if GAP-43 interacts with APP in a way that alters its metabolic processing. As previously discussed, the reasons we selected a potential APP-GAP-43 interaction for investigation were multiple. Briefly, Norstrom and colleagues found that GAP-43 immunoprecipitates with human APP_{695} fused to a C-terminal affinity tag *in vivo* from mouse brain (Norstrom *et al.*, 2010). Additionally, APP and GAP-43 have several overlapping physiological functions (**Table 1**) and are known to localize to the same subcellular compartments (**Figure 5**). Furthermore, GAP-43 expression is known to be altered in AD brain

and has been found to colocalize in the brains of those with AD (Coleman *et al.*, 1992; Masliah *et al.*, 1992; de la Monte *et al.*, 1995; Cheetham *et al.*, 1996; Masliah *et al.*, 2001). For these reasons, we hypothesized that GAP-43 may interact with APP in a bona fide protein complex and that this interaction may influence the processing fate of APP. We wanted to determine the effect of GAP-43 expression on the formation of APP metabolites as well as to confirm a potential novel APP-GAP-43 interaction. The following paragraphs will summarize our findings and their implications. Additionally, any discrepancies between our data and that found in existing literature will be addressed.

Summary of findings, implications, and relation to established literature

In order to confirm and characterize a potential APP-GAP-43 interaction, we selected an *in vitro* model system. The primary reason we opted to utilize an *in vitro* system was to minimize variables so as to afford us greater experimental control and reduce interpretation errors. For our *in vitro* system, we initially selected a mouse neuroblastoma cell line, N2a cells. N2a cells were selected at the onset of our journey because their neuronal origin offered us the ability to study the potential interaction in a more native setting. Although N2a cells were our first choice for our *in vitro* system, we were not able to utilize them for the duration of our studies due to transfection inefficiencies. This obstacle was discovered while investigating if silencing GAP-43 expression in N2a cells influences APP processing (**Figure 6**). After repeating the experiment several times, we noted our results were not consistent between trials. While several variables could account for such an observation, we realized that it was our inability to standardize the percentage of successfully transfected cells per experimental group that hindered our ability to successfully interpret our data as our read out was relative change in

the level of APP byproducts. Thus, we opted to utilize a cell line well known for its readily transfectable nature, HEK293 cells (Thomas & Smart, 2005).

Although not of neuronal origin, HEK293 cells display several neuronal markers and importantly, possess all of the cellular machinery necessary for complete APP metabolism; consequently, HEK293 cells are used extensively within the AD research community (Shaw *et al.*, 2002; Norstrom *et al.*, 2010; Gordon *et al.*, 2013; Stepanenko & Dmitrenko, 2015). Moreover, they are known for their readily transfectable nature and consistent behavior in culture systems, serving as one of the most widely used cell culture model systems (Thomas & Smart, 2005). Once we switched our *in vitro* model system from N2a cells to HEK293 cells, our results became consistent and we were thus able to begin interpreting our data. However, since HEK293 cells do not express detectable levels of GAP-43, we could not repeat the siRNA experiment in this cell line (**Figures 7 and 9**). Therefore, we chose to investigate the potential impact of GAP-43 overexpression on APP metabolism.

When we overexpressed GAP-43 in HEK293 cells along with either wild type APP or APP_{SWE}, we observed a reduction in APP metabolites, both α-CTF and β-CTF, as compared to the GFP control group (**Figure 7**). Since the reduction in APP byproducts was noted in both the wild type APP- and APP_{SWE}-transfected groups when co-transfected with GAP-43, we concluded that this effect was independent of the Swedish mutation (**Figure 7**). To the best of our knowledge, no paper has been published as of this writing in which a functional relationship between GAP-43 and APP was investigated. However, correlative data between APP and GAP-43 expression has been published. This collective body of data indicates that GAP-43 expression is reduced in AD brain; more specifically, it indicates that GAP-43 expression is reduced in areas of the AD brain associated with heavy plaque burden such as the hippocampus (Coleman *et al.*,

1992; Masliah *et al.*, 1992; de la Monte *et al.*, 1995; Cheetham *et al.*, 1996; Masliah *et al.*, 2001). Thus, by extrapolation, if GAP-43 expression is reduced in areas of AD brain associated with increased Aβ deposition, then it is reasonable to argue that increasing GAP-43 expression in these areas may have the opposite effect. This line of reasoning is further supported by the findings of Routtenberg et al. in which GAP-43 overexpression was shown to result in enhanced long-term potentiation and learning, processes negatively impacted in AD (Routtenberg *et al.*, 2000; Selkoe, 2011). Again, by extrapolation, since there is reduced GAP-43 expression in areas of the brain impacted in AD, which is a disease characterized by impaired learning and memory, then it is logical to assume that enhanced expression of GAP-43 in these areas may enhance long-term potentiation and learning (Coleman *et al.*, 1992; Masliah *et al.*, 1992; de la Monte *et al.*, 1995; Cheetham *et al.*, 1996; Masliah *et al.*, 2001; Selkoe, 2011). Whether or not the impact of GAP-43 overexpression on long-term potentiation and learning is due to its impact on APP processing is unknown and should be explored further. However, our findings are in line with the aforementioned literature.

Conversely, our findings are not in line with those published by Inoue et al. This group found that silencing GAP-43 expression in HEK293 cells results in a decrease in Aβ production (Inoue *et al.*, 2015). A logical explanation for this difference could be that GAP-43 expression, within a set expression range, results in a decrease in Aβ production. However, more studies will need to be performed to better address this discrepancy. Although our data was not in line with Inoue's findings, it does not detract from the fact that we consistently observed a reduction in α-CTF and β-CTF fragments when we overexpressed GAP-43 along with APP in hEK293 cells. Our finding is significant because it reveals a novel APP protein interaction that influences the processing destiny of APP. Furthermore, this finding highlights the complexity of this

disease and the importance of understanding each individual player in the onset and progression of AD. We clearly have much more work that needs to be done in order to truly understand the pathology of the disease. In order to try and further elucidate this new player in AD pathology, we next sought to characterize the parameters required for GAP-43 to exert this effect.

Since APP is a type-I transmembrane protein and since GAP-43 is targeted to the membrane as a result of palmitoylation of cysteine residues 3 and 4, we wanted to determine if palmitoylation of these residues is necessary for GAP-43 to influence APP processing (Skene JH, 1989; Wasco *et al.*, 1992; Wasco *et al.*, 1993). To this end, point mutagenesis was carried out to mutate Cys_3 and Cys_4 to alanines, residues that cannot be palmitoylated. When Cys_3 and Cys_4 of GAP-43 were mutated to alanines, we noted a change in the distribution of GAP-43 (**Figure 8**). Typically, GAP-43 fluctuates between the membrane and cytosolic fractions with the majority localized to the membrane compartment (El-Husseini *et al.*, 2001; Liang *et al.*, 2002). However, upon mutation of Cys_3 and Cys_4 to alanines, the membrane targeting of GAP-43 was completely abolished as indicated by the complete absence of signal in the membrane fraction (**Figure 8**). With the GAP-43 CCAA double mutant, we were only able to detect GAP-43 in the cytosolic fraction (**Figure 8**). This is in agreement with other studies that have verified the necessity of the palmitoylation of GAP-43 at Cys_3 and Cys_4 for the membrane targeting of GAP-43 (El-Husseini *et al.*, 2001; Liang *et al.*, 2002). Abolishing the targeting of GAP-43 to the membrane fraction was important for our studies as we wanted to determine if the impact of GAP-43 overexpression on the metabolic processing of APP could potentially be due to a physical interaction between the two proteins. Although it is conceivable that GAP-43 could effect a change in APP processing via targeting to a soluble target, as later determined,

membrane association was essential for the modulation of APP processing by GAP-43 overexpression.

When GAP-43 CCAA was co-expressed with APP_{SWE} in HEK293 cells, the impact of GAP-43 overexpression on APP processing was abolished (**Figure 9**). This result implied that the palmitoylation of Cys_3 and Cys_4 of GAP-43 is necessary for a functional interaction between APP and GAP-43. We further deduced that GAP-43 may influence the processing fate of APP through a protein-protein interaction, either directly or indirectly via a multi-protein complex. This is the first known report to show that GAP-43 is not only able to influence the processing of APP, but that this effect is dependent upon the palmitoylation of Cys_3 and Cys_4 of GAP-43. Since we noted that GAP-43 CCAA did not impact the processing fate of APP, we wanted to determine if its inability to do so could be due to a lack of a physical interaction between the GAP-43 CCAA double mutant and APP.

To test our hypothesis, we co-expressed FLAG-tagged versions of either wild type GAP-43, GAP-43 CCAA, or GFP along with APP_{SWE} in HEK293 cells and, using an anti-FLAG antibody, immunoprecipitated complexes. We then probed for the presence of APP in the complexes using an anti-APP antibody after running the immunoprecipitates via SDS-PAGE. When APP_{SWE} was co-expressed with FLAG-tagged versions of either GAP-43 CCAA or GFP, we were not able to detect APP in the immunoprecipitate, thus indicating that the proteins do not interact *in vitro* (**Figure 10**). However, when APP_{SWE} was co-expressed with FLAG-tagged GAP-43 in HEK293 cells, we did detect APP in the immunoprecipitate (**Figure 10**). These findings agree with those published by Norstrom et al. as they found that GAP-43 co-immunoprecipitates with APP *in vivo* from mouse brain (Norstrom *et al.*, 2010). Although no other studies have demonstrated an interaction between APP and GAP-43, Inoue et al.

demonstrated an interaction between GAP-43 and γ-secretase (Inoue *et al.*, 2015). The findings of Inoue et al. do not contradict ours as it may be that GAP-43 interacts with APP in a multi-protein complex, a complex which may include γ-secretase.

Significance

As a result of our studies, we were able to successfully confirm and characterize a novel protein-protein interaction between APP and GAP-43. These studies reveal that through the palmitoylation of Cys_3 and Cys_4, GAP-43 is targeted to the membrane and moreover, that the targeting of GAP-43 to the membrane is essential for an interaction between APP and GAP-43. Furthermore, we found that the interaction of GAP-43 with APP reduces the accumulation of byproducts of APP metabolism. These findings represent the first report of a functional APP-GAP-43 protein-protein interaction. Additionally, these findings indicate that GAP-43 may play a relevant role in AD pathogenesis by modulating APP processing. Although this discovery is very important, much work still needs to be done.

Future directions

Although we have shed light on a new factor that influences the processing of APP as it relates to AD pathology, it is important to continue to test the mechanics and significance of this interaction. It will be important to determine how GAP-43 expression influences the other byproducts of APP metabolism. By determining the impact of GAP-43 expression on the other metabolites of APP, we will gain a better understanding of how GAP-43 influences APP processing. Additionally, it will be important to determine at which subcellular sites the two proteins interact. Do they interact at more than one site? Is the physiological outcome the same

at different sites? Based on the reduction of both α- and β–CTF levels, the subcellular location of a potential APP-GAP-43 interaction is likely to be early in the secretory pathway. That is, since both α- and β–CTF levels are reduced, the interaction seems to be guiding APP towards sites capable of both α- and β-secretase activity, and possibly γ-secreatse activity, which is primarily the early secretory pathway, and away from sites predominantly responsible for either α- or β–secretase activity, which is the plasma membrane and late endosomal pathway respectively (Chow *et al.*, 2010). However, this hypothesis needs experimental validation. Further tests will need to be conducted in order to determine if GAP-43 interacts directly with APP and/or α- or β–secretase or, as the findings of Inoue indicate, γ-secreatse (Inoue *et al.*, 2015). Additionally, it will be important to determine if this effect is cell line specific. If the impact of GAP-43 overexpression on APP processing is not limited to HEK293 cells, testing should be conducted in an *in vivo* system. It will be interesting to see if this interaction does indeed have any relevance to the onset and progression of AD.

While we do not currently have the answers to these questions, it is an exciting possibility that should be explored further as this finding illuminates a means by which APP processing is influenced. Once we can paint a clear picture of all the factors that influence APP processing and Aβ formation and deposition, we can then design a therapy to combat the disease.

Conclusions

As a result of the experiments conducted throughout the course of this study, we determined: 1) GAP-43 overexpression reduces APP processing. 2) Palmitoylation of Cys_3 and Cys_4 of GAP-43 is required for membrane targeting of GAP-43. 3) Membrane targeting of GAP-43 is required for GAP-43 to impact APP processing and to form a complex with APP *in vitro*. Due to

the sum total of these findings, we concluded that GAP-43 interacts with APP in a manner that alters the prcoessing fate of APP and that this functional interaction is dependent upon the palmitoylation of Cys_3 and Cys_4 of GAP-43 (**Figure 11**).

Figure 11: Study Conclusions

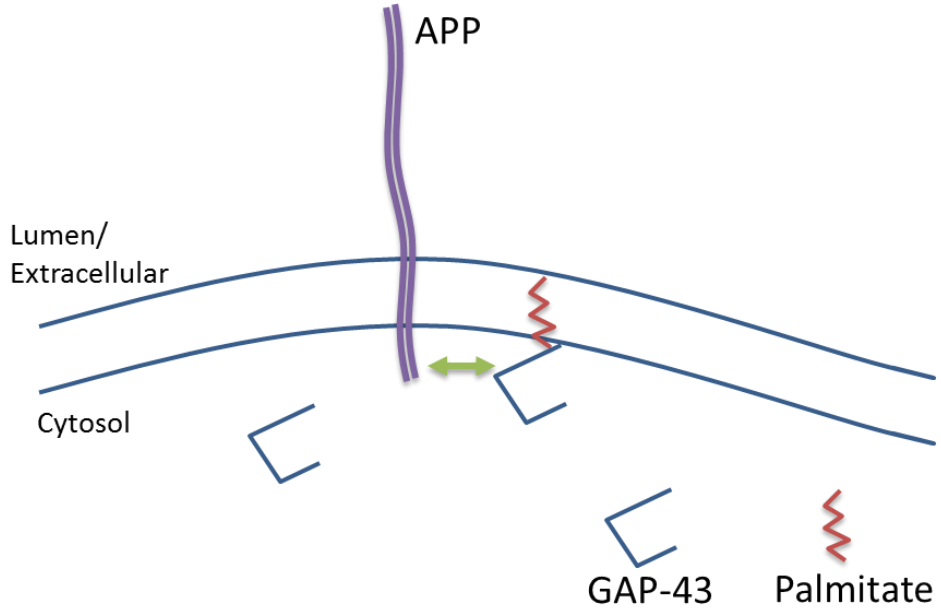


Figure 11: The above figure graphically depicts the conclusions from our studies: 1) GAP-43 functionally interacts with APP in a way that alters its processing fate. 2) The APP-GAP-43 interaction is dependent on the palmitoylation of Cys3 and Cys4 of GAP-43.

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