Familial Alzheimer's Disease Mutations Decrease Gamma-secretase Processing of Beta Amyloid Precurson Protein

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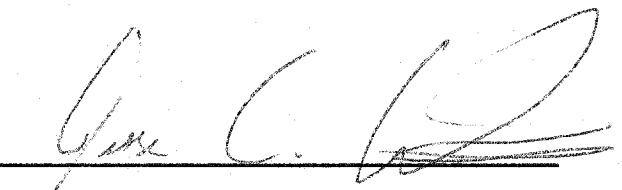
Signature 

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Abstract

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Gamma-secretase mediated cleavage of the β-secretase processed β-amyloid precursor protein leads to the extracellular release of Aβ40 and Aβ42, the more amyloidogenic form of the β-amyloid peptide, which subsequently forms the amyloid-plaques in the brains of Alzheimer's Disease patients. Mutations in β-amyloid precursor protein, Presenilin-1 and Presenilin-2 associated with forms of Familial Alzheimer's Disease (FAD) increase release of Aβ42, suggesting that FAD may directly result from increased γ-secretase activity. Here we show that FAD mutations clustered near the sites of γ-secretase cleavage actually decrease γ-secretase mediated cleavage of the β-amyloid precursor protein, as assessed by release of the intracellular fragment (CTFγ). Concordantly, Presenilin-1 mutations that result in Alzheimer's Disease also decrease the release of CTFγ. These data indicate that Alzheimer's mutations in β-amyloid precursor protein and Presenilin-1 may be categorized as loss of function mutations, which would result in decreases in signaling associated with normal transcriptional regulation by β-amyloid precursor protein.

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Chapter I: Introduction

History of Alzheimer's Disease: a short overview

Overview

Alzheimer's Disease (AD) bears the name of the neuropathologist and psychiatrist, Alois Alzheimer, who originally characterized a unique pathology associated with a patient demonstrating presenile dementia in 1907. The brain of this patient, refered to as Auguste D., was examined by Nissl-staining post-mortem and found to possess diffuse cortical atrophy, nerve cell loss, plaques and tangles(Alzheimer 1907); findings which to this day are considered definitional attributes of Alzheimer's Disease. In the 1930's it was observed that this disorder ran in families; a similar type of dementia affected members of a four generation pedigree, and autopsy revealed the disorder to be what is now refered to as Alzheimer's Disease(Schottky 1932). Shortly thereafter, similar findings were observed by other researchers(Lowenberg 1934; McMenemy 1939), setting the stage for later genetic explorations of the molecular etiology of what became known as Familial Alzheimer's Disease (FAD).

In the 1950s, a large study in Sweden examining inheritance patterns of Alzheimer's pathology concluded that the etiology was multifactorial, and that, particularly in "presenile" early-onset cases, was likely to be a dominantly inherited disorder(Sjogren 1952). Several studies in the 1980s confirmed familial clusters of AD, including pedigrees indicating autosomal dominant inheritance(Breitner and Folstein 1984; Breitner et al. 1988). In many instances, however, heritable causes of the disease

were less clear(Fitch et al. 1988). In the later study, an examination of 91 families of patients with AD, only 43% of the patients demonstrated a familial history of the disorder, suggesting that Alzheimer's pathology could be either 'genetic' or 'sporadic', with no clear pathological differences between the two. The distinction between the familial and nonfamilial emergence of the disorder led to the classification of AD as familial (FAD) and sporadic. Estimates of the proportion of AD instances in each class vary. The majority of cases, however, are 'sporadic', suggesting a multifactorial etiology(St George-Hyslop 2000; Warwick Daw et al. 2000).

By the 1960's Alois Alzheimer's original depiction of the neuropathology associated with 'presenile' dementia had been observed in senile forms of dementia as well. Some 50-70 percent of cases of AD occur in aged members of the population with pathologic findings indistinguishable from those with "presenile" dementia(Selkoe and Podlisny 2002). Less than 1 percent of the population between the ages of 60-64 suffer from AD, but the age specific incidents increases precipitously to 35-40 percent of the population by age 85(Breteler et al. 1992). Hence, only ~2-3 percent of AD cases reflect the original 'presenile' definition(Ott et al. 1995). The distribution of FAD onset age and inheritance patterns was found to be bimodal, suggesting divergent genetic mechanisms(Farrer et al. 1990). In a group of 70 families in which 541 individuals had inherited the disorder and 1066 had not, the distribution of inheritance was found to segregate around 58 years of age. The probability of inheritance was lower for the early-onset than the late-onset form the disease. Early-onset AD demonstrated an inheritance pattern that was consistent with an autosomal dominant mode of transmission. However,

late-onset AD had a higher probability of transmission than could be explained by an autosomal dominant model alone. Consequently, the bimodal distribution of inheritance probabilities suggested distinct etiological explanations for the early-onset and late-onset forms of the pathology.

Mapping and isolation of AD genes

The "multifactorial" nature of AD presented difficulties in initial attempts to genetically map associated gene loci. One of the early problems was the failure to distinguish between early and late-onset forms of the disorder. The presumption was that presenile and senile forms of dementia would have a common molecular etiology. However, even within studies looking at the early-onset form of AD, there were discordant observations about genetic loci. Genetic mapping using anonymous DNA markers established linkage between AD and a specific region of chromosome 21(St George-Hyslop et al. 1987). This study examined 4 kindred groups in which FAD was prevalent. The linkage to chromosome 21 was intriguing, as Downs Syndrome patients develop an AD pathology early in life(Coyle et al. 1986). Downs Syndrome results from Trisomy 21, in which an extra copy of chromosome 21 is propagated. Consequently, the linkage of FAD to this loci suggested a potential genetic explanation for the similarity of the AD pathology in the two disorders.

The isolation of the β-amyloid peptide from plaque cores(Glenner and Wong 1984a; Glenner and Wong 1984b) led to the identification of the β-amyloid precursor protein (APP) gene, and its subsequent mapping to chromosome 21(Kang et al. 1987; Tanzi et al. 1987b). However, the identification of the APP gene lead to disparate reports

about its putative role in AD pathogenesis. The absence of duplication of the APP gene within sporadic AD patients suggested a different mechanism of disease propagation than observed in Downs syndrome(Tanzi et al. 1987a). Examination of the APP loci in FAD families by RFLP analysis concluded that there was recombination between AD and the APP loci, suggesting that APP could not be the "Alzheimer's gene"(Tanzi et al. 1987c; Van Broeckhoven et al. 1987). Subsequent investigation used more chromosomal markers and distinguished between early and late-onset forms of the disorder. These efforts led to the discovery that, within many families, the AD mutation did map to chromosome 21 in early-onset cases, but that late-onset forms of the disorder had a divergent etiology(St George-Hyslop et al. 1990). This study concluded that AD may not be a genetically homogenous disorder with a single etiological derivation. Reports that the Volga Germans, classic instances of the early-onset form of the disorder, had no linkage to chromosome 21 bolstered the idea that multiple molecular etiologies may underlie AD(Schellenberg et al. 1988).

Multiple genetic mechanisms playing a role in the development of AD pathology fit emerging data that other chromosomal loci may be linked to AD. An examination of predominantly late-onset patients in an extensive mapping study found strong linkage between a loci on chromosome 19 and heritable AD(Pericak-Vance et al. 1991). The contribution of the apolipoprotein E (ApoE) alleles to AD pathogenesis was already emerging, demonstrating that the epsilon 4 allele may be the predominant genetic risk factor for the late-onset form of AD(Corder et al. 1993). Observations that ApoE4 (ApoE epsilon 4 allele) binds to β-amyloid in an allele specific manner suggested a potential

mechanism whereby ApoE4 could contribute to AD pathogenesis(Schmechel et al. 1993; Strittmatter et al. 1993a; Strittmatter et al. 1993b). The role of specific alleles of ApoE in AD was extended by the observation that the E2 allele confers protection against the development of late-onset AD pathology(Corder et al. 1994). Involvement of the ApoE allele in AD pathogenesis had been observed in sporadic forms of AD as well. Over 60 percent of sporadic AD patients had the E4 allele compared with 20 percent of the control group(Rebeck et al. 1993). The role of ApoE in AD and earlier mapping studies converged when the ApoE gene was mapped at high resolution to the position of chromosome 19 associated with increased risk of late-onset AD(Chartier-Harlin et al. 1994). These findings, coupled with countless reports in recent years, have demonstrated that ApoE may be the predominant genetic risk factor in both sporadic and familial late-onset AD.

By the early 1990s, it was observed by multiple groups that a major genetic risk factor for the early-onset form of AD mapped to chromosome 14(Mullan et al. 1992b; St George-Hyslop et al. 1992; Van Broeckhoven et al. 1992). The identification of this locus, and the previous work discussed above on the ApoE allele, established the genetic heterogeneity of AD. While the locus on chromosome 14 had been recognized as conferring risk for the early-onset form of AD in the early 1990s, it was not until 1995 that it was mapped to S182 by unbiased linkage analysis and utilization of positional cloning techniques(Levy-Lahad et al. 1995b; Rogaev et al. 1995; Sherrington et al. 1995). Contemporaneously, mapping studies also identified another locus on chromosome 1, the genetic locus associated with familial AD within the Volga German population(Levy-

Lahad et al. 1995a; Levy-Lahad et al. 1995b; Rogaev et al. 1995). The identification of these two genes, both linked to early-onset forms of heritable AD, and demonstrating high levels of homology, led to the renaming of both genes as Presenilin-1 and Presenilin-2. In the same year, sel-12 was isolated in a screen of mutants for perturbation of lin-12 (Notch homologue) function(Levitan and Greenwald 1995), opening the door to use of *C. elegans* as models for the study of the pathogenesis of AD.

Discovery of mutations in APP, Presenilin-1 and Presenilin-2

The previous mapping of early-onset AD to the APP gene on chromosome 21 within some kindred groups was confirmed by the discovery of individual mutations in the APP gene within afflicted families. The first observation of a novel specific mutation associated with heritable AD was in families demonstrating cerebral hemorrhage and amyloidosis(Levy et al. 1990; Van Broeckhoven et al. 1990). This "Dutch" mutation had a single cytosine to guanine tranversion at codon 693 resulting in an amino acid conversion from Glu to Gln. While this group of patients predominantly suffered from microvascular amyloidosis and consequent cerebral hemorrhage, the genetic linkage with the APP gene, and excessive production of β-amyloid deposits, was clear. The Dutch mutation resides within the extracellular portion of the Aβ peptide sequence. Other mutations at this position have since been discovered. The 'Arctic' mutation, Glu693Gly, demonstrated that other mutations at this codon position could result in more classic manifestations of AD(Kamino et al. 1992). Shortly after the discovery of the 'Dutch' mutation, another missense mutation was found in APP within a group of early-onset AD patients in London. This mutation mapped to codon 717 and resulted in a shift

from Val to Ile(Goate et al. 1991). The identification of this mutation among a kindred group that demonstrated genetic linkage to chromosome 21 reconfirmed the previous observations that in some forms of early-onset heritable AD, the APP gene is the disease locus. Simultaneously with the discovery of the London mutation, two other groups identified individual mutations at codon 717 of the APP gene. The Val717Phe and Val717Gly mutations were found by separate groups to be associated with AD pathology(Chartier-Harlin et al. 1991; Murrell et al. 1991). In contrast to the 'Dutch' mutation, the 'London' mutation mapped to the transmembrane region of the APP gene near the carboxy-terminal region of the Aβ peptide.

The most famous APP mutation was identified the following year within an early-onset familial form of AD in a group in Sweden. The mutation entailed a dual nucleotide transversion at codon 670/671, resulting in the substitution of two amino acids from Lys and Met to Asn and Leu(Mullan et al. 1992a). This "Swedish" mutation occurs in the extracellular domain of APP adjacent to the amino-terminal region of the Aβ peptide. Accordingly, it appeared that mutations in APP throughout the Aβ peptide region could cause early-onset AD. However, the biochemical mechanism of how these mutations couple to elevations in Aβ deposition had yet to be explored. Over the next decade, multiple mutations in the APP gene had been isolated that occur within, or adjacent to, the portion of APP encoding the Aβ peptide. The topology of these mutations distributed to both the extracellular domain (Iowa mutation(Grabowski et al. 2001)) and proximal to the C-terminal portion of the Aβ peptide within the transmembrane domain (Austrian

Thr714Ile(Kumar-Singh et al. 2000a), Iranian Thr714Ala(Pasalar et al. 2002), French Val715Met(Ancolio et al. 1999), German Val715Ala(De Jonghe et al. 2001), Florida Ile716Val(Eckman et al. 1997), Ile716Thr(Terreni L 2002), Indiana Val717Leu(Murrell et al. 2000) and Australian Leu723Pro(Kwok et al. 2000)). The last member of the list, the "Australian" mutation, is of particular interest as it falls within a region of the transmembrane domain C-terminal to the Aβ coding sequence. Interestingly, the "Australian" mutation is adjacent to the "epsilon" cleavage site that will be discussed later in this work. Multiple mutations mapping to the APP gene in early-onset AD unequivocally established the APP locus as one of the key genetic determinants of AD pathogenesis.

The identification of APP mutations associated with early-onset AD fit well with the data coming from Down syndrome patients. It had been observed in Down syndrome patients that the disorder correlated with elevated levels of Aβ in both the cerebrospinal fluid and plasma(Tokuda et al. 1997; Teller et al. 1996). Within the Down syndrome population it was observed that diffuse plaques composed mostly of a longer form of Aβ (Aβ42) deposit first, followed by the deposition of Aβ40. Over many years, often decades, the pathology becomes more severe, ultimately leading to neuritic plaque formation(Lemere et al. 1996). This suggested a progressive pathology in Down syndrome. However, strong evidence that Down syndrome AD-like pathology is specifically associated with the APP gene came from a case study. A patient with partial Trisomy 21 who was disomic for the APP gene had no noticeable mental decline in later

years, nor were any AD pathologies noted at autopsy(Prasher et al. 1998). Given the almost complete penetrance of AD pathology in Down syndrome subjects, this case supported a pathology based upon an APP gene-dosage effect.

Several Presenilin-1 missense mutations were isolated within its coding region, all of them associated with early-onset FAD(1995; Campion et al. 1995; Clark et al. 1995; Cruts et al. 1995; Perez-Tur et al. 1995; Rogaev et al. 1995; Sherrington et al. 1995; Sorbi et al. 1995; Tanahashi et al. 1995; Wasco et al. 1995). The number of Presenilin-1 missense mutations has grown to over 80(Selkoe and Podlisny 2002). Multiple missense mutations associated with early-onset FAD have also been mapped to the Presenilin-2 gene(Levy-Lahad et al. 1995a; Levy-Lahad et al. 1995b; Rogaev et al. 1995), including the Volga German mutation. The total number of Presenilin-2 mutations is currently 6(Selkoe and Podlisny 2002). The number of mutations mapping to the Presenilin-1 and Presenilin-2 genes may relate to their relative role in determining genetic predisposition to AD. A large study of early-onset FAD patients in 34 families in France examined the prevalence rates of mutations in the APP, Presenilin-1 and Presenilin-2 genes. They found that approximately 56 percent of families had mutations in Presenilin-1 and 15 percent had mutations in APP(Campion et al. 1999). No mutations were identified in the Presenilin-2 gene. Accordingly, as much as 71 percent of heritable early-onset AD may be attributed to mutations in Presenilin-1 and APP. Presenilin-2 mutations may just be more rare. However, the generally more severe AD progression in patients with Presenilin-1 mutations also suggests that Presenilin-1 may more potently promote AD pathogenesis(Selkoe and Podlisny 2002).

Pathology of AD

The genetic heterogeneity associated with heritable forms of AD that manifest no distinguishable pathological characteristics led to the speculation that AD may be a "family of diseases" with many etiologies and a common pathology(Hardy 1997). The primary characteristics of AD pathology are the presence of amyloid plaques and neurofibrillary tangles localized to the cerebral cortex (primarily within the association cortices), hippocampus, amygdala and entorhinal cortex(Selkoe 1996). The amyloid depositions can be of more than one type, from the pathologically less significant 'diffuse plaques', which demonstrate lower levels of amyloid deposition, to 'neuritic plaques', which demonstrate a more pronounced degree of fibrillar amyloid deposition and are associated with dystrophic neurites (degenerating dendrites and axons)(Selkoe 1996). The plaques are primarily composed of 8 nm filaments of multimeric Aβ. It is has been suggested that the different plaque types may represent progressive stages of the disorder(Selkoe 2001). Often the deposition of Aβ occurs within the microvasculature of the brain, resulting in amyloid deposition in the basement membranes and smooth muscles of the microvessels in cortical and meningeal vasculature—a pathology referred to as congophilic amyloid angiopathy (CAA)(Selkoe and Podlisny 2002). Amyloid depositions may result in hemorrhage of the microvasculature as Aβ has been shown to be cytotoxic to vascular endothelial cells(Eisenhauer et al. 2000), and to play a role in microvessel damage and cerebral hemorrhage(Wang et al. 2000).

Another cardinal feature of AD pathology are neurofibrillary tangles (NFT). These are composed of aggregates of hyperphosphorylated tau. NFTs may prevent tau from binding and stabilizing microtubules(Mudher and Lovestone 2002), and thus could interfere with neuronal axonal transport. At the electron microscope level, one observes approximately 20 nm paired helical filaments (PHF) of aggregated hyperphosphorylated tau, sometimes at synapses, and often associated with 'dystrophic neurites'(Selkoe 1996). There is an ongoing debate over which pathological feature of AD—amyloid plaques or NFTs—is more relevant to the manifestations of the disease(Mudher and Lovestone 2002).

Elucidation of the aggregation of Aβ as a multi-step process, from monomeric form to soluble fibrils to amyloid plaques, has cast more complexity into the determination of the role of amyloid in AD pathology. Amyloid fibrils, or 'protofibrils', are soluble 6-8 nm by 200 nm aggregates of oligomerized amyloid monomers(Walsh et al. 1999; Walsh et al. 1997; Ward et al. 2000). Protofibrillar amyloid has been implicated as the physiologically relevant form in AD pathogenesis due, in part, to the observation that soluble protofibrils result in shifts in the electrophysiological responsiveness of cultured neurons, and can induce neuronal cell loss(Hartley et al. 1999). Further, amyloid protofibrils may be involved in the depression of electrophysiological mechanisms of memory formation as protofibrillar amyloid depresses the induction of long-term potentiation (a model of synaptic plasticity associated with memory formation)(Walsh et al. 2002). The involvement of protofibrillar amyloid in AD

pathology was supported by the biochemical observation that Aβ42 aggregates into protofibrillar form more rapidly than does Aβ40(Harper et al. 1997; Jarrett et al. 1993).

The potential role of protofibrillar Aβ in AD pathogenesis may partially resolve conflicting reports on the relevance of amyloid plaque formation to the development of dementia. In work examining dementia severity and amyloid plaque formation observations both supporting(Di Patre et al. 1999) and refuting(Nagy et al. 1995) the correlation have been reported. While the above studies suggest that Aβ can cause neuronal toxicity, it has been reported that there is poor correlation between neuronal cell loss and amyloid plaques in AD patients(Carter and Lippa 2001). The latter study challenges the idea that amyloid deposition triggers the cascade of events leading to the degeneration of cortical neurons observed in AD pathology. Further, neurofibrillary tangles have been reported to be a better diagnostic marker than amyloid plaques(Nagy et al. 1996; Nagy et al. 1995). How some individuals with high levels of neuritic plaques and neurofibrillary tangles fail to demonstrate any dementia is a point of confusion that is currently unresolved(Price 1997; Troncoso et al. 1996).

Processing of APP, Presenilin-1 and Presenilin-2

The discovery of the APP gene and the genetic identification of this gene locus as a risk factor for the development of AD immediately led to the observation that the pathogenic Aβ peptide was derived from a small portion of the whole APP gene product. The holoAPP protein has three major splice variants encoding proteins of 695, 751 and 770 amino acids. The latter two forms are distinguished from the APP695 form in two

respects. First, these two isoforms contain a region of the extracellular domain (Kunitz inhibitory domain) that is highly homologous to serine protease inhibitor peptides, the most notable member of the family being bovine pancreatic trypsin inhibitor (BPTI)(Selkoe 2001). Second, while APP751 and APP770 are more ubiquitously expressed, the APP695 form is expressed at much higher levels in neurons(Haass et al. 1991; LeBlanc et al. 1991; Yamada et al. 1993). The lack of the Kunitz inhibitor domain within APP695 may also suggest specific functions of neuronal APP. Some APP binding partners, such as the low-density lipoprotein receptor-related protein (LRP), may depend on this region for association(Kinoshita et al. 2001). However, all of the isoforms of APP contain the Aβ peptide sequence.

Since the extracellular accumulation of β-amyloid a priori necessitates the excision of this fragment of the protein from the holo-form, an exploration of APP processing began almost immediately following the cloning of the APP gene. The initial insight into APP proteolysis, from two separate groups, was that a large extracellular region of APP was cleaved and released(Esch et al. 1990; Sisodia et al. 1990). The soluble extracellular domain of APP was observed in the medium of cultured cells, yet the cleavage was internal to the amyloid portion of APP. Consequently, both groups speculated that generation of β-amyloid may reflect a perturbation of "normal" processing(Esch et al. 1990; Sisodia et al. 1990). However, the idea that Aβ production was due to aberrant APP processing was disputed by the observation that cultured cells release the Aβ peptide, and a smaller fragment (since referred to as p3), under normal

culture conditions(Haass et al. 1992). This also opened the door to utilizing cell culture models to study the pathogenic effects of FAD mutations upon Aβ production. The originally observed 'normal' processing site has since been identified as the alpha-secretase cleavage site. While it was originally ambiguous that proteolytic activity was responsible for this cleavage event, it was later recognized that a matrix metalloproteinase (gelatinase) could cleave APP and liberate the soluble extracellular domain(Miyazaki et al. 1993). Subsequently, many different proteolytic models were proposed. Identification of a specific member of the metalloproteinase family as the physiologically active alpha-secretase finally came about with the observation that in null mutant mice for the TNF alpha converting enzyme (TACE) the majority of alpha-secretase cleavage of APP was abolished(Buxbaum et al. 1998). ADAM9 and ADAM10, other members of the metalloprotease family, have since been implicated in the alpha-secretase cleavage of APP as well(Kojro et al. 2001; Asai et al. 2003). The potential multiplicity of alpha-secretases involved in APP processing suggests that events differentially regulating ADAM9, ADAM10 and ADAM17 may control the liberation of the soluble ectodomain in a broader set of circumstances and tissues. Consequently, this may provide a panoply of mechanisms to promote alpha-secretase cleavage of APP, which is thought to potentially play a mitigatory role in AD pathogenesis, as alpha- and beta-secretase cleavage of APP are reciprocally prohibitive(Skovronsky et al. 2000).

While the identification of alpha-secretase has contributed greatly to the understanding of APP processing, it did not address the issue of how the pathogenic Aβ peptide was formed since alpha-secretase cleavage occurs internal to the Aβ sequence.

Original speculation about the nature of the elusive beta-secretase suggested that the matrix metalloprotease gelatinase may function as the beta-secretase(LePage et al. 1995). However, this possibility was refuted when it was observed that mice homozygous null for gelatinase did not have any detectable decrease in Aβ production(Itoh et al. 1997). It had also been postulated that members of the lysosomal cathepsin family of metalloproteases may function as the beta-secretase within endocytosed fractions of APP(Brown et al. 1996; Chevallier et al. 1997; Marks et al. 1995). However, the real identification of the beta-secretase did not occur until the aspartyl-protease BACE was isolated(Vassar et al. 1999), an atypical member of the pepsin family(Haniu et al. 2000). The role of BACE as the long-sought after beta-secretase was demonstrated by showing that over-expression of the BACE protein led to increases in amyloid production, that endogenous amyloid secretion could be drastically diminished using RNAi knockdown of BACE expression, and that purified BACE protein cleaves APP with the same site specificity as beta-secretase(Vassar et al. 1999). The proverbial nail in the coffin was driven in when BACE homozygous null mutant mice demonstrated no beta-secretase activity in brain(Roberds et al. 2001).

Elucidation of the alpha- and beta-secretases explains the proteolytic mechanisms by which the soluble ectodomain is generated, but it leaves open a formidable question: what is the gamma-secretase? Initial attempts to characterize the gamma-secretase suggested that perhaps it was the chymotrypsin associated activity of the 'multicatalytic enzyme', more commonly known as the proteasome, as it appeared to cleave fluorescently labeled substrate analogues(Mundy 1994). Other studies suggested that

gamma-secretase activity was consistent with lysosomal cathepsins, as was speculated for the beta-secretase(Marks et al. 1995). While the exact role of these two general classes of proteolytic complexes in the processing of APP is not fully resolved, it soon became clear that the transmembrane proteolysis predicted for the gamma-secretase was due to neither complex, but instead to the early-onset FAD genes Presenilin-1 and Presenilin-2.

The initial insight leading to the idea of the presenilins as the 'gamma-secretase' was the observation that APP and the presenilins physically associate. Immunoprecipitation studies demonstrated that a non-covalent attachment between either Presenilin-1 or Presenilin-2 and the holoAPP protein was established early in the processing pathway as predominantly immature/partially-glycosylated forms of APP were detected(Weidemann et al. 1997; Xia et al. 1997). The original supposition was that presenilins functioned as modifiers of the ultimate proteolytic gamma-secretase cleavage. The predicted topology of the presenilins, owing to the exploration of *C. elegans* homologue sel-12, suggested that they form an 8 pass transmembrane protein with small extracellular loops and two large cytosolic loops occurring at the N-terminus and between transmembrane domains (TM) 6 and 7(Li and Greenwald 1996). The high degree of homology between *C. elegans* and mammalian presenilins suggested a similar structure for the human presenilin genes. Given that the gamma-secretase was known to cleave in the transmembrane domain of APP, this topology fit with the hypothetical notion of an intramembranous protease. The vast majority of Presenilin-1 FAD mutations mapped to the putative transmembrane regions, suggesting that key functionality was embedded in

the transmembrane domains. However, irrefutable association between gamma-secretase activity and presenilins came about with the demonstration that presenilin null cells exhibit a build-up of the alpha- and beta-secretase cleavage products and have undetectable levels of secreted Aβ(De Strooper et al. 1998). These observations were extended with the demonstration that presenilin modified processing of APP in the ER and Golgi compartments(Xia et al. 1998)(Murayama et al. 1999). The observation that Aβ formation could be modified by presenilin in the ER and Golgi fit with early observations of intracellular accumulation of Aβ in cell culture models(De Strooper et al. 1995; Martin et al. 1995; Yang et al. 1995) and fed the speculation about the relative pathogenicity of the secreted and intracellular forms of Aβ.

Characterization of sel-12 in *C. elegans* suggested that post-translational proteolytic processing occurred in the cytosolic loop between TM6 and TM7. The similarity in processing between the *C. elegans* and mammalian presenilins was demonstrated as human presenilins were found to undergo endoproteolysis to form an N- and C-terminal fragments(Thinakaran et al. 1996). Later, endoproteolytic cleavage of mammalian presenilin was shown to map to the cytosolic loop spanning the junction between TM6 and TM7, just as had been observed with the worm. Interestingly, almost no uncleaved presenilin was detected in either cell culture models or within brain extracts generated from normal or AD patients. This observation was supported by the characterization of temporal processing of presenilin in neuroblastoma cells. Pulse-chase experiments demonstrated that the half-life of the unprocessed form of presenilin was

approximately 1.5 hours, while the half-life of the endoproteolyzed N- and C- terminal fragments was 24 hours(Ratovitski et al. 1997). This suggested that endoproteolysis happens rapidly and may play a role in the stabilization of the resultant fragments.

The notion that endoproteolysis may be critical for presenilin function was supported by the observation that there are two conserved aspartate residues within transmembrane domain 6 and 7. Use of peptidomimetic compounds developed as transition state analog inhibitors of gamma-secretase had already been employed to suggest that gamma-secretase was an intramembranous aspartyl protease(Wolfe et al. 1999a). The mutagenesis of these two aspartate residues demonstrated that Asp to Ala conversion of either residue resulted in both loss of gamma-secretase activity and a shift towards the holoprotein form(Wolfe et al. 1999b). In addition to suggesting a link between endoproteolysis and gamma-secretase activity, this evidence also strongly suggested that presenilin was not a co-factor of the gamma-secretase but was itself the intramembranous aspartyl protease requisite for the final stage of APP processing. Subcellular localization of the endoproteolysis suggested it was an early processing event as presenilin endoproteolysis was observed in the ER and Golgi(Zhang et al. 1998), demonstrating a potentially similar locale to where presenilin-dependent modification of APP processing had been observed to occur. The ER and Golgi localized processing of presenilin and APP led to the "spatial paradox", the conundrum that presenilin, and consequently gamma-secretase, localized to early events in protein sorting while gamma-secretase activity was believed to occur at the cell surface(Annaert and De Strooper 1999; Neve et al. 2000).

Whether the endoproteolytic processing of presenilin is required for its subsequent biological function is not clear. One early-onset FAD mutation resident within Presenilin-1 results in the elimination of a splice junction between exon 8 and exon 9 (deltaexon 9 mutant)(Selkoe and Podlisny 2002). The splice junction deletion results in the elimination of exon 9, and an in-frame splice junction between exon 8 and exon 10. Since exon 9 contains the site for endoproteolysis, the mutant protein is not cleaved into N- and C-terminal fragments. The deltaexon 9 mutant, like the other presenilin FAD mutations, results in elevated Aβ42 production. However, to address whether it was the impact of the missense mutation on the coding sequence or the lack of endoproteolysis that accounts for the elevation in Aβ, the mutant Cys residue at position 290 was reverted to the normal Ser residue(Steiner et al. 1999). The surprising finding was that restoring the mutant Cys residue to Ser did not impact the level of endoproteolysis of the deltaexon 9 mutant. However, it did reduce Aβ production to normal levels. What general conclusions can be drawn about the impact of the endoproteolytic processing of presenilin upon its subsequent biological activity is not clear. Yet, it does suggest that endoproteolysis may not be necessary for biological function, at least not in the absence of the portion of presenilin encoded by exon 9.

Characterization of the gamma-secretase complex

The 'spatial paradox' of APP processing in subcellular domains of protein trafficking that are discordant with the requisite order of cleavage events has confused

AD research for many years. However, the recent characterization of the gamma-secretase complex as a multimeric association has elucidated not only the biochemistry of gamma-secretase cleavage but also the subcellular targeting of the components. The role of Nicastrin as an essential component of the gamma-secretase complex was suggested by its original isolation in which it was demonstrated that genetic ablation of Nicastrin in *C. elegans* produces a phenotype indistinguishable from the genetic ablation of the two presenilin homologues(Yu et al. 2000). The importance of these findings for AD was concretized within the same work, wherein it was demonstration that Nicastrin associates with presenilin and can modify presenilin-dependent processing of APP(Yu et al. 2000). Subsequently, multiple groups have demonstrated that Nicastrin was essential for the gamma-secretase processing of Notch in Drosophila(Chung and Struhl 2001; Hu et al. 2002b; Lopez-Schier and St Johnston 2002), potentially through stabilization of presenilin, suggesting a clear role for Nicastrin as an essential component of the gamma-secretase complex.

Glycosylation of Nicastrin was reported in the initial observation of Nicastrin as a component of the gamma-secretase complex(Yu et al. 2000), yet the functional significance of this modification was not known. Later, it was shown that Nicastrin glycosylation in the golgi is dependent upon association with presenilin, resulting in elevated stability of the glycosylated form(Herreman et al. 2003; Tomita et al. 2002). The over-expression of Nicastrin in the absence of presenilin results in endoglycosylase H sensitivity and retention, and subsequent degradation, of Nicastrin in the ER(Herreman et al. 2003). Previous reports had already suggested that the Nicastrin interaction with

Presenilin was involved in targeting both proteins to the plasma membrane(Kopan and Goate 2002; Leem et al. 2002). Consequently, this suggests that the Presenilin/Nicastrin complex functions to reciprocally stabilize the two gamma-secretase components which subsequently travel, potentially in association with APP, to the plasma membrane where sequential proteolysis of APP occurs. The reciprocal stabilization model was confirmed by the observation that inhibitory RNA knockdown of Nicastrin resulted in dramatic decreases in the levels of presenilin and that presenilin-/- cells demonstrated decreased levels of Nicastrin, both of which block processing of the alpha- and beta-secretase processed APP(Edbauer et al. 2002b). The 'spatial paradox' previously discussed seems to be resolved by these observations as well. This also explains mechanistically what had been observed repeatedly, presenilin levels seem to have a biochemical set-point. Over-expression of presenilin does not result in elevated levels(Thinakaran et al. 1997), but rather a switch from the endogenous to the exogenously expressed form.

The discovery of Nicastrin as a component of gamma-secretase was followed by what now appears to be a complete characterization of the multimeric gamma-secretase complex. Using a screen of *C. elegans* mutants, two additional genes were discovered that appear to be critical for Notch processing which interact with sel-12 and aph-2, the *C. elegans* homologues of Presenilin and Nicastrin(Francis et al. 2002; Goutte et al. 2002). The identified genes are aph-1 and pen-2, which appear to code for integral membrane proteins. Inhibitory RNA approaches by the same group demonstrated that knockdown of either gene leads to an abrogation of Notch signaling and gamma-secretase processing of APP. Other groups simultaneously discovered that Pen-2 is an essential

component of the gamma-secretase complex and extended the finding with the observation that RNAi knockdown of Pen-2 results in decreases in presenilin levels and increases of the non-glycosylated "immature" form of Nicastrin(Steiner et al. 2002). The observation that Aph-1 interacts with Nicastrin and presenilin was extended to mammalian systems showing again that RNAi inhibition of Aph-1 decreases the gamma-secretase processing of Notch and APP, leading to elevated levels of the gamma-secretase precursor substrate(Lee et al. 2002b). Shortly thereafter it was observed that Aph-1 and Pen-2 bind to the immature and unprocessed forms of Nicastrin and presenilin, and that the functional ablation of these co-factors decrease the amount of processed presenilin. This suggests that these two cofactors may be critical in the initial processing steps involved in the formation of the functional gamma-secretase complex(Gu et al. 2003; Luo et al. 2003). The sufficiency of these four factors to form a functional gamma-secretase complex was demonstrated by introducing human Presenilin-1, Nicastrin, Pen-2, and Aph-1 into yeast, that possess no endogenous gamma-secretase activity. The result was that over-expression of these four genes conferred gamma-secretase processing of APP and Notch to yeast(Edbauer et al. 2003). These data have led to the conclusion, combined with informal reports of failed attempts to isolate other *C. elegans* genes requisite for gamma-secretase activity (J. Nye, Neuroscience Conference, 2002), that these four genes form the complete gamma-secretase complex.

Biochemical Characterization of the FAD Mutations

The identification of discrete early-onset mutations in the APP, Presenilin-1 and Presenilin-2 genes began the search for how disparate genetic mutations could lead to a common pathology. While both neurofibrillary tangles and amyloid plaques are definitional components of the AD pathology, the examination of amyloid states and levels was the more tractable approach in the search for common biochemical mechanisms at the etiology of the disease. Further, the ability to determine amyloid levels from patients using radioimmunoassays with antibodies against the internal Aβ sequence had been demonstrated(Pardridge et al. 1987). The ability to examine Aβ levels in patients also provided a method of accessing potential correlates of the disease pre-mortem. Further, the capacity to examine amyloid secretion from cultured cells(Haass et al. 1992) provided in vitro systems for examining the potential biochemical differences between expression of wild-type and FAD mutants in APP and the presenilins.

The initial observation that an individual APP mutation impacted the levels of Aβ generated came from studies with the KM670/671NL "Swedish" mutation. Over-expression of the mutant form of APP was found to dramatically increase the levels of secreted Aβ40 and Aβ42 in culture models by 6-8 fold(Citron et al. 1992). The two observed forms of Aβ, a shorter Aβ40 that corresponded to the original characterization of the peptide discussed previously, and a longer 42 residue form containing two additional carboxy-terminal hydrophobic amino acids of the APP transmembrane

domain(Selkoe 2001). While the distinction was not significantly noted at this time, the potential difference between long and short forms of Aβ has produced camps of etiologists debating the biological role played by each in AD pathology. Following on the heels of the Swedish mutation, it was determined that the V717I "London" mutation resulted in a 1.5-1.9 fold increase in the level of Aβ42 when over-expressed in neuroblastoma cells(Scheuner et al. 1996). The observation that two specific APP mutations demonstrated a rise in Aβ42 specifically suggested that the FAD mutation may function as a 'gain of function' mutation shifting the gamma-secretase cleavage site down by two residues. The notion of a specific pathology associated with the longer form of Aβ fit with the observations made in Down syndrome in which the initial deposition of amyloid was primarily composed of the longer Aβ42 form. (Lemere et al. 1996)Further, this also coincided with results from reverse polymerization experiments in which AD plaques were resolubilized and fractionated with each solubilization cycle. The observation was that Aβ40 solubilized first, followed by increases in the level of Aβ42(Tamaoka et al. 1994). This promoted the concept of serial deposition of amyloid species wherein Aβ42 deposits first due to its increased hydrophobicity. Further, as previously mentioned, the long Aβ42 form of amyloid has been shown by multiple groups to homo-aggregrate into protofibrillar assemblages more rapidly than the Aβ40 form(Harper et al. 1997; Jarrett et al. 1993). Consequently, these studies suggest that elevated Aβ42 production may lead to increased rates of polymerization into fibrillar form and subsequent deposition onto specific cortical and subcortical areas.

The role of FAD mutations in APP upon subsequent amyloid generation was shown to be a general phenomena. One group demonstrated the positive correlation between FAD mutations and increases in Aβ42/Aβ40 ratios following expression of the mutants in neuronal culture(De Jonghe et al. 2001). This study examined six different transmembrane APP FAD mutations and found that in each instance the ratio of Aβ42/Aβ40 increased, suggesting that a shift in the gamma-secretase cleavage site from Aβ40 to Aβ42 may account for increases in amyloid deposition. Further, the same study went on to demonstrate that there was an inverse correlation between the age of onset of the pathology associated with each individual mutation and the ratio of Aβ42/Aβ40. However, it must be noted that while the Aβ42/Aβ40 count consistently increased, it was not clear, in many instances, that the total level of amyloid increased. In several mutations the level of total Aβ produced appeared to drop. This is a critical study that will be addressed again in the discussion section of this work.

The observation from in vitro cell culture models fits well with the studies examining patients with FAD mutations in APP, Presenilin-1 and Presenilin-2. Plasma Aβ levels taken from individuals with mutations in each of the above mentioned genes consistently showed increased Aβ42(Scheuner et al. 1996). FAD mutations in Presenilin-1 and Presenilin-2 have also consistently resulted in increases in Aβ42 levels in multiple model systems(Citron et al. 1997). In this work it was shown that Aβ42 increases in fibroblasts taken from patients with specific FAD mutations in Presenilin-1 or Preseniln-2. Additionally, this study showed the same finding can be recapitulated in

cell culture models in which exogenous Presenilin-1 or Presenilin-2 was introduced to cell lines stably expressing APP. Further, these observations were extended to transgenic models in which either wild-type or FAD mutant Presenilin-1 or Presenilin-2 constructs were introduced into mice. The FAD presenilin transgenic lines demonstrated elevated levels of Aβ42 production in brain, suggesting that a common mechanisms of presenilin FAD pathogenesis in AD may be novel gain of function for gamma-secretase cleavage at the site liberating Aβ42. Similar results have been repeatedly reported by other groups(Duff et al. 1996). (Sudoh et al. 1998)One report demonstrated consistent elevation of Aβ42/Aβ40 levels in 20 FAD presenilin mutants in which the degree of increase in the ratio correlated with age of onset(Murayama et al. 1999). The mouse modeling of FAD mutants also demonstrated that crossing the Swedish mutant transgenic lines with Presenilin-1 M146L expressing transgenics resulted in substantially elevated Aβ42 levels compared to the single transgenic progenitors(Holcomb et al. 1998). Interestingly, it was noted in both the single Swedish transgenic line and the Presenilin-1/Swedish cross that behavioral impairments preceded the formation of histologically detectible Aβ depositions. This suggested that some behaviorally pathological aspects of the mutations may not be directly associated with amyloid plaque formation. This observation is consistent with previously discussed results suggesting that perhaps 'protofibrillar' amyloid, or some other aberrant component of APP processing associated with the mutations, may underlie early events in the progression of dementia associated with AD.

While gamma-secretase cleavage of APP is the final event prior to the liberation of Aβ, it also releases the intracellular portion of APP into the cytoplasm. The intracellular fragment (referred to as CTFγ for the remainder of this work) should be composed of either 57 or 59 amino acids, depending on whether the cleavage event occurs at the Aβ42 or Aβ40 site respectively. Yet, recent studies suggest that neither site may be the physiologically relevant cleavage point, as CTFγ appears to result from cleavage at position resembling the S3 site of Notch(Sastre et al. 2001; Weidemann et al. 2002; Yu et al. 2001a). Also, resident within CTFγ is a caspase cleavage site that cuts between two potentially binding moieties. The characterization of the resulting product suggests that a 31 amino acid fragment is generated, probably by caspase-8, which appears to be cytotoxic to cultured neurons(Lu et al. 2000). This fragment, referred to commonly as C31, may be increased following BACE cleavage, which is dramatically upregulated in the Swedish mutation(McPhie et al. 2001). Consequently, there appear to be two cytotoxic fragments of APP, both of which may be affected by the FAD mutations in APP and the presenilins.

Genetic Mouse Models of AD

APP transgenic mouse models of AD

Multiple transgenic mouse lines over-expressing individual FAD APP and presenilin mutations have been generated and were briefly discussed in the previous section. This section will review the literature on transgenic and null mutant animals for

APP, Presenilin-1 and Presenilin-2 with respect to the emergent AD like pathology and some of the issues that have arisen in modeling AD in mice over the last ten years. The initial observation that FAD APP mutations could produce critical aspects of AD pathology came about with the generation of a transgenic mouse expressing an FAD variant at the London locus. High levels of expression of APP V717F demonstrated some of the hallmark features of AD pathogenesis including: Aβ deposition, amyloid plaque formation, and microgliosis(Games et al. 1995). The similarity between the neuropathology associated with FAD APP expression in these animals and classical AD neurodegenerative pathology supported the idea that a single point mutation within the APP gene could sufficiently alter APP processing to predispose individuals harboring the mutation to cultivation of the disorder. These observations were followed shortly by similar observation in transgenic mice expressing the Swedish (KM670/671NL) mutation. In the Swedish transgenics, comparative studies of neuropathology and spatial memory were performed to correlate the two components of the disorder in human. Over-expression of the Swedish mutation was found to be sufficient to produce both spatial memory deficits and elevated levels of Aβ deposition in the cerebral and limbic cortices in an age dependent fashion(Hsiao et al. 1996). Increases in both forms of Aβ were observed, with an approximate five-fold increase in Aβ40 and over a ten-fold increase in Aβ42 in brains of transgenic mice relative to controls. The seemingly strong correlation between dementia-like cognitive decline and elevated levels of amyloid deposition suggested that FAD APP mutations were sufficient to drive the generation of

extracellular amyloid deposition and strengthen the perspective that 'amyloid plaques' are the etiological explanation of AD. Similar results were observed by others using both Swedish and London (V717I) transgenic mice, wherein predominantly 'diffuse' amyloid plaque formation was noted(Sturchler-Pierrat et al. 1997). Further, point mutations at the TACE cleavage site, internal to the Aβ sequence, that were shown to decrease APP processing by alpha-secretase also produces neurotoxic effects in hippocampal and cerebral cortices(Moechars et al. 1996). Interestingly, the observed neuronal apoptosis induced by the non-FAD mutant APP did not correlate with increases in Aβ levels suggesting more than one type of disruption in APP processing could lead to neuropathological degeneration in the cortex.

The simple correlation between amyloid deposition and the cognitive decline in AD was originally challenged with behavioral studies of the V717F transgenic mice. Radial arm maze studies demonstrated the behavioral impairment in the task preceded the time point at which amyloid plaque deposition occurred(Dodart et al. 1999). In other studies employing object recognition tasks, the time line between amyloid sedimentation and decreased performance was more closely aligned. However, the observation that behavioral deficits could be produced from FAD APP models in the absence of plaques suggested that the amyloid plaques may not be the initiating event in the pathological progression. This is consistent with the results reported from the APP Swedish/Presenilin-1 mutant discussed at the end of the previous section. Additional work with the APP Swedish mice crossed to Presenilin-1 M146L transgenics explored the temporal correlation of behavioral memory performance tasks and amyloid plaque

formation. The results demonstrated a lack of correlation between the two as the double transgenic animals developed early deficits in the Y-Maze task at time points prior to plaque formation, yet the severity failed to increase appreciably at time points when plaque formation became more pronounced(Holcomb et al. 1999). Further, studies using Morris water maze testing of the dual transgenics failed to demonstrate high levels of impairment in either the acquisition phase or the reprobe trials, even at time points when the animals demonstrated elevated levels of plaque formation.

The conclusion that the authors drew from this work was that the presence of amyloid plaques alone was not sufficient to produce the full pathology associated with AD. Notably, these observations fit with the report from neuropathologists describing the presence of amyloid plaques in non-demented individuals. While the apparent lack of direct temporal correlation may mark the early phase of the disorder, given that time points were only taken until 9 months of age, it also suggests the possibility that amyloid plaque formation is not sufficient to precipitate the whole cluster of traits within AD neurodegeneration and dementia. This conclusion was buttressed by findings comparing several lines of transgenic animals in which it was noted that APP E693Q animals ("Dutch" mutation) bore a striking similarity in behavioral assays to mice carrying the Swedish and London transgene, despite a complete absence of detectable amyloid plaque formation(Kumar-Singh et al. 2000b). Pathological characterization of the animals also revealed similarities with respect to neuronal apoptosis in the cortex. Consequently, correlative studies suggests that one can have both plaques in the absence of detectable behavioral impairment, and behavioral impairments in the absence of plaques. These observations,

combined with the difficulties that individuals have had replicating all of the pathological markers of AD in one model, continue to leave the question open about exactly what aberration in APP processing engenders the highly penetrant pathological progression associated with FAD mutations in human AD.

The prospect of another etiological mechanism of AD progression was postulated by one group that observed that expression of multiple FAD APP mutants in primary neuronal culture produced a shift in products within the FAD mutants towards what is commonly known today to be the BACE cleavage product(McPhie et al. 1997). Transgenic mice over-expressing the BACE C100 fragment of APP demonstrated neuronal degeneration consistent with some of the pathological markers of AD, most notably a disordering of neuritic structure and profound neuronal apoptosis throughout areas of the hippocampus, in the absence of amyloid plaque formation(Oster-Granite et al. 1996). Subsequent work has demonstrated that these animals also have progressively impaired spatial memory capacities(Berger-Sweeney et al. 1999). These results have recently been disputed suggesting that over-expression of C100 alone is not sufficient to produce quantitative changes in the number of pyramid and granule neurons in the hippocampus nor the number of synaptophysin immuno-positive synaptic boutons(Rutten et al. 2003). However, irrespective of the emergent winner between the C100 camps it is clear that transgenic models of APP FAD mutations have yet to produce the endgame of AD pathogenesis.

Presenilin-1 and Presenilin-2 transgenic mouse models

Over-expression of early on-set FAD mutant Presenilin-1 and Presenilin-2 constructs have been repeatedly observed to lead to the development of elevated Aβ42 production in cortical tissues(Duff et al. 1996; Oyama et al. 1998; Siman et al. 2000). While this aspect of aberrant presenilin function is grossly similar to that observed in APP mutant transgenic

lines, there is little evidence supporting that mutant presenilin transgenics produce either amyloid plaque deposition or the impaired memory performance as observed by some groups with the APP transgenic models. This issue is obfuscated by the fact that many of the studies using FAD presenilin transgenics look for effects by back-crossing the animals onto FAD APP mutant lines. In such models it is difficult to disentangle what effects are directly attributable to the presenilin mutation and which are due to modulation of APP mutant effects by FAD mutant presenilin. The lack of behavioral memory deficits in FAD Presenilin-1 transgenic animals was noted in the work examining dual APP/Presenilin-1 mutants. In their assays, animals singly transgenic for M146L did not demonstrate any measurable impairment in the Y-maze task at any age tested(Holcomb et al. 1999).

Despite the lack of clear association between presenilin transgenics and memory impairment, electrophysiological changes associated with expression of a single mutant presenilin transgene have been observed. Examination of the electrophysiological responsiveness of hippocampal slice preparations have demonstrated that M146L and M146V FAD mutant Presenilin-1 lines have elevated excititory glutamatergic response (Barrow et al. 2000; Schneider et al. 2001). These works show that increased calcium efflux from intracellular stores localized to the ER mediated the increased electrophysiological responsiveness. While this facilitates the production of long-term potentiation, it also engenders a proclivity toward excitotoxic response to glutamatergic stimulation. This analysis is partially contended with observations from the P264L mutant Presenilin-1 knock-in mouse in which it is observed that glutamatergic

stimulation does not result in differential toxicity compared to wild-type Presenilin-1 animals(Siman et al. 2000). Crossing the P264L knock-in mice to APP FAD mutant animals results in elevated Aβ42 formation selectively and facilitates amyloidogenesis, as was reported previously with dual FAD APP/Presenilin-1 lines. Consequently, the emerging picture of the role played by FAD mutant presenilins in AD pathogenesis is not clear, but suggests that complicated alteration of synaptic physiology may be a contributing factor.

APP, APLP-1,2 and Presenilin null mutant models

Genetic studies of AD have been extended to the generation of homozygous null mutant mice for APP, other members of the APP family known as the APP-like proteins 1 and 2 (APLP1 and APLP2), and both presenilin genes. The APP null mutant mice show a relatively mild phenotype characterized by cerebral gliosis and locomotor deficiencies in aged animals(Zheng et al. 1995). In vitro neuronal culture models generated from the APP-/- postnatal animals have demonstrate a decrement in survival and potentially concomitant reductions in dendritic arborization(Perez et al. 1997). While it is unclear initially what to anticipate from the absence of a protein believed to lead to a pathogenic state based on excessive processing, multiple roles have been implicated for the physiological role of APP—a full discussion of which is beyond the scope of this work. However, early work demonstrated that APP is initially targeted to axons and subsequent localization to dendritic compartments(Simons et al. 1995). Extracellular cleavage by the alpha- or beta-secretase results in the liberation of the soluble ectodomain of APP which has been multiply reported to possess neurotrophic

capacities(Araki et al. 1991; Bowes et al. 1994; Ninomiya et al. 1994; Roch et al. 1994; Yamamoto et al. 1994). This will be discussed more in the section on APP Signaling.

The lack of a more pronounced phenotype in APP null homozygous mice may suggest a non-essential role for the APP gene or some functional redundancy between APP and other members of the gene family. In support of the latter hypothesis animals that carry mutations for both APP and APLP2 are embryonic lethal suggesting that some degree of physiologic overlap in function exists between these two members of the gene family(Heber et al. 2000). Yet, the APP-/- animals crossed to double null homozygosity with APLP1 did not appear to have a more pronounced phenotype than that observed with the single APP deletion. This may support greater conservation of function between the APP and APLP2 members of the gene family. The relevance of APP, and related family members, playing an essential role in the viability of an murine development during embryogenesis to AD progression late in life is not clear. However, the necessity of APP and APLP2 in embryonic development likely demonstrates an essential physiological role of APP family members that is maintained throughout the life of mammalian organisms whose disruption may have profoundly deleterious consequences. The potential implications of the necessity for APP and related homologues will be discussed more in the following section on APP signaling.

The generation of Presenilin-1 and Presenilin-2 null mutant animals demonstrated the essential function subserved by these two genes. Presenilin-1 mutants had gross pathological malformations throughout the skeletal structure and profound neuronal defects. The cortical characterization of the null mutant phenotype showed elevated

levels of cerebral hemorrhage, decreases in neurogenesis and decreases in neuronal viability pervasively throughout cortical and subcortical structures resulting in what the authors referred to as "cerebral cavitation"(Shen et al. 1997). The phenotype observed in the Presenilin-2 null mutant mouse is comparatively subtle, demonstrating low-level pulmonary fibrosis and subsequent hemorrhage with age, and no apparent change in APP processing(Herreman et al. 1999). However, the severity of the developmental disruption leading to embryonic lethality within the double Presenilin-1 and Presenilin-2 knockout is more severe. While the essential role served by these genes that have now been characterized as an essential component of the gamma-secretase complex can be of little surprise, it does suggest the potential complexity in determining the exact molecular and physiological defects associated with the progression of a neurodegenerative disease that occurs late in life may be extremely difficult to unequivocally attribute to any single aberration of molecular function.

APP Signaling: Getting RIPed

Ever since the discovery of APP in 1987 it has been postulated that it may function as a cell surface receptor(Kang et al. 1987). Yet, in the decade and a half following its cloning there has been very little success in identifying any potential ligands for APP. While numerous studies have demonstrated the association of proteins with the amyloid plaques, including ApoE2-4 and a2 macroglobulin(Selkoe 2001; Selkoe and Podlisny 2002), little work has been completed identifying relevant binding partners for the soluble ectodomain portion of APP which could function in a receptor-ligand complex. Originally it was shown that the Kunitz inhibitory domain

within the ectodomain of APP functioned as a potent chymotrypsin-like protease inhibitor (Oltersdorf et al. 1989; Van Nostrand et al. 1989). The soluble ectodomain from APP751, which contained the Kunitz inhibitory domain, interacted stably with trypsin while APP695, the splice variant lacking the Kunitz inhibitory domain, did not. This prompted the observation that the soluble domain of APP751 appeared to be indistinguishable from protease nexin-II, suggesting that they were in fact the same molecule. While this portends a role for the secreted form of APP in the regulation of extracellular proteolysis, it does not address its function as a receptor as the primary neuronal form of APP lacks this binding domain.

Subsequently, it was observed that APP bound to collagen in the extracellular matrix(Breen et al. 1991). The association of APP and collagen was later suggested to be mediated through a heparin-bridge mechanism, which correlated with the trophic role of APP in neuritic extension(Breen 1992; Small et al. 1993; Small et al. 1994). The portion of APP binding to heparin was mapped to the N-terminus of the molecule between residues 96-110(Small et al. 1996). Peptides mimicking this region of APP blocked some of the growth promoting effects upon neurite outgrouwth observed with ectopic treatment of the soluble APP ectodomain(Araki et al. 1991). A second heparin binding domain has been mapped to a region immediately carboxy-terminal to the Kunitz inhibitory domain(Small et al. 1999). Intriguingly, this second heparin binding domain overlaps the region of the soluble APP ectodomain to which trophic function has been mapped(Bowes et al. 1994; Roch et al. 1994). While it has been suggested that APP binding to collagen at a site adjacent to its second HBD may play a more important role in APP extracellular adhesion than do the HBDs themselves(Beher et al. 1996), it does not appear that the association of APP with the extracellular matrix is the key to unlock the door to discovering APP's role as a receptor.

The notion that APP functions as a receptor makes the presumption that it possess the capacity to signal. Such a capacity necessitates a defined pathway by which the intracellular domain can complex with other cytosolic factors in a functional manner. The relatively short intracellular domain of APP, less than fifty amino acids, would naively suggest that the number of interacting proteins that bind to this portion of the molecule must be small. Unfortunately, in this case, the naiveté attributed to this assumption is justified as over half a dozen proteins or protein complexes have been suggested to associate with the short cytoplasmic tail of APP. To discuss all of the pathways and their potential effects upon glial and neuronal physiology is vastly beyond the scope of this work. Consequently, a circumscribed description of the major interacting complexes will be discussed briefly; but in most instances the potential signaling mechanism and detailed impact will not be covered where it extends beyond the boundaries of the thesis topic.

The cytosolic domain of APP is only 47 amino acids long, which suggests that the protein complexes that are formed with the cytosolic tail must be competitive. While no systematic study has been done demonstrating which of the known binding partners can co-interact with the cytosolic tail of APP, one can infer certain mutually exclusive relationships based on the site of interaction. There are, fundamentally, two primary binding sites within the APP intracellular domain (ICD). The first binding site is at the amino-terminal region of the APP ICD adjacent to the membrane. This is the region to which Go, kinesin light chain (KLC), and PAT1 have been found to associate. While it appears that diverse associative binding mechanisms may tether proteins to this region of the APP ICD, several binding interactions have been mapped to this portion of the molecule. These will be discussed in more detail shortly. The second binding domain is the YENPTY motif spanning amino acids 682-687 of APP695, which is the mostly commonly utilized site of interaction within the APP ICD. The litany of proteins that bind to the YENPTY motif (Fe65, X11, APP-BP1, JIP1b, and mDab1) do so through the presence of a

phosphotyrosine interacting domain (PID). While this would suggest that interaction at this site is strictly regulated by tyrosine kinases, in many instances the association of the APP ICD binding factors occurs in a tyrosine-phosphorylation independent manner (Fe65 and X11)(Van Gassen et al. 2000). These binding interactions shall all be discussed briefly in turn.

The first report of a signaling mechanism associated with APP came with the observation that there was a G-protein binding site in the APP ICD. The Go class G-protein was found to associate with the amino-terminal region of the ICD between residues 657-676 of APP695(Nishimoto et al. 1993). Since the Go class of heterotrimeric G-protein antagonizes the activity of adenylyl cyclase, this suggested a potential mechanism for APP to counter-regulate the formation of cAMP. The possibility of Go signaling playing a role in the pathogenesis of AD arose from the discovery that FAD mutations at the London locus result in constitutive activity of the Go complex(Okamoto et al. 1996), specifically resulting in the down-regulation of CRE mediated transcription(Ikezu et al. 1996). The notion that constitutive activity of Go would repress the activation of CRE mediated transcription is consistent with the enormous literature describing the role of PKA in the activation of CREB-mediated transcription. While the generality of disruption to the cAMP pathway from multiple FAD mutations has not been established, the known role of CRE-mediated transcription in neuronal survival(Bonni et al. 1999; Riccio et al. 1999) suggests a potential compelling link between at least some FAD mutations and neuronal degeneration associated with AD pathology.

The concept of APP functioning to regulate transcriptional activation by specific classes of response elements was furthered with the recognition that APP associates with

PAT1(Zheng et al. 1998). PAT1 is a microtubule binding protein that interacts with APP through the basolateral sorting signal(Van Gassen et al. 2000), and consequently may play a role in cellular trafficking events associated with APP. Following the characterization of PAT1 binding to the APP ICD, the role of this complex in transcriptional regulation was explored. Using recombinant APP- CTFγ constructs tagged with GFP it was demonstrated (perhaps weakly) that the PAT1-APP- CTFγ complex migrates to the nucleus wherein it counter-regulates retinoic acid mediated transcriptional activation(Gao and Pimplikar 2001). While this initial observation awaits further confirmation, this does suggest that the APP-ICD may be involved in transcriptional signaling in a fashion dependent on proteolytic processing. The theme of transcriptional activation following intramembranous cleavage is central to this work and will be discussed later as a central tenet of what has come known as regulated intramembrane proteolysis, or RIP, as a generalized mechanism of cellular signaling.

PAT1 bears limited sequence similarity to the kinesin light chain (KLC) of the microtubule motor protein complex kinesin-1(Van Gassen et al. 2000), which has recently been identified as a direct binding partner of the APP-ICD. KLC binding to the amino-terminal region of the APP ICD has been shown to be critical for the anterograde targeting of APP to axon terminals(Kamal et al. 2000). Consequently, this suggests that one signaling function of APP may involve the subcellular trafficking of associated vesicles to the presynaptic nerve terminal. Further, the association of APP with KLC appears to be required for the targeting of BACE and presenilin to axon terminals, wherein proteolytic processing generates Aβ and CTFγ, and leads to dissociation of KLC

from the vesicle membrane(Kamal et al. 2001). This leads to the notion that APP may play a general role in the regulated targeting of vesicles to axonal compartments, wherein gamma-secretase cleavage of APP may mark the completion of delivery. These ideas have been discussed(Sisodia 2002), suggesting APP may play a role as a "cargo receptor". However, one significant implication of this interaction is that it occurs exclusively outside of the nucleus. This suggests that the APP binding partners may not only compete for available binding sites, but may also be involved in antagonistic regulation of subcellular localization of APP/APP-ICD. This point will be revisited in the discussion section of this work.

The remaining known binding partners of APP associate with the carboxy-terminal YENPTY motif. One of the first proteins to be discovered to bind to the carboxy-terminal region (this interaction is not specifically mapped to the YENPTY motif) of APP is the APP binding protein 1 (APP-BP1) (Chow et al. 1996). APP-BP1 associates with both the APP-ICD and hUba3. The complex formed with the later results in a ubiquitin activating complex that may be involved in the post-translational attachment of both ubiquitin and NEDD8 to associated substrate proteins(Chen et al. 2000). The role of APP-BP1 in APP signaling is not completely clear; however, the above work did demonstrate that over-expression of APP-BP1 can lead to the restoration of cell cycle progression in temperature sensitive S-phase blocked cells. The relevance of this to AD research comes from repeated observations that anomalous markers for neuronal progression into the cell cycle accompany AD pathology(Ding et al. 2000; Husseman et al. 2000). The observation that APP-BP1 over-expression in cultured

neurons drives the re-entry into the cell cycle and subsequent induction of caspase-mediated apoptosis suggested that regulation of the activity associated with APP-BP1 may be involved in some component of the observed AD pathology(Chen et al. 2000).

Potential induction of neuronal apoptosis by APP binding partners is a theme that is revisited by the discovery that JIP1b binds directly to the APP carboxy-terminus and functions as a scaffold for the JNK stress activated kinase(Scheinfeld et al. 2002b). Given the known role of JNK in the activation of neuronal apoptosis(Xia et al. 1995), this suggested yet another potential link between APP associated signaling and the observed AD neuropathology. The model becomes more compelling with the observation that JIP1b interaction with JNK seems to play an activating role in other non-neuronal systems(Tawadros et al. 2002). Interestingly, JIP1b not only stimulates the general activation of JNK, but has also the JNK mediated phosphorylation of APP at Thr668 (relative to APP695)(Inomata et al. 2003). Further, this JNK mediated phosphorylation increases the interaction between APP and KLC suggesting a possible role for JNK in the regulation of the 'cargo receptor' role of APP.

The concept of APP coordinating signaling through activation of cytosolic kinases is repeated with the discovery that APP associates with the human homologue of the *Drosophila* Disabled protein mDab1(Trommsdorff et al. 1998). Further, the same work demonstrated that mDab1 can interact with LRP, a protein which has since been shown to be involved in the regulation of proteolytic processing of APP(Pietrzik et al. 2002). The interaction between mDab1 and APP suggests a potential signaling mechanism coupling APP to nonreceptor tyrosine kinases, as activated mDab1 can stimulate both src and

abl(Trommsdorff et al. 1998). The physiological relevance of the interaction between APP and abl signaling is particularly interesting given the role played by abl in promoting both cytoskeletal rearrangement, neuritogenesis and axonal outgrowth(Kadlec and Pendergast 1997; Maru et al. 1996; Wills et al. 1999). Further, abl may stimulate the tyrosine phosphorylation of APP at the amino-terminal tyrosine of the YENPTY motif which appears to confer association of abl with APP(Zambrano et al. 2001). While it is not clear whether the interaction between APP and abl is directly mediated through the abl SH2 domain or by the association between abl and mDab1, it does suggest that abl may be involved in both downstream signaling events and the regulation of the tyrosine phosphorylation of the YENPTY motif. Since the YENPTY motif is the binding site for multiple proteins, not all of which depend on it being tyrosine phosphorylated, tyrosine phosphorylation at this site may be involved in the competitive association of the APP binding factors. Further, the activated form of abl has been shown to complex with Fe65 through its WW domain(Zambrano et al. 2001).

The interaction between Fe65 and APP was originally identified using a Y2H screen implementing the carboxy-terminal region of APP as the bait(Bressler et al. 1996). The same work found that the interaction between Fe65 and APP generalized to the other members of the APP family, APLP1 and APLP2. The significance of the interaction between these two factors is the topic of continuous exploration, including work presented within this thesis. The interaction between APP and Fe65 was later mapped to one of the two phosphotyrosine interaction domains (PID) of Fe65 and the YENPTY motif of APP(Zambrano et al. 1997). While this suggests direct regulation of the

association by either receptor or non-receptor tyrosine kinases, the association between APP and Fe65 (as well as the APP binding protein X11) appears to occur in a phosphorylation-independent manner(Borg et al. 1996; McLoughlin and Miller 1996). The role of the interaction between the APP YENPTY motif and X11 will not be discussed, except to point out that X11 forms a heterotrimeric complex with Cask and Veli which is involved in synaptic vesicle exocytosis through the association with Munc18-1 and beta-neurexin(Van Gassen et al. 2000). Following on the earlier discussion of APP as a 'cargo receptor', the interaction with X11 may confer a role for APP in the regulation of presynaptic vesicular exocytosis. This would suggest that APP may play a dual role in the coordination of presynaptic vesicular physiology—targeting vesicles to the presynaptic terminal and the subsequent exocytotic release.

Multiple physiological roles have been attributed to Fe65 based on its interaction with other factors. The association between Fe65, through its WW domain, and the cytoskeletal protein Mena suggested a role in actin remodeling in areas such as the growth cone(Gertler et al. 1996; Van Gassen et al. 2000). This speculation of an APP-Fe65-Mena complex involved actin remodeling at synaptic sites may contribute to the observation that APP null mutant cultured neurons demonstrate a deficiency in neurite outgrowth, as discussed earlier in this work(Perez et al. 1997). It was later discovered that Fe65 also binds to the CP2 transcription factor (also known as LSF and LBP1) through its PID1 domain and coordinately migrates to the nucleus(Zambrano et al. 1998). The nuclear localization of Fe65 was found to depend on a region of the protein containing the WW domain, suggesting that the migration of Fe65 to the nucleus may

require association with factors(Minopoli et al. 2001). The nuclear migration of Fe65 was blocked by over-expression of APP, suggesting that APP can function to localize Fe65 to the membrane and presumably disrupt its regulation of CP2. While the exact regulatory role of Fe65 upon CP2 transcription has not yet been characterized, it has been demonstrated that Fe65 over-expression leads to the repression of the thymidylate synthase gene, which is driven by CP2(Bruni et al. 2002). This suggests that Fe65 play a repressive role in CP2 transcription, at least with the thymidylate synthase gene. Further, the repressive effect of Fe65 was mitigated by the concomitant over-expression of APP. Since it has already been demonstrated that APP can prevent Fe65 translocation to the nucleus, the authors took this as demonstration that APP can regulate Fe65 repression of CP2. However, CP2 has been found to bind to different DNA elements in distinct heteromeric states(Shirra and Hansen 1998). Consequently, it is possible that Fe65 plays divergent roles in the context of different DNA binding elements. However, while the exact role of Fe65 in the regulation of CP2 mediated transcription has yet to be completely characterized, the likelihood that this pathway may play a role in AD is supported by the potential genetic protective effects of a polymorphism in the CP2 3'UTR that leads to the elevation in CP2 protein levels(Lambert et al. 2000).

The observation, discussed above, that APP can prevent Fe65 nuclear translocation, suggests that the association between these two factors may play a fundamental physiological role in CP2 transcription—as well as other APP/Fe65 signaling mechanisms. Intriguingly, phosphorylation at Thr668 of APP695 results in decreases the interaction between APP and Fe65(Ando et al. 2001). While this site is not

the primary binding site for Fe65, NMR characterization of different APP ICD phosphorylation states suggests that phosphorylation at this site may shift the conformation of the APP ICD from an all trans to cis-conformation(Ramelot and Nicholson 2001). Consequently, this phosphorylation site may regulate the association of the different APP binding partners. Consistent with this notion, as it was discussed earlier, phosphorylation at this site appears to promote the interaction between APP and KLC. Hence, APP Thr668 phosphorylation may function as a general switching mechanism governing the association of different APP coordinating factors. This is particularly interesting as multiple protein kinases have been shown to phosphorylate APP at this residue. SAPK, GSK3beta, cdk5, MAPK, and potentially JNK have all been implicated in the phosphorylation of APP at this site(Standen et al. 2001; Aplin et al. 1996; Aplin et al. 1997; Iijima et al. 2000; Inomata et al. 2003). Consequently, the interaction between APP and Fe65, in addition to other binding factors, may be highly regulated.

Perhaps the most compelling model of APP signaling has arisen recently with the discovery that APP has nuclear signaling capacities analogous to Notch. The preliminary suggestion that such a mechanism may exist came from the numerous observations that the APP CTFγ can migrate to the nucleus in association with Fe65(Cupers et al. 2001; Kimberly et al. 2001; Kinoshita et al. 2002b; Sudol et al. 2001). The combined observations that APP can restrict Fe65 from the nucleus and ability of Fe65 to shuttle the gamma-secretase cleaved APP product to the nucleus suggested a regulated signaling mechanism for membrane to nucleus translocation. The plausibility that such a

mechanism could be involved in transcriptional regulation was subsequently investigated using APP-Gal4- CTFγ constructs in which the Gal4 DNA binding element was interposed in between the stop transfer sequence and the rest of the carboxy-terminal extent of the APP ICD. The results of this exploration demonstrated convincingly that the APP ICD complexes with Fe65 and Tip60 to promote induction of gene transcription(Cao and Sudhof 2001).

Tip60, which was originally identified as an HIV-1 Tat coactivator(Kamine et al. 1996), has subsequently been shown to be a histone acetyltransferase targeting specific amino-terminal lysine residues in histones H2-4(Kimura and Horikoshi 1998; Yamamoto and Horikoshi 1997). Subsequently it was also established that this histone acetylation, which was originally speculated to play a role in chromatin rearrangement, did function to increase the transactivation of endogenous mammalian transcriptional systems(Brady et al. 1999). This work describing Tip60 as a transcriptional coactivator specifically addressed the role of Tip60 in nuclear hormone receptor signaling, as it promoted the activity of the androgen, estrogen and progesterone receptor gene induction. Since then, however, it has been found that Tip60 may also play a repressive role in other transcriptional systems as it appears to decrease the activity associated with both CREB and Stat3 signaling(Gavaravarapu and Kamine 2000; Xiao et al. 2003). Interestingly, it appears that Tip60 enzymatic function as a histone acetyltransferase may be modulated through phosphorylation by cyclinB/cdc2 kinase at the G(2)/M stage of the cell cycle(Lemercier et al. 2003). The phosphorylation was blocked by roscovitine, which is

a well known cdk5 inhibitor, suggesting that perhaps cdk5 may play a role in the modulation of both the APP Thr668 and Tip60.

The model for APP signaling to the nucleus described a heterotrimeric complex consisting of APP, Fe65 and Tip60. In this model, Fe65 functions as a linker bridging the association between APP and Tip60 through its two phosphotyrosine interaction domains(Cao and Sudhof 2001). While the paper convincingly demonstrated that the activation of a Gal4-reporter was dependent on the presence of Fe65, it was not demonstrated that gamma-secretase cleavage of the APP substrate was absolutely necessary for this process. However, this is the most parsimonious conclusion that can be drawn. Further, the depiction of an essential role for Tip60 is also not entirely clear as over-expression of Fe65 seems to be sufficient to drive activation of the system. While it is demonstrated that Tip60 forms a complex with APP and Fe65, this group did not show that mutations in Tip60 that abrogate the interaction with Fe65 attenuate signaling associated with APP-Gal4/Fe65. However, despite these methodological shortcomings, this work does make a strong case that the association between the APP ICD and Fe65 has transactivation potential. The role of APP and Fe65 in nuclear signaling has recently been extended to include the other members of the APP family, APLP1 and APLP2, using the same methodology(Scheinfeld et al. 2002a). This suggests that the nuclear signaling role may be conserved across the APP family, and may explain the potential redundancy of function seen within the crosses of null mutant mice for APP family members discussed previously.

A second model for transcriptional activation via the APP-ICD/Fe65/Tip60 complex has since been proposed involving the modulation of NF-kappaB signaling. By this account MEKK phosphorylation of Tab2 leads to the displacement of the N-CoR/Tab2/HDAC3 repressor complex from the NF-kappaB p50 homodimer. This is followed by the binding of Bcl3 to p50. Bcl3 promotes the association of the heterotrimeric complex composed of APP- CTFγ, Fe65 and Tip60 which stimulates transactivation of the KAI1 tetraspanin promoter(Baek et al. 2002). The KAI1 promoter was used merely to exemplify a potential mechanism that could be more broadly relevant in CNS signaling. This account demonstrates a more specific biochemical mechanism whereby the APP/Fe65/Tip60 complex can lead to transcriptional activation, while also validating the original observation of a nuclear signaling role for APP. The portion of this story that remains to be elucidated is what the APP- CTFγ function is within the nucleus. This work demonstrates the necessary role played by the histone acetyltransferase (HAT) activity of Tip60. However, it is not clear what the role of the short APP gamma-secretase cleavage product is in this mechanism. As was discussed earlier, the APP ICD has two binding regions associated with the amino-terminal region and the carboxy-terminal YENPTY motif. Since Fe65 binds to the YENPTY motif and to Tip60, the requisite nature of the APP ICD suggests that the amino-terminal region is likely involved in complexing with another, as of yet unidentified, component of the RNApolII transcription complex. Given the caspase proteolytic processing of the APP ICD(Lu et al. 2000), elucidation of its role may be critical in defining a potential key

point of APP transcriptional regulation. This issue will be revisited in the discussion section.

One key concept that is emerging from the study of regulated intramembrane proteolysis (RIP), gamma-secretase substrates send their intracellular domain into the nucleus wherein they activate transcription. Several reviews have discussed RIP as a conserved mechanism of cellular signaling(Bothwell and Giniger 2000; Brown et al. 2000; Ebinu and Yankner 2002; Hoppe et al. 2001; Huppert and Kopan 2001; Rawson 2002; Urban and Freeman 2002). The original characterization of this phenomenon occurred with Notch, wherein it was observed that following association with its transmembrane ligand Delta, gamma-secretase cleavage was induced, and the intracellular domain translocates to the nucleus activating transcription(Berezovska et al. 2000b; Struhl and Adachi 1998). Several reports in both Drosophila and mammalian systems have demonstrated the requisite role of presenilin in mediating Notch signaling(Jack et al. 2001; Struhl and Greenwald 1999; Struhl and Greenwald 2001). While other reports have suggested that gamma-secretase inhibition may not completely attenuate Notch signaling(Berechid et al. 2002; Berezovska et al. 2000a), general consensus, even among these studies, is that presenilin associated gamma-secretase processing of Notch plays a key role in its post-proteolytic signaling. Further, it has been demonstrated that Notch and APP function as competitive substrates for gamma-secretase cleavage(Berezovska et al. 2001), strongly suggesting a common mechanism shared in the processing of these two proteins. The concept of RIP as a general signaling mechanism was extended to ErbB4, as it was identified that ErbB4 is also gamma-

secretase cleaved leading to nuclear signaling(Lee et al. 2002a). Further, work in our lab has suggested that the neurotrophin receptor p75 and related homologues NRH1 and NRH2 are also gamma-secretase targets. While this represents an exciting new mechanism for the regulation of cellular signaling (Kanning, et al, in press), it also offers many challenging questions about the interaction and coordinated regulation of such diverse signaling pathways.

The hypothesis: is it rate or ratio?

β-amyloid participation in AD pathogenesis has been attributed to the relative increase in Aβ42. However, there are several problems with this explanation. First, many of the ratio changes observed are very small; as little as a 7% shift in the relative Aβ42/ Aβ40 has been associated with some Presenilin-1 mutations. Second, the relativistic account of β-amyloid modulations leaves the question of how much total cleavage occurs completely open to speculation. Given the physical proximity of the FAD mutations to the γ-secretase cleavage site, it seems likely that these mutations will directly impact the overall level of enzymatic activity as well as potentially shifting the cleavage site to favor Aβ42 formation. The recent advances suggesting a RIP-mediated Notch-like cleavage mechanism regulating transcriptional signaling through the liberation of the intracellular domain necessitates a resolution to one of the central questions remaining in γ-secretase processing of APP: is it rate or ratio? Given the difficulty of quantitating all β-amyloid species and the inherent biochemical instability of the CTFγ,

we present a model here using an APP-Gal4VP16 transcriptional screen of γ-secretase activity suggesting that rate, as well as ratio, is impacted by FAD mutations.

Chapter II: Methods

Plasmid generation and mutagenesis

Construction of APP-Gal4VP16 Screen System

The human APP695 cDNA was PCR amplified with 36 nucleotide primers containing a HindIII site in the 5' primer and a SpeI site in the 3' primer, which also deleted the stop codon. The reaction was run with a one minute melting time at 95C, an annealing temperature of 55 C for 90 seconds, and an extension temperature of 68C for 2 minutes. The PCR reaction was run for 15 cycles. Standard PCR reaction components were used. The 2 kb PCR product was purified, digested and subcloned into pCEFL (expression vector driven by EF1α promoter, gift of Dr Silvio, NIH). pSGVP (a gift from Dr Mark Ptashne, Sloan-Kettering Institute) contains the Gal4-VP16 coding sequence. This construct was used as a template to amplify the Gal4-VP16 coding sequence with a SpeI site engineered into the 5' primer and an XbaI site built into the 3' primer. The Gal4-VP16 coding sequence was subcloned into the EF1α-APP695 vector creating an in-frame APP695-Gal4VP16 fusion construct (referred to as APPGV16). The normalization vector, EFnLACZ was generated by PCR amplifying the nuclear targeted NLS-LacZ (gift of R. Palmiter, University of Washington) with a 5' primer containing a NheI site and 3' primer containing a HindIII site. The purified PCR product was digested and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, California). The CMV promoter was subsequently removed and the EF1α promoter was subcloned into the NruI and NheI sites resulting in EF1α-NLS-LacZ (referred to as EFnLacZ).

The mutagenesis was performed using an adaptation of the standard QuikChange mutagenesis procedure (Stratagene, La Jolla, California). 200 ng of APPGV16 DNA was used in each reaction with mutagenesis primers into which silent restriction site were employed to diagnose successful mutagenesis by restriction digest analysis. The 45 nucleotide mutagenesis primers were designed to overlap with one another by 20-25 nucleotides, leaving 20-25 nucleotides of unhybridized sequence per primer. An initial extension reaction was run for 4 cycles of PCR prior to the addition of the template APPGV16 DNA, yielding an approximately 65 nucleotide 5' and 3' mutagenesis primer. The standard QuikChange conditions were used for the mutagenesis reactions, with the exception that 5% DMSO was added to the mutagenesis reactions. All APPGV16 mutations were restriction digestion mapped to ensure the presence of the silent restriction site incorporated into the primers. All mutations were confirmed by sequencing.

CMV-Gal4VP16 was generated by using the Gal4VP16 fragment of APPGV16. The SpeI-XbaI Gal4VP16 coding sequence was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, California). The CTFγ-Gal4VP16 construct was generated by amplifying the carboxy-terminal fragment of APP between the β-amyloid cleavage site and carboxy-terminus using primers that included a BamHI site in the 5'-primer and a SpeI site in the 3'-primer. The APP PCR product was used to make a fusion with CMV-Gal4VP16 cut with BamHI and SpeI, resulting in APP-CTFγ being subcloned into the 5'-end of Gal4VP16 in frame with the coding sequence.

Construction of GFP-tagged APP and APP-CTFγ

GFP-tagged versions of wild-type and three FAD mutant APP were generated by using the same APP PCR primers described above with wild-type APP, V642G, T639I, and KM595/596NL cDNA templates. The PCR conditions used were identical to those used to originally amplify the APP cDNA discussed above. The resulting PCR products were purified, cut with HindIII and SpeI, and subsequently band purified. The isolated product was subcloned into GFP XLT (obtained from the laboratory of Dr Randy Moon), which had been cut with HindIII and XbaI generating an in-frame subcloning site at XbaI. The products were then subjected to extensive restriction mapping, using the SpeI entry into the 3' XbaI site in GFP XLT as a diagnostic—successful subcloning of SpeI into XbaI destroys this restriction site.

APP-CTFγ was subcloned into GFP XLT using an almost identical strategy, except that PCR primers were made that amplify the short carboxy-terminal domain of the human APP695 cDNA. The 36 nucleotide primers had a built in 5' BamHI site in the 5' primer and a 5' XbaI site in the 3' primer. The PCR reaction was maintained for 15 cycles and the approximately 200 nucleotide product was purified, cut with BamHI and XbaI and subcloned into GFP XLT which had been identically digested. The resulting constructs were mapped and sequenced to verify their identity. Both GFP-CTFγ and CTFγ-GFP were made (referred to in the figures as C57GFP). Site-directed mutagenesis on CTFγ-GFP was performed as described above using 36 nucleotide primers that induced a shift in nucleotide sequence at the stop transfer sequence from the tripartite lysine (KKK) to either Thr-Ser-Ala (TSA) or a tripartite RRR and included a silent

restrict site for mapping diagnostics purposes. The resulting constructs were restriction mapped and positive clones were sequence verified.

Gal4-APP-CTFγ constructs

The Gal4-APP-CTFγ constructs were made by one of two methods. First, the Gal4 coding sequence was PCR amplified and subcloned into pcDNA3.1 with an in-frame EcoRI restriction site at the 3' end of Gal4. Primers were generated that amplify different length portions of the carboxy-terminus of APP resulting in sequences coding for the C59, C57, C50 and C31 portions the carboxy-terminal domain of APP with an EcoRI site at the 5' end. The original Gal-C59 construct was generated by subcloning it into the above described CMV-Gal4 construct. This construct was verified by restriction mapping and was subsequently sequenced. This construct was used for the original comparative studies with APPGal4. The low-level of observed transactivation from this construct led to it being remade using the CMV-Gal4VP16 construct as the template. The CMV-Gal4VP16 had an additional EcoRI site in the polycloning site that was removed by digesting the vector with flanking unique restriction sites, blunting the vector, and religating it to itself. This version of CMV-Gal4VP16 with a verified single EcoRI site was used as the parent vector for the generation of the Gal4-C59, -C57, -C50 and -C31 constructs. These constructs are used in subsequent comparative examination of transactivation potential of the distinct hypothetical gamma-secretase generated carboxy-terminal cleavage products.

Vectors

Fe65 expression vectors

The Fe65 constructs used in these experiments were obtained from the laboratory of Dr George Martin. The neuronal, non-neuronal and a2 isoforms of Fe65 had been subcloned into pcDNA3.1myc, wherein the non-tagged versions of the construct contained an introduced stop codon prior to the poly-myc tag to stop translation of this portion of the resulting transcript.

APPGal4 and Tip60

The APPGal4 and Tip60 constructs were provide by the laboratory of Dr Thomas Sudhof. The APPGal4 construct was made by interposing the Gal4 coding sequence in frame in between the stop-transfer sequence (KKK) and the remaining cytosolic portion of the APP-CTFγ. The Tip60 construct was generated by subcloning the human Tip60 cDNA into pcDNA3.1 CMV expression vector.

Cell Culture and transfection conditions

Primate kidney Cos7 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 1X penicillin and streptomycin (Invitrogen, Carlsbad, California) at 37C in 5% CO_2. Primary mouse embryonic fibroblasts, both the $PS1^{-/-}PS2^{-/-}$ and wildtype, were grown under the same conditions (obtained from Dr. B. de Strooper, Flanders Interuniversity Institute for Biotechnology, Belgium). Rat adrenal pheochromacytoma PC12 cells were grown in

Dulbecco's Modified Eagle's Medium supplemented with 10% horse serum (Hyclone, Logan, Utah), 5% fetal bovine serum (Hyclone, Logan, Utah) and 1X penicillin/streptomycin (Invitrogen, Carlsbad, California) at 37C in 5% CO_2. Stock plates were grown to approximately 50-80% confluence prior to splitting cells for transfection. PC12M cells were grown in DMEM supplemented with 10% horse serum and 5% fetal bovine serum (both from Hyclone).

The Presenilin-1stable cell lines were generated by transfecting Cos7 cells with the dual Zeo cassette Presenilin-1 constructs (a gift from Dr Selkoe, Harvard University) and placing the cells under Zeomycin (Invitrogen) selection, starting 48 hours post-transfection. The cells were grown under selection for several passages over a three week period and then used for experiments.

All transactivation assays were done using cells seeded onto 24-well plates. In subcellular localization studies, the cells were plated onto glass slides that had been antecedently treated with poly-D lysine to facilitate cellular adhesion. The plates were grown to 80-90% confluence and transfected using Lipofectamine 2000 (Invitrogen). Cos7 cells were transfected using 1.5 μl of Lipofectamine 2000 reagent per well. Control experiments were done to determine this concentration of Lipofectamine was optimal for this system in Cos7 cells. PC12 cells and PMEFs were transfected using 3 μl of Lipofectamine 2000 per well. The transfection mix was incubated on the cells for 2-4 hours, prior to a replacement of the medium of fresh growth medium.

In all transactivation assays, 1 μg DNA was used per well of the 24 well plate. APPGV16 concentrations are defined in each experiment in which titration assays are

done. If it is not specified, then 100 ng/well of APPGV16 DNA was used. In all experiments, the promoter concentration was held constant by the addition of either pcDNA3.1 (Invitrogen) to normalize CMV-promoter concentrations (PS1 experiments) or pCEFL to normalize the amount of EF1α promoter present in each condition. In experiments in which APPGV16 was titrated across the experiment, pCEFL was counter titrated to ensure that there would not be any promoter competition or variation due to promoter saturation across conditions. The Gal4-luciferase reporter used was pFRluc (Invitrogen), which was used at 400 ng/well. In experiments also over-expressing PS1 isoforms, 250 ng/well of the reporter construct was used and 150 ng/well of the PS1 construct was used (unless concentrations provided in the figure). Normalization was done using 50 ng/well of the EFnLACZ construct.

Transfections for Western blot were done in 6 well plates at approximately 90% confluence. 1 μg of APPGV16 DNA was used for each isoform (corresponding to approximately 250 ng/well of DNA in the 24 well plate assay). The cells were incubated with transfection mix for 4-5 hours prior to replacing the media with fresh growth media. The cells were lysed 48 hours post-transfection for examination by Western blot.

Cleavage assay and inhibitors

Luciferase and βGal assays were performed by standard methods as previously reported(Wiley et al. 1999). The cells were lysed on ice 36-48 hrs post-transfection and luciferase assays were performed and point to point normalized to βGal values. For the assays in which parallel protein concentration levels were assessed, 1X proteasome inhibitor mix (Sigma, St Louis, Missouri) was added. In comparative studies, the

addition of proteasome inhibitor mix was found to have no effect on the luciferase assay results. All measurements were performed on a EG&G Berthold LB 96V luminometer.

The γ-secretase inhibitors used in these experiments were generously provided by Dr. M Wolfe (Harvard University). The WPE-II compound had been found to be 10-fold more effective at blocking β-amyloid secretion with an IC50 of 100 nM, while DAPT had an approximate IC50 of 1 μM (Wolfe, personal communication). These compounds were used at the concentrations described in the figures. For experiments in which explicit concentrations are not given WPE-II was used at 1 μM and DAPT was used at 10 μM. The cells were treated with WPE-II and DAPT γ-secretase inhibitors starting approximately 24 hours post-transfection and remained in the inhibitors for 16-20 hours prior to lysis.

Subcellular localization of APPGFP and APP-CTFγ-GFP constructs

Subcellular localization studies were performed using 500ng of the APP expression construct. In studies performing co-transfection with CMV-Fe65, 250ng of the Fe65 expression vector was used. Co-transfection of the parent CMV expression vector was done to maintain promoter levels and total DNA in the transfection. For the CTFγ-GFP transfections, Cos7 and PC12M cells were transfected for 24 hours followed by the addition of 10 μM MG-132 (a potent proteasome inhibitor, Calbiochem) or 10 μM PPI (a second proteasome inhibitor, Calbiochem). In initial experiments comparing the effects of the presence and absence of MG-132, no signal was detect in the absence of MG-132. The constitutive CMV-GFP expression vector, GFP BE (obtained from the laboratory of Dr Randy Moon), was used in control conditions. The transfected and

treated cells were washed once in cold 1XPBS and subsequently fixed in 4% paraformaldehyde for 10-15 minutes. The cells were then mounted to slides and covered with Vectashield containing propidium iodide to label the nuclear compartment. These slides were imaged using the Bio-Rad MRC 600 dual scanning confocal microscope taking thin section (approximately 1 μM sections) images. The APPGFP images were obtained the same way except that no pre-treatment with MG-132 was utilized and the total transfection time was only 24 hours. The counter-stain used in APPGFP to localize the nuclear compartment was DAPI and these slides were examined under the Leica Spectral Confocal Microscope at the Keck Center imaging facility at the University of Washington.

Immunoprecipitations, Western blots and antibodies

Protein expression level comparisons were made from lysates taken from the 6 well plates transfected with just the APPGV16 isoforms. The cells were lysed using standard RIPA buffer, protein concentrations were quantitated, and 50 μg of protein was loaded from each condition. The blots were probed using Rabbit anti-Gal4 DNA binding domain antibody (Zymed, San Francisco, CA). Comparison of APPGV16 expression levels in the transactivation assays were done by removing 50 ul of lysate from each sample and transferring it to 65C 1XPBS/1% SDS. The samples were run on 15-4% gradient gels and probed using the Rabbit anti-Gal4 DNA binding domain antibody (US Biologicals, Swampscott, MA).

Immunoprecipitation studies of the carboxy-terminal gamma-secretase cleavage products generated in the APP-Gal4VP16 screen were done in Cos7 cells which were

plated in 6-well plates at 300,000 cells/well and subsequently transfected for 24 hours using 4 μg of the APP-Gal4GV16 DNA. The cells were subsequently treated with 1 μM WPE for 24 hours. Lysis was performed using TNT lysis buffer (20 mM Tris, 200mM NaCl, 1% Triton X-100 at pH 7.5) with 1X proteases inhibitor cocktail (Sigma). 5 μg of the Zymed Gal4 (rabbit) antibody was used for each immunoprecipitation reaction which was performed at 4C for 4 hours. The samples were run on SDS PAGE and blotted to nitrocellulose. The blots were blocked in 5% milk for one hour and probed with the Clontech Gal4 (mouse) antibody at 1:1000.

Western blots of the CTFγ-GFP (C57GFP and $C57GFP_{TSA}$) proteins were performed in Cos7 and PC12M cells following a 24 hour transfection and an overnight treatment with either 10 μM MG-132 or PPI (Calbiochem). The lysis was done in 65C SDS lysis mix (1% SDS in 1XPBS) and normalized by H-stain to quantities corresponding to approximately 50 μg of protein per condition. All blots were Ponseau S stained to verify equivalent protein loading. The resulting blots were probed with either the rabbit GFP living colors antibody (Clontech) or the rabbit APP carboxy-terminal antibody (Cell Signaling) as specified. The GFP antibody was used at 1:100 dilution and the APP carboxy-terminal antibody was used at 1:500 dilution. In both cases, resulting in a working antibody concentration of 1-3 μg/ml.

Chapter III: Results

APPGV16 Screen Detects γ-secretase Activity

Following the completion of the APP-Gal4VP16 (APPGV16) screen preliminary experiments were performed to assay the transcriptional activation following transfection of both PC12M and Cos7 cells. High levels of luciferase output suggested that transactivation potential had been maintained through the construction of the APPGV16 fusion construct. However, the possibility that the observed transactivation resulted from some event other than γ-secretase cleavage, necessitated the exploration of the γ-secretase dependence of the assay system. In order to verify that gamma-secretase dependent intramembrane cleavage was necessary, two basic approaches were adopted: pharmacologic inhibition of γ-secretase and use of genetic ablation of key genes in the gamma-secretase complex. The impact of each manipulation was assessed upon the relative luciferase output from the APPGV16 system.

The pharmacological approach was implemented by titrating concentrations of two well characterized γ-secretase inhibitors, WPE-II-89 and DAPT. Both compounds effectively inhibited APPGV16-dependent luciferase activity with differential efficacy, achieving greater than 80% inhibition at 10 μM (Figure 2A). WPE-II-89 had an IC50 of approximately 10 nM.. WPE-II-89 was effective at lower concentrations than DAPT, the later of which had an approximate IC50 between 100 nM and 1 μM. The effective inhibitory concentrations for both WPE and DAPT were consistent with results obtained assaying inhibition of Aβ release (personal communication, Dr M Wolfe, Harvard University). Similar results were obtained in Cos7 cells (data not shown). Thus, most of the APPGV16-dependent luciferase activity observed results from cleavage of APPGV16 by γ-secretase.

Figure 1. Diagram of APPGV16 with FAD mutations mapped relative to secretase cleavage sites.

The human neuronal 695 isoform of APP was used to construct the APP-Gal4VP16 fusion protein. The position of the FAD mutations is shown at the right hand side, where the mutational nomenclature relative to APP695 is provided first in black followed by the common name associated with that mutation. The FAD position relative to the APP770 isoform is given in red in brackets. The class I mutations are designated as those occurring in the extracellular region, while class II mutations are those falling within the transmembrane region adjacent to the γ-secretase cleavage sites.

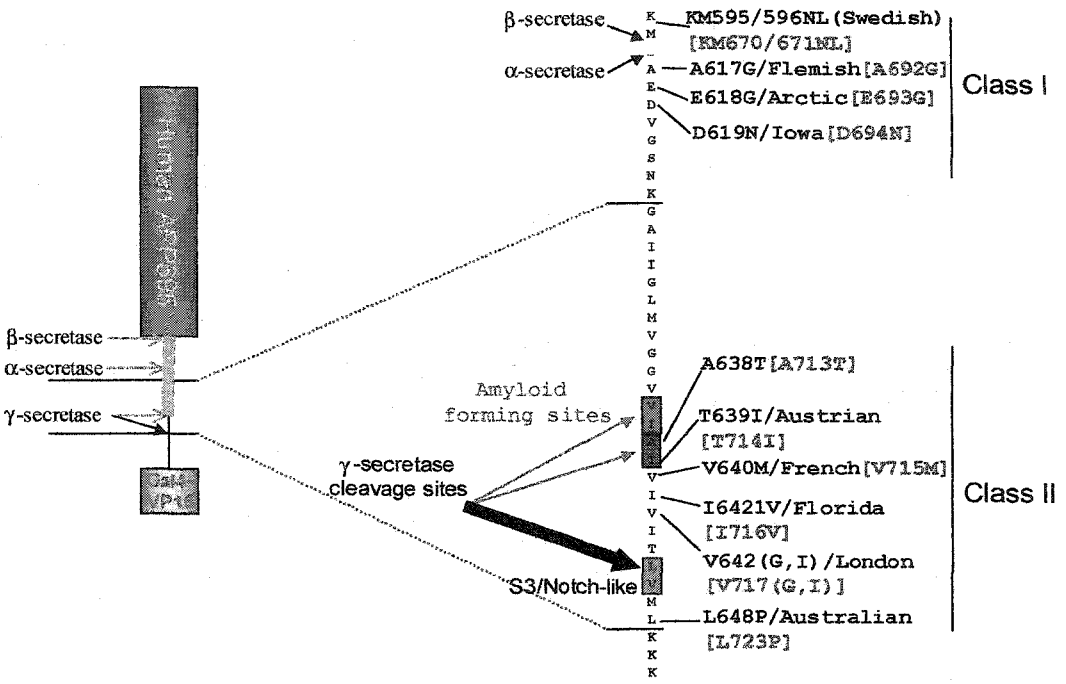
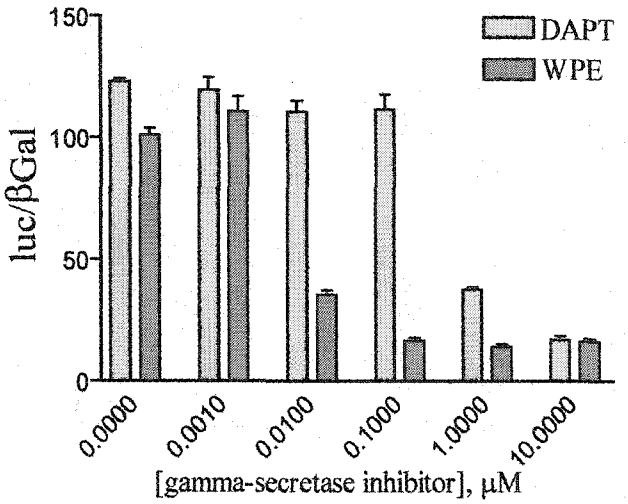


Figure 2. APPGV16 assay detects γ-secretase activity.

The efficacy of the APPGV16 reporter system to detect γ-secretase activity was tested pharmacologically using known γ-secretase inhibitors and genetically using PS1/PS2 null PMEFs. (A) PC12 cells were transfected with the APPGV16 construct and dual titrations were performed using two known γ-secretase inhibitors (WPE-II-89 and DAPT). (B) PS1-/-PS2-/- and wild-type PMEFs were transfected with various quantities of APPGV16 plasmid. Generation of luciferase activity in the wild-type and PS1-/-PS2-/- PMEFs differed 8-10 fold, demonstrating statistical difference (two-way ANOVA $p<0.001$). (C) PS1-/-PS2-/- cells were transiently transfected with 100 ng/well of APPGV16 and increasing amounts of PS1 wt DNA.

A.



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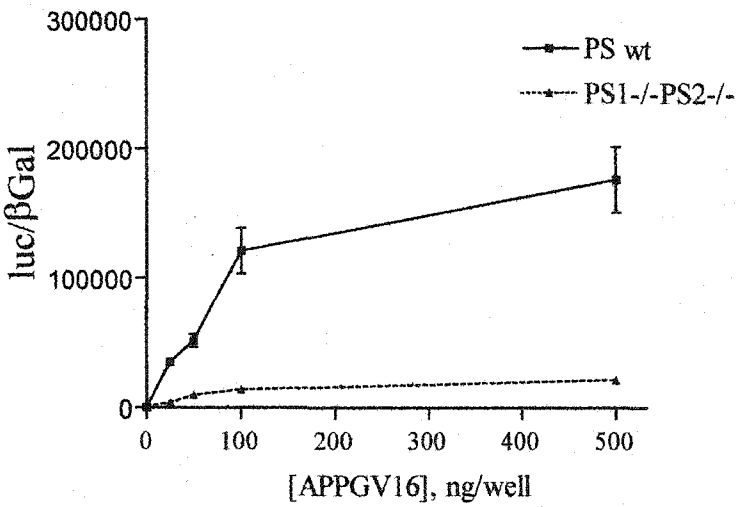

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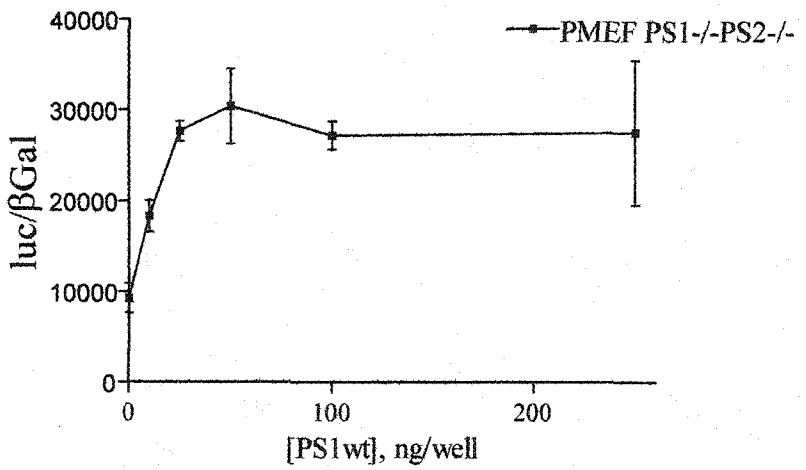


This conclusion was confirmed genetically by experiments employing SV40 T-antigen immortalized primary mouse embryonic fibroblasts (PMEFs) in which targeted deletion of both the Presenilin-1 and Presenilin-2 genes, essential components of the γ-secretase protease complex, had been performed. The $PS1^{-/-}PS2^{-/-}$ primary mouse embryo fibroblasts (PMEFs) produced much less Gal4VP16-dependent activity than did wild-type PMEFs (Fig. 2B) across a broad range of APPGV16 concentrations. These results were confirmed in multiple experiments. Further, the relative luciferase activity in the $PS1^{-/-}PS2^{-/-}$ PMEFs, following transfection of a set concentration of APPGV16 and titrated quantities of Presenilin-1, increased many-fold with the re-introduction of exogenous wild-type Presenilin-1 (Fig. 2C). While exogenous Presenilin-1 failed to rescue relative luciferase activity to the level observed in wild-type PMEFs, the Presenilin-1 dependence of APPGV16 output strongly supports the notion that intramembranous cleavage mediated by γ-secretase is necessary within this screening system. Thus, both pharmacological and genetic elimination of γ-secretase activity prevents generation of relative luciferase activity, confirming that this assay system provides a valid measure of γ-secretase-mediated cleavage of Gal4VP16-tagged APP.

Familial Alzheimer's Disease Mutations in APP decrease γ-secretase mediated cleavage

The APP mutations associated with FAD generally lie in the extracellular region proximal to the α-secretase and β-secretase cleavage sites (Class I mutations) or within the transmembrane domain adjacent to the γ-secretase cleavage sites (Class II mutations). We generated a number of these mutations in APPGV16 to assess the effect on γ-secretase-mediated cleavage (see Figure 1). All experiments titrated the concentration of transfected plasmid to assess whether any effects of mutations on cleavage reflect

differences in cleavage at plateau concentrations (loosely comparable to enzymatic Vmax) or differences in concentration of plasmid required to reach plateau (loosely related to enzymatic Km). Contrary to our expectations of increased APPGV16 processing in the FAD mutants, all Class II β–APP mutations significantly decreased APPGV16-dependent luciferase activity (Fig. 3A-F), while the single Class I mutation tested (Swedish) did not differ significantly from wild-type (Fig. 3G).

Observed differences between mutant and wild-type APPGV16 are not likely to be due to unintended discrepancies in expression level since mutant and wild-type activities differ in the plateau region of the plasmid titrations. This is the region of the titration where concentrations of APPGV16 substrate are apparently saturating. To ensure that this saturation was not merely due to maximal Gal4-promoter activation, the titration experiment was performed comparing a pre-cleaved form of the activator to the holo-APPGV16. The precleaved CTFγ-Gal4VP16 gave much greater activation across the titration than did the holo-APPGV16, confirming that the saturation phenomenon was not due to maximal promoter activation (Fig 3H). The difference between APPGV16 and CTFγ-Gal4VP16 was more profound at higher ranges of the titration, which supports the interpretation that the plateau effect observed with APPGV16 is due to saturation of the proteolytic enzymes leading to liberation of the CTFγ-Gal4VP16 moiety from the membrane.

Further, to verify that protein levels were increased in proportion to elevating concentrations of the APPGV16 DNA, western blot analysis was employed to assess APPGV16 protein concentrations within the lysate used to perform the transactivation assay. There were comparable amounts of wild-type and mutant APPGV16 expressed

for each quantity of plasmid (Fig. 4A). For wild-type, Austrian and Florida mutant isoforms of APPGV16, the concentration of protein expressed increased roughly linearly with increasing amounts of plasmid transfected. No plateau in APPGV16 protein expression was observed over a range of plasmid quantities that yielded a plateau of transactivation. This observation is consistent with our conclusion that the transactivation plateau reflects generation of APPGV16 levels sufficient to saturate cleaving proteases.

To ensure that the different isoforms of APPGV16 were expressing equivalently, several FAD mutant forms of APPGV16 were compared to wild-type following transfection of a single quantity of plasmid (Fig. 4B). No significant difference in quantity of APPGV16 expressed between mutants and wild-type was observed, with the possible exception of the Swedish mutation for which the protein levels appeared slightly higher. Consequently, the decreases among Class II APP mutations in production of the transcriptionally active carboxy-terminal fragment of APPGV16 do not reflect differences in the amount of APPGV16 expressed but rather, appear to result from decreases in efficiency of γ-secretase-mediated processing of the mutant protein. To ensure that differences were in fact do to cleavage of the APPGV16 construct, immunoprecipitation experiments were performed using the Gal4 antibody from cells transfected with either the wild-type or T639I mutant version of APPGV16. The analysis revealed a small but detectable band at the correct molecular weight (approximately 32.5 kD) for the wild-type isoform which was absent in cells treated with the WPE γ-secretase

Figure 3. Comparison of wild-type and FAD mutant $APP_{695}GV16$.

Direct comparisons were made of wild-type and individual FAD mutant versions of APPGV16. In each case, titrations of wild-type and FAD APPGV16 were used to assess potential differences in γ-secretase-mediated cleavage at multiple APPGV16 expression levels. All mutants, except V642G and V642I, were made using the standard quick-change method (Stratagene) with a preceding round of primer extension to increase the size of the mutagenic primers prior to the addition of template. All mutations were sequence verified. V642G and V642I were generously provided by Dr. R. Neve, Harvard University, and subcloned into the EF1-GV16 cassette. The human APP695 isoform (provided by Dr. G. Martin, University of Washington) was used in all instances. Consequently the positional nomenclature is relative to the APP695 isoform. The FAD mutations compared to wild-type are (A) T639I/Austrian mutation, (B) V640M/ French mutation, (C) I641V/Florida mutation, (D) V642I/ London mutation, (E) V642G/ London, (F) L648P/ Australian mutation, and (G) KM595/596NL / Swedish mutation. A comparison of the APPGV16 and the CTFγ-Gal4VP16 activation across a titration of expression vector concentrations was performed to ensure that the plateau effect observed was not due to maximal activation of the Gal4-luciferase reporter.(H).

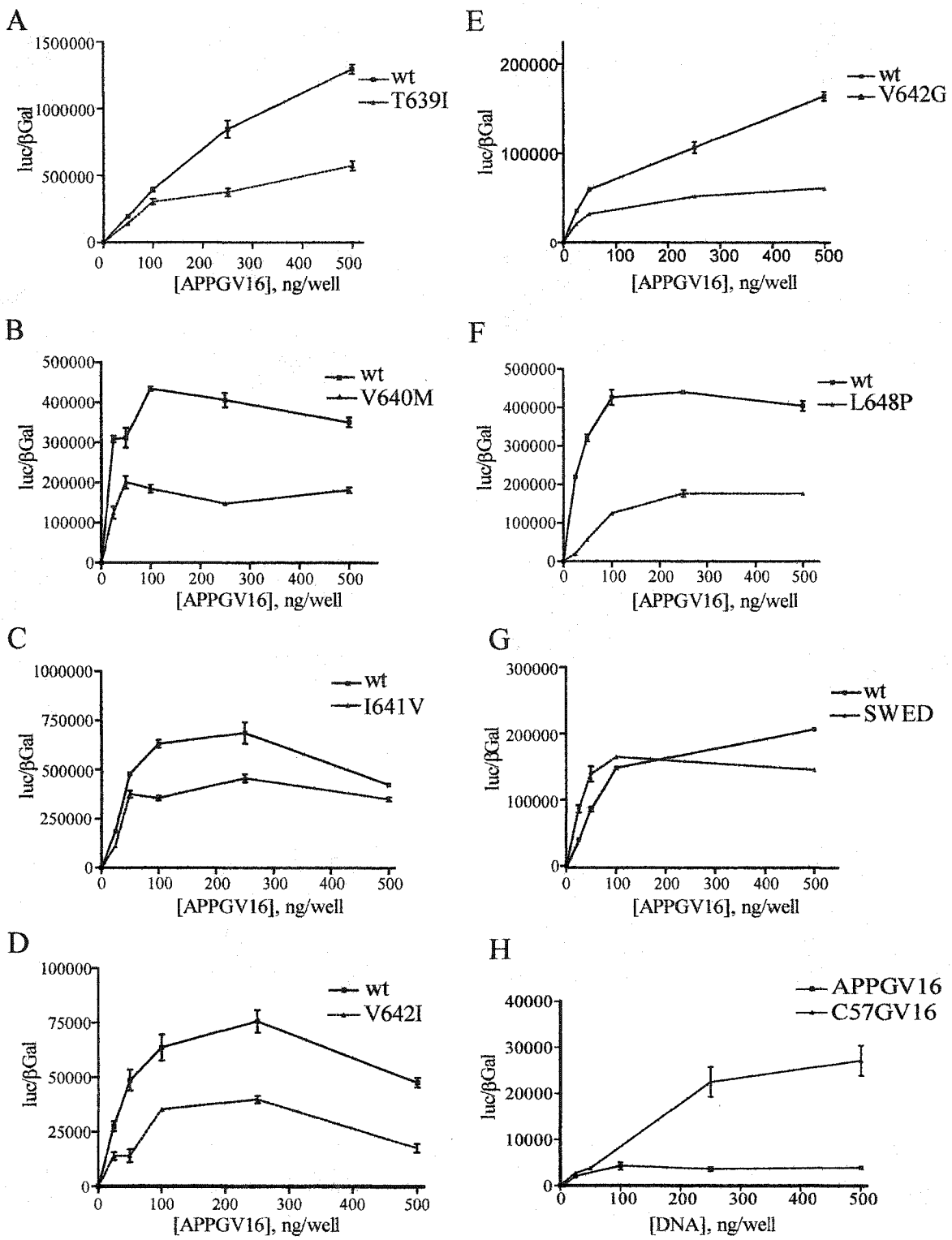
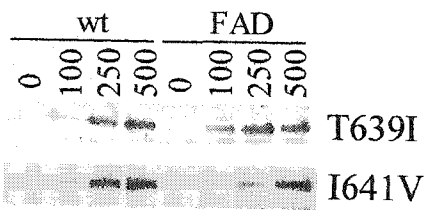


Figure 4. Comparison of APPGV16 protein expression levels

(A) APPGV16 protein levels were examined in the same lysates used for the transactivation assays (from Fig 3) for the Austrian and Florida mutants relative to wild-type. Protein levels were found to increase to very similar levels in both instances. (B) Similarity of protein expression levels resulting from equivalent amounts of transfected APPGV16 DNA was explored using the wt, T639I, V642G, V642I, and KM595/596NL isoforms. All the constructs gave bands of indistinguishable size running at the predicted molecular weight (~117 kDa). (C) Immunoprecipitation of the CTFγ-Gal4VP16 moiety from Cos7 cells expressing wild-type and T639I APPGV16 in the presence and absence of 10 μM WPE to ensure that the product observed corresponded to γ-secretase activity.

A.



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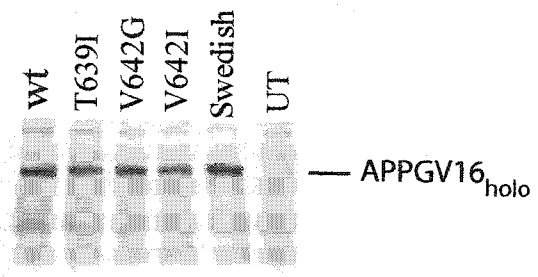

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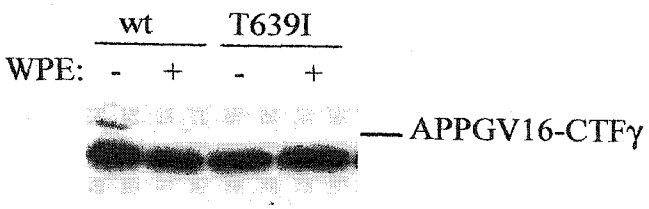


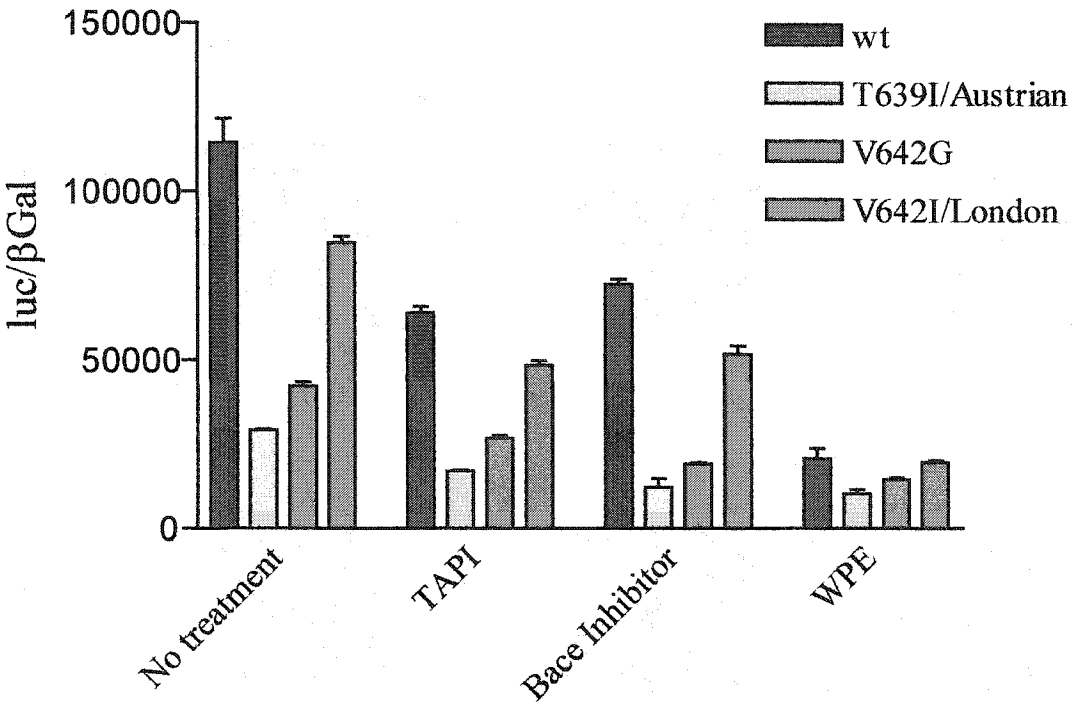
inhibitor (Fig. 4C). No CTFγ-Gal4VP16 band was detectable in the presence or absence of γ-secretase inhibitor with the T639I mutant. This further confirms that the difference between wild-type and Class II APP FAD mutants is due directly to decreases in γ-secretase mediated cleavage of mutant APPGV16.

Normal α- and β-secretase processing of wild-type and mutant APPGV16

In view of these surprising findings, we wished to confirm that APPGV16 behaves like native APP in the manner in which it is processed. γ-secretase-mediated cleavage of APP requires prior cleavage by α- or β-secretase. We assessed whether cleavage of APPGV16 was subject to a similar requirement by examining the effect of inhibitors of these enzymes. For wild-type and mutant APPGV16, inhibition of either α- or β-secretase diminishes γ-secretase-mediated release of the carboxy-terminal fragment (Fig. 5). Thus, APPGV16 processing does not differ from APP in requiring α- or β-secretase-mediated cleavage prior to γ-secretase-mediated cleavage. The different APPGV16 isoforms were inhibited equivalently by α- and β-secretase inhibitors. This indicates that the decreased γ-secretase-mediated transactivation by mutant forms of APPGV16 does not reflect diminished processing by the α- and β-secretases. The significant attenuation of wild-type and mutant APPGV16 cleavage caused by the γ-secretase inhibitor demonstrates the γ-secretase dependence of processing in each case. Further, the statistical difference maintained between wild-type and FAD mutant APPGV16 in the presence of α- and β-secretase inhibitors, disappears in the presence of γ-secretase inhibitor. These results indicate that the differences observed reflect intrinsic differences in γ-secretase processing of wild-type and mutant substrate.

Figure 5. Effects of inhibition of α-, β– and γ-secretase activity on GV16-dependent luciferase expression in wild-type and FAD mutant APPGV16.

The contribution of α-, β– and γ-secretase to the differential processing of wild-type and FAD mutant APPGV16 was assessed pharmacologically using inhibitors for each secretase. Cos7 cells were transfect with wild-type, KM595/596NL (Swedish), T639I, V642G and V642I APPGV16 constructs. TAPI, a widely used α-secretase inhibitor, was used at 50μM; β–secretase inhibitor II (Calbiochem) was used at 10 μM; WPE, the γ-secretase inhibitor, was used at 1 μM. For simplicity of analysis, a statistical threshold of significance was set at p=0.01. Gal4-dependent luciferase expression from wild-type APPGV16 was statistically higher than from any of the FAD mutations, except Swedish, in the absences of treatment. The statistical difference between wild-type and the Class II FAD mutations persisted in the presence of α- and β-secretase inhibitors. However, there were no statistically significant differences between wild-type and any of the FAD mutations in the presence of the WPE-II-89 γ-secretase inhibitor.



Identical subcellular localization of mutant and wild-type APP-GFP

While the above observations strongly suggest a direct difference in proteolytic processing between mutant and wild-type APP, it does not rule out the possibility that the mutations affect protein trafficking which results in subcellular partitioning of the substrate APP from its processing enzymes. In order to address this point, mutant and wild-type APP-GFP fusion constructs were generated. These constructs were transfected into Cos7 cells, and high-resolution confocal microscopy was undergone to assess the subcellular localization of wild-type, T639I and V642G isoforms of APP-GFP. In all cases, the localization of the protein occurred around the nucleus in a subcellular locality greatly resembling the ER/TGN. No colocalization markers were implemented to confirm that this subcellular region is the ER/TGN, but this localization pattern was consistent with numerous reports discussed earlier in this work targeting localization of APP to the ER and Golgi. However, irrespective of what subcellular organelle hosts the APP-GFP constructs there was clearly no demarcated difference between mutant and wild-type isoforms, ruling out the possibility of subcellular partitioning of APP from proteolytic secretase processing.

FAD mutations in Presenilin-1 lead to decreases in APP cleavage by γ-secretase

This finding led us to ask whether Presenilin-1 mutations associated with FAD also decrease the cleavage efficiency of APPGV16. Wild-type APPGV16 plasmid was titrated into cells transiently over-expressing either wild-type or FAD mutant forms of Presenilin-1. All FAD mutations of Presenilin-1 tested resulted in a decrement in release

Figure 6. Subcellular localization of wild-type and FAD mutant APP-GFP. Potential rearrangements in subcellular localization due to FAD mutations were tested by generating APP-GFP fusion constructs. The Human APP695 coding sequence from APPGV16 wild-type, Austrian (T639I), and V642G was subcloned into the GFP XLT fusion vector. APP_{wt}-GFP (A and B), APP_{T639I}-GFP (C and D) and APP_{V642G}-GFP (E and F) were all over-expressed in Cos7 cells. The localization pattern of the APPGFP constructs is presented 24 hours post-transfection in (A), (C) and (E). The over-expressed APP-GFP constructs are shown relative to DAPI stained nuclei in (B), (D) and (F). The scale bar in (B) represents 100 μM.

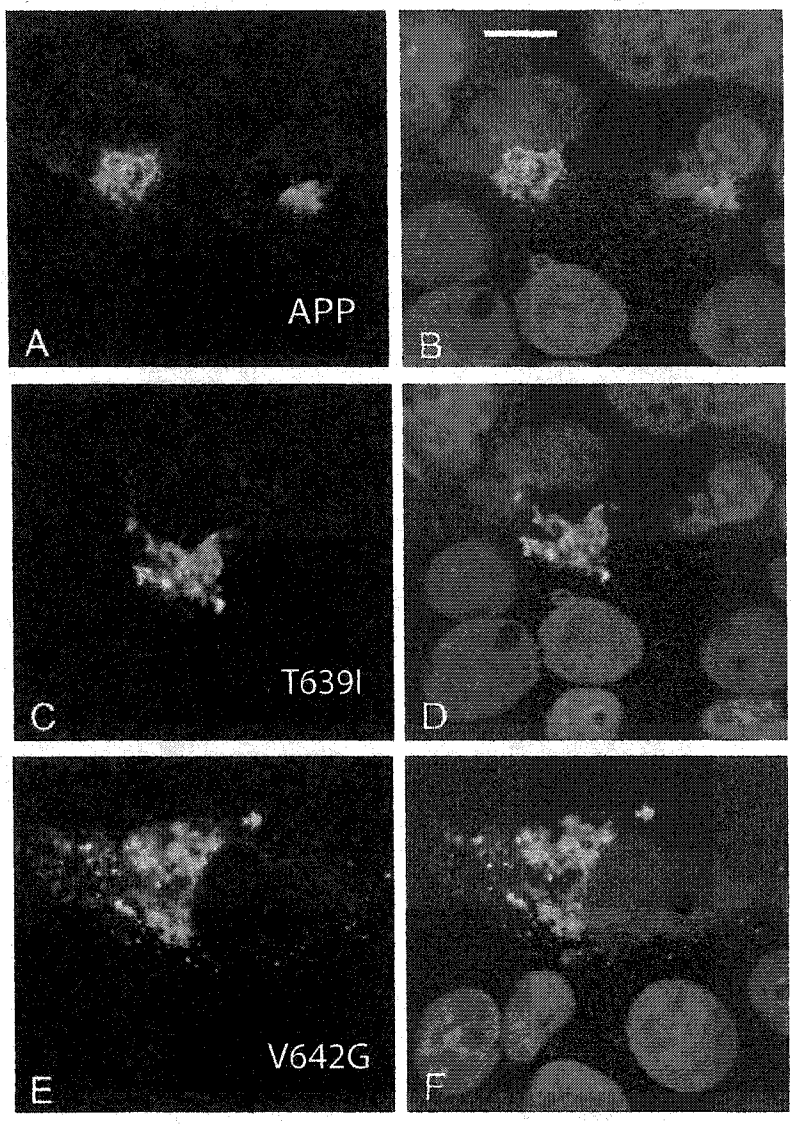
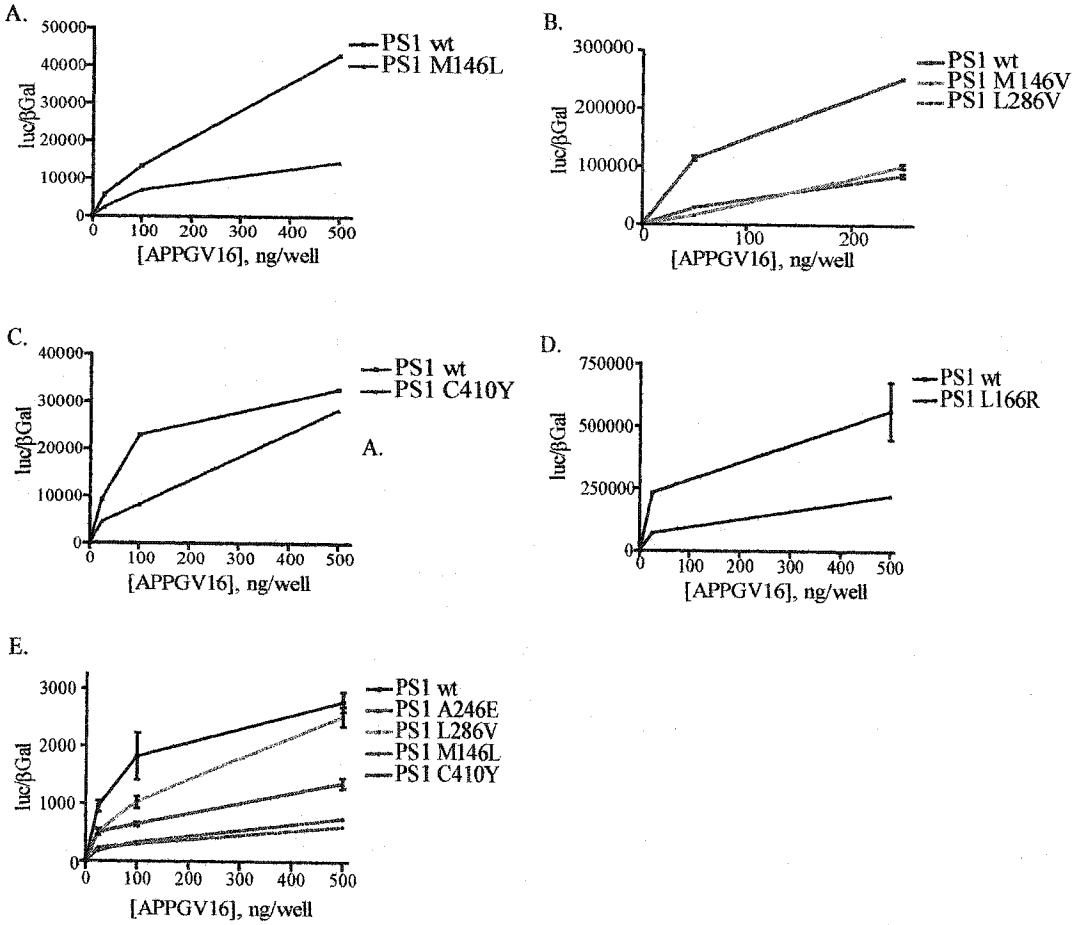


Figure 7. Comparison of cleavage of APPGV16 by wild-type and FAD mutant Presenilin-1.

γ-secretase mediated cleavage of APPGV16 was tested in both transiently and stably transfected Cos7 cells over-expressing either wild-type or FAD mutant isoforms of Presenilin-1 (constructs provided by Dr. D. Selkoe, Harvard University). 500 ng/well of Presenilin-1 vector DNA was used in all transient transfection experiments (A-C) and assays were performed 48 hours following transfection. Two-way ANOVA analysis of the wild-type and FAD mutant Presenilin-1 transiently transfected cells was performed with the following results (L286V<wt, $p<0.08$; M146L<wt, $p<0.0001$; C410Y<wt, $p<0.001$). (D) Stable Presenilin-1 transformants were generated using the same DNA vectors. We employed mixed populations of stably transfected clones to avoid clonal bias between the different Presenilin-1 lines. The Cos7 Presenilin-1 stable lines were all transfected with various quantities of APPGV16 (range 25ng-500ng APPGV16) and harvested for assay 48 hours post-transfection with APPGV16. Two-way ANOVA analysis of the comparative titration shows that GV16-dependent luciferase expression generated by all the FAD mutant forms of Presenilin-1 is significantly lower than that resulting from wild-type Presenilin-1 (A246<wt, $p<0.0001$; L286V<wt, $p<0.01$; M146L<wt, $p<0.0001$; C410Y<wt, $p<0.0001$).



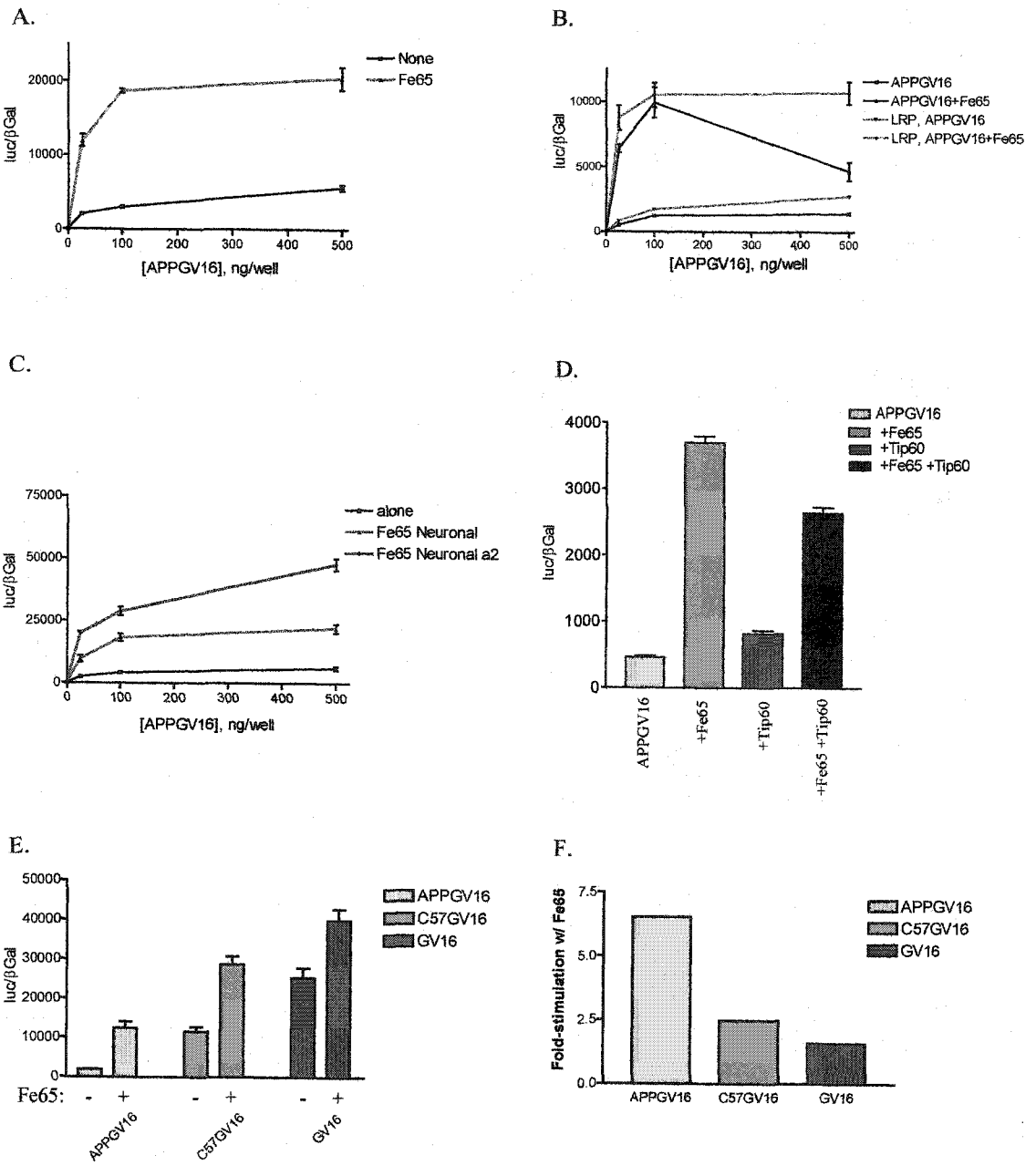
of CTFγ from APPGV16 (Figure 7A-D). It is important to note that this technique of over-expressing presenilin has been shown to result in a replacement of exogenous for endogenous presenilin (see Introduction section on Presenilin-1 processing). One of the Presenilin-1 mutations tested here has already been characterized as a loss of function mutations (Fig. 7D). (Moehlmann et al. 2002)These results were confirmed using stable cell lines expressing the same Presenilin-1 constructs, for which even greater differences between wild-type and mutant Presenilin-1 were observed (Fig. 7E). Intriguingly, the two Presenilin-1 mutations that lead to the earliest onset of FAD(Czech et al. 2000) cause the greatest decrement of APPGV16 processing (M146L, C410Y).

The Role of Fe65 in APP Processing

Since Fe65 is known to be a predominant intracellular binding partner and signaling co-factor for APP, the possibility of Fe65 modulating γ-secretase mediated APP cleavage was explored. In initial experiments, it was observed that Fe65 had a profound effect increasing APPGV16 cleavage by 8-10 fold over APPGV16 alone in Cos7 cells (Fig. 8A). Recent work demonstrating the Fe65-dependent role of LDL receptor-related protein (LRP) in APP processing(Pietrzik et al. 2002) suggested that Fe65 and LRP may play a coordinated role in APP processing. To test whether coordinate interaction between LRP and Fe65 further impacts γ-secretase mediated cleavage of APP, stable LRP expressing Cos7 cells were generated. Comparative APPGV16 titrations were performed in the presence and absence of co-transfected Fe65 in both LRP stables and naïve Cos7 cells. The results suggested that there was little or no added effect of LRP

Figure 8. Modulation of APPGV16 cleavage by Fe65

Cos7 cells were transiently transfected with titrated amounts of APPGV16 (A-C). (A) 250 ng/well of CMV-Fe65 (neuronal isoform) was transiently transfected into one set of cells and compared to cells transfected with pcDNA3.1 control expression vector. (B) Stably transformed Cos7 cells expressing the LRP protein were compared to naïve Cos7 cells in the presence or absence of transient co-transfection with CMV-Fe65 (neuronal isoform). (C) The pcDNA3.1 control expression vector was compared to over-expression of either the Fe65 neuronal or a2 isoform. (D) Tip60 and Fe65 were transiently transfected, 250 ng/well of each, to assess potential interactive effects of the two factors in promoting APPGV16 cleavage. (E) APPGV16, CTFγ-GV16 and GV16 were transiently transfected in the presence or absence of Fe65 to determine stimulatory effect of Fe65 in each context. (F) The results in (E) were plotted as a function of Fe65 stimulation by dividing the Fe65 over-expressing values by the non-expressing values for APPGV16, CTFγ-GV16 and GV16.



upon the APPGV16 processing (Fig 8B). However, the level of endogenous expression of LRP may be sufficient to mediate coordination with Fe65. Further, it is still possible that LRP association with extracellular ligand (potentially ApoE2-4) may result in different coordinative association with Fe65.

The discovery that one allele of Fe65 confers a neuroprotective effect against sporadic late-onset AD suggested it may play a specific role in APP processing. To test the efficacy of the neuronal Fe65 to the protective Fe65a2 isoform, Cos7 cells were transfected with a APPGV16 titration and the two Fe65 isoforms. Both Fe65 and Fe65a2 resulted in marked increases in signal relative to APPGV16 alone, yet the protective Fe65a2 isoform had an approximate 2-fold greater stimulatory capacity than Fe65 (Fig. 8C). This supports the notion that γ-secretase mediated cleavage of APP correlates well with AD pathogenesis. Since Tip60 is also known to associate with APP and Fe65 forming the pre-nuclear signaling complex, it was speculated that it may also play a role in regulating APP cleavage. However, in contrast to expectation Tip60 had little impact when expressed alone and appeared to either decrease APPGV16 output or do nothing, when co-transfected with Fe65 (Fig. 8D).

To ensure that Fe65 was regulating γ-secretase mediated cleavage, rather than stabilization or nuclear targeting, Fe65 effects upon APPGV16 were compared to the pre-cleaved CTFγ-Gal4GV16. The effects of Fe65 upon the nascent Gal4VP16 were also tested to rule out non-specific transcriptional effects. While Fe65 had little effect upon Gal4VP16 activity, it did increase both the APPGV16 and CTFγ-Gal4GV16 signal (Fig.

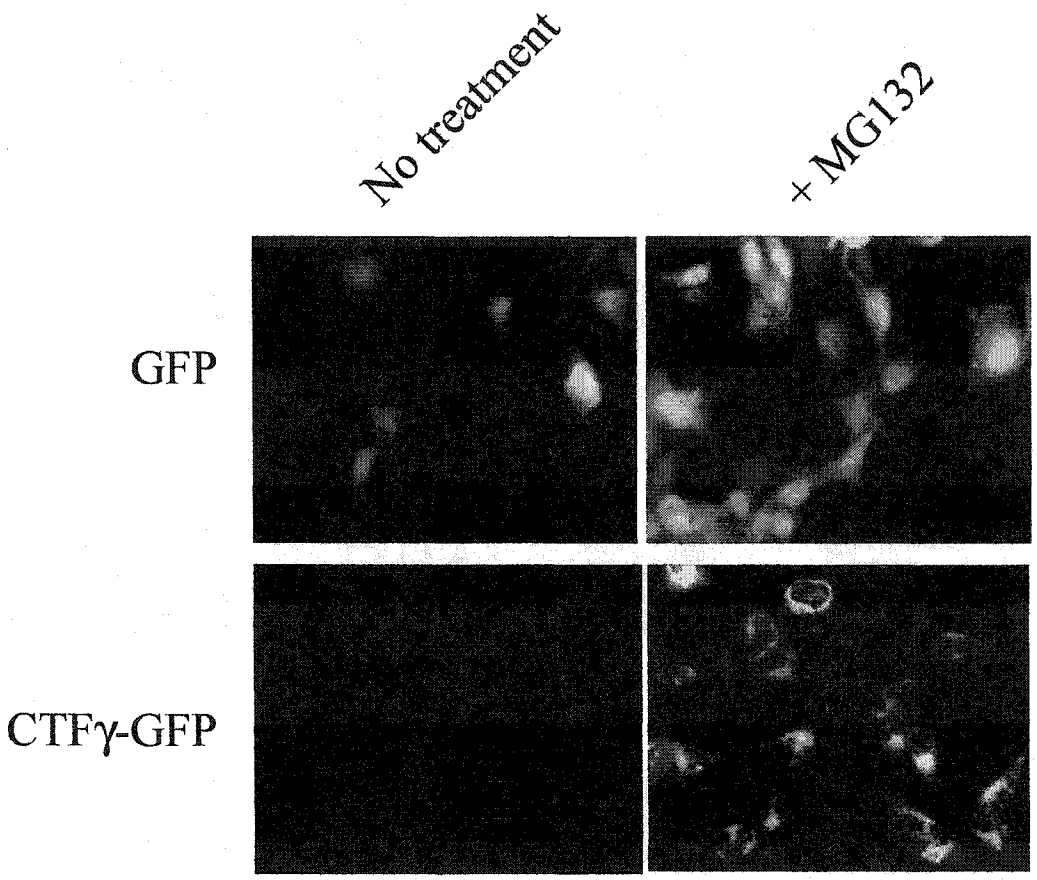
8E). However, it was observed in repeated experiments that the Fe65 effect upon APPGV16 was considerably greater than the effect observed upon CTFγ-Gal4GV16 (Fig. 8F). Plotting fold-stimulation by Fe65, it is clear that Fe65 effects upon APPGV16 are greater than 6-fold while the stimulation upon CTFγ-Gal4GV16 is barely above 2-fold. This suggest that Fe65 may play a small role in stabilization of the intracellular moiety and a greater role in the regulation of APPGV16 cleavage.

Subcellular localization of CTFγ-GFP

To assess the localization pattern of the γ-secretase cleaved fragment, CTFγ-GFP constructs were transfected into PC12M and Cos7 cells. In all instances tested no signal was detectable in the absence of proteasome inhibitors. However, with the addition of either MG-132 or PPI (both potent proteasome inhibitors) the CTFγ-GFP localized to the perinuclear region of the cell (Fig. 9). The perinuclear localization pattern was distinct from the ubiquitous distribution observed with GFP, suggesting the perinuclear localization was due exclusively to the CTFγ portion of the fusion construct. These results were performed in Cos7 cells, but identical observations have been made repeatedly in both Cos7 and PC12M cells. Since Fe65 has been reported to promote nuclear targeting of the post-cleavage APP-CTFγ fragment, CTFγ-GFP was assessed in the presence and absence of Fe65. Strikingly, as others have reported, addition of Fe65 promoted nuclear targeting of the CTFγ-GFP protein (Fig. 10). Given the proteasome sensitive expression of both CTFγ-GFP and GFP-CTFγ (data for the latter not shown), it was hypothesized that ubiquitinization of the N-terminal lysines in the stop-transfer

Figure 9. Subcellular localization of GFP-CTFγ

PC12M cells were transiently transfected with either GFP or GFP-CTFγ for 24 hours and subsequently treated with 10 uM MG-132 overnight in designated conditions. The images on the left represent PC12M cells not treated with MG-132. The column on the right shows GFP and GFP-CTFγ expressing cells that were treated with MG-132. The imaging was done using thin-section confocal microscopy.



sequence was responsible for the rapid degradation of CTFγ-GFP. In order to test this hypothesis, the KKK stop-transfer sequence was mutated to TSA. While this had little impact on the stability of the protein, as expression was still undetectable in the absence of proteasome inhibitor, it did result in a disparate localization pattern as the CTFγ-GFP_{TSA} mutant had diffuse cytoplasmic localization (Fig. 11).

Post-translational modification targeted to the KKK stop-transfer sequence

Given the unexpected findings with the CTFγ-GFP_{TSA} mutant, the possibility that an alternate lysine mediated the poly-ubiquitination associated with targeting to the proteasome was assessed. CTFγ-GFP and CTFγ-GFP_{TSA} were expressed in the presence and absence of MG-132 and PPI. Curiously, it was observed that neither protein demonstrated the well characterized molecular weight laddering associated with poly-ubiquitination (Fig. 12A). However, CTFγ-GFP was found to run as a doublet, while CTFγ-GFP_{TSA} ran as a singlet, suggestive of a N-terminal lysine directed post-translational modification. While this is the most direct explanation, it remains possible that the observed post-translational modification may be due to indirect steric effects resulting from the mutation of the lysine residues. Since the APP Thr668 residue is known to undergo phosphorylation by cdk5, the activation co-factor of which is proteasome regulated, it was sought to determine whether the doublet was due to proteasome activated cdk5 phosphorylation at this site. This explanation was dismissed since the commonly utilized cdk5 inhibitor Roscovitine failed to eliminate the upper band (Fig. 12C). Further, probing blots of lysate run from cells expressing CTFγ-GFP in the

Figure 10. Fe65 Targeting of CTFγ-GFP to the nucleus

Cos7 cells were transiently transfected with either GFP or CTFγ-GFP in the presence or absence of Fe65. All cells were treated with 10 uM MG-132 over-night. The top row is cells in the absence of Fe65. The bottom row shows images of Cos7 cels co-transfected with Fe65. All images were done using thin-section confocal microscopy.

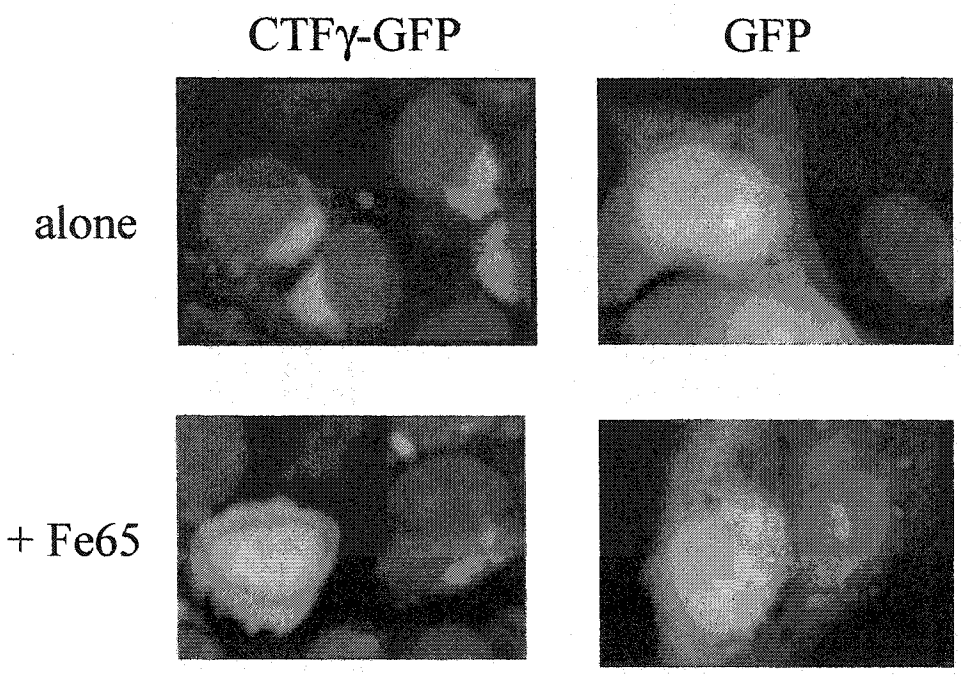


Figure 11. Subcellular localization of CTFγ-GFP and CTFγ-GFP_{TSA}

Cos7 cells were transiently transfected with either GFP, CTFγ-GFP, or CTFγ-GFP_{TSA}, or not transfected. The top row shows cells over-expressing GFP in the presence or absence of two proteasome inhibitors, each used at 10 uM (MG-132, PPI). The second row depicts cells over-expressing CTFγ-GFP in the presence or absence of proteasome inhibitors. The third row is images of cells over-expressing CTFγ-GFP_{TSA} in the presence or absence of proteasome inhibitors. The bottom row is untransfected controls. The transient transfection was performed for 24 hours and subsequently cells were treated with proteasome inhibitors in designated conditions. All images were collected using thin-section confocal microscopy.

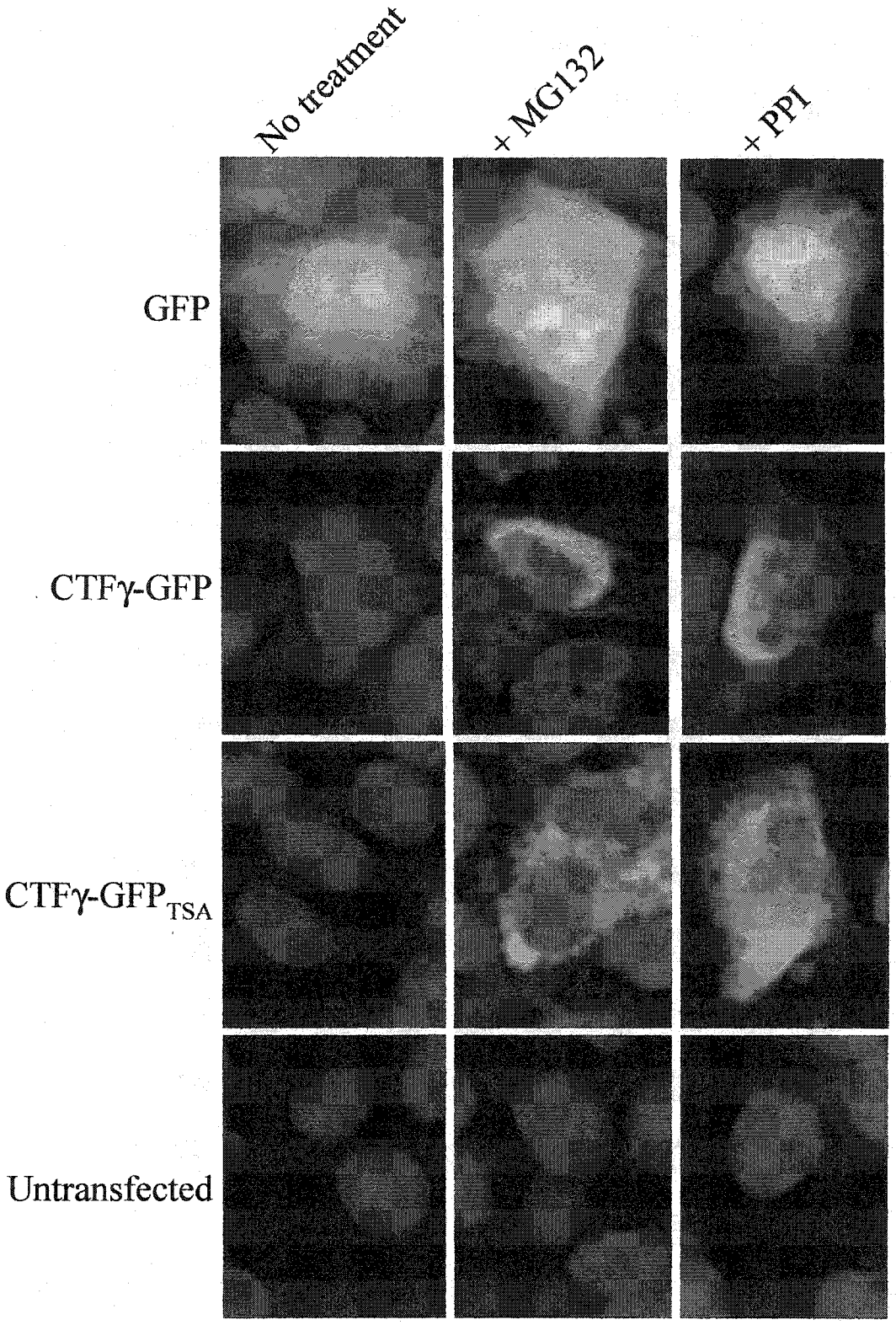
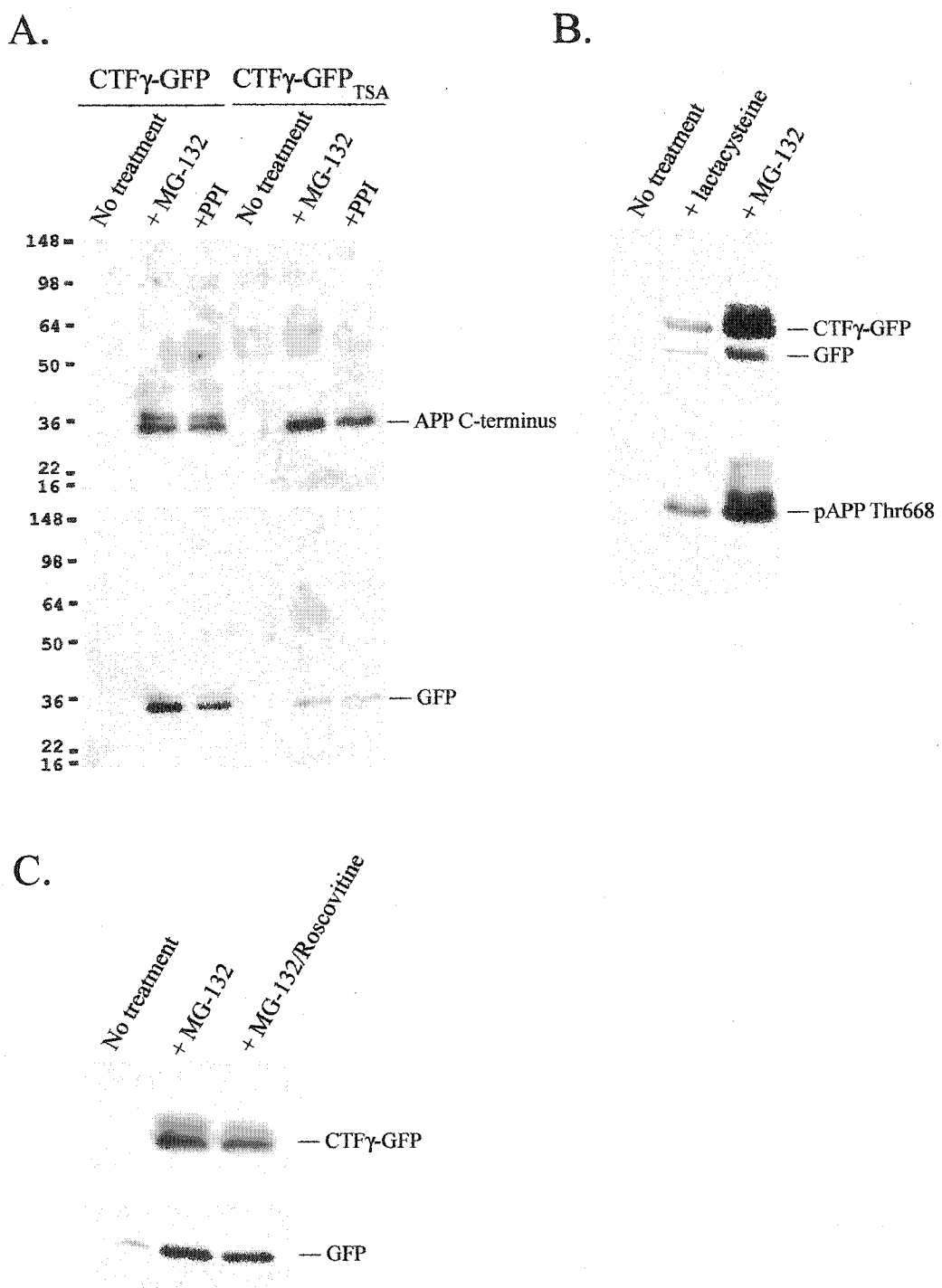


Figure 12. Post-translational modification of CTFγ-GFP at the N-terminal KKK motif (A) Cos7 cells were transiently transfected with either CTFγ-GFP or CTFγ-GFP_{TSA} in the presence or absence of 10 uM MG-132 or PPI (two proteasome inhibitors). Following 24 hours of transfection and over-night treatment with proteasome inhibitors (in designated conditions), the cells were lysed and Western blots were performed. The blots were probed with both APP carboxy-terminal (top) and GFP antibodies (bottom). (B) Cos7 cells were co-transfected with CTFγ-GFP and GFP and subsequently treated with the lactacysteine or MG-132 proteasome inhibitors in designated conditions. Western blotting was performed with the lysates and probed with either the GFP antibody (top) or the phosphor-specific APP pThr668 antibody (bottom). (C) Transient co-transfection with CTFγ-GFP and GFP followed by treatment with either MG-132 alone or MG-132 in combination with 5 uM Roscovitine (a known Cdk5 inibitor). Lysates were used in Western blotting. The blots were probed with GFP antibody. The upper panel shows the bands corresponding to CTFγ-GFP expression. The lower panel shows the bands corresponding to GFP expression.



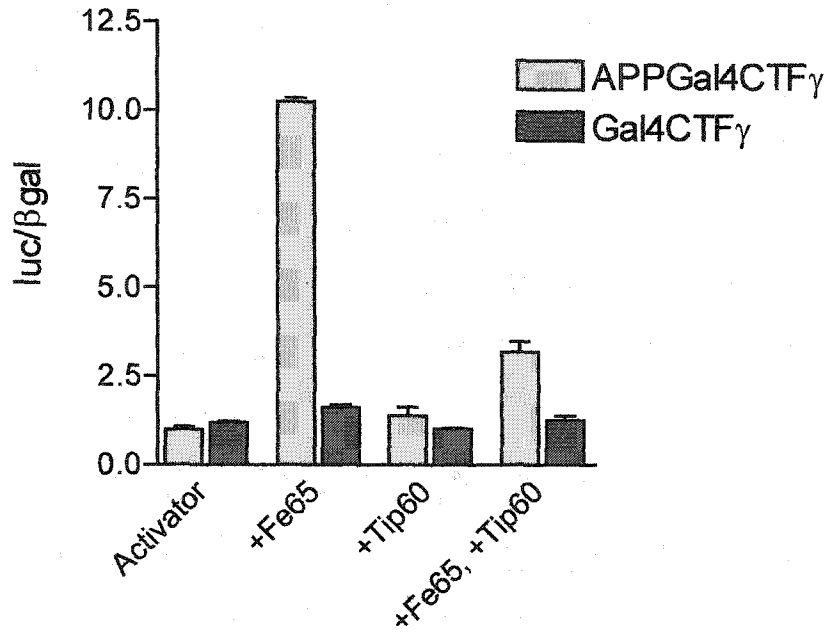
presence of proteasome inhibitor with the phospho-Thr668 antibody demonstrated recognition of both members of the doublet, suggesting the doublet was not due to phosphorylation at Thr668 (Fig. 12C). This suggests that there is an N-terminal post-translational modification that is most likely target directly to the KKK sequence.

Endogenous transactivation by APP-Gal4-CTFγ and Gal4-CTFγ

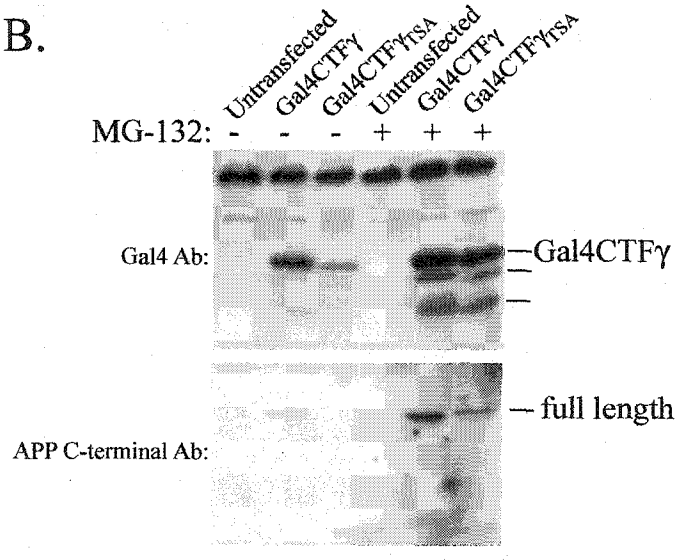
Given the previous reports of inherent transactivation potential of the CTFγ moiety of APP following γ-secretase cleavage, a comparison of the Fe65 dependence of both full-length APP and the putative C59 γ-secretase product (not what was used in previous work) transactivation capacity was explored. Consistent with work already done, full-length APP-Gal4-CTFγ stimulated transactivation in a Fe65 dependent fashion, yet no transactivation was observed using the soluble Gal4-CTFγ in the presence or absence of Fe65 (Fig. 13A). No activity was observed with the Gal4-CTFγ_{TSA} mutant either (data not presented here). Since the vector had already been sequenced verifying that the C59 moiety was in-frame with the amino-terminal Gal4 coding region, protein expression levels for both the Gal4-CTFγ and Gal4-CTFγ_{TSA} mutant were assayed in the presence and absence of the MG-132 proteasome inhibitor. Surprisingly, both constructs were easily detectable in the absence of proteosome inhibitor by probing with the Gal4 antibody (Fig 13B). A second unanticipated result was that with both the Gal4-CTFγ and Gal4-CTFγ_{TSA} mutant multiple bands were detected wherein the uppermost band corresponded to the predicted molecular weight and the lower bands were suggestive of

Figure 13. Comparative transactivation mediated by APP-Gal4CTFγ and Gal4CTFγ (A) Cos7 cells were transiently transfected with the APP-Gal4CTFγ or Gal4CTFγ constructs in the presence or absence of Fe65 and Tip60, along with the Gal4-luciferase and EF-BGal reporter system. The Gal4CTFγ used the C59 APP cleavage product. (B) Western blots were performed using lysates from cells over-expressing either Gal4CTFγ or Gal4CTFγ TSA mutant in the presence and absence of proteasome inhibitors. The blots were probed with the Gal4 antibody (top) and subsequently stripped and reprobed with the APP carboxy-terminal antibody (bottom). The single band recognized by the APP carboxy-terminal antibody corresponded exactly to the upper band in the blots probed with the Gal4 antibody.

A.



B.



cleavage products as they were not present in the untransfected controls. The lower molecular weight bands were specifically stabilized by the addition of the MG-132 proteasome inhibitor. Reprobing the blot with the APP carboxy-terminal antibody demonstrated that only the upper band was recognized, suggesting that the lower bands had lost the APP carboxy-terminal epitope. Further, neither in the presence or absence of proteasome inhibitor was any doublet detected as with the CTFγ-GFP construct, perhaps suggesting that CTFγ moiety must be at the N-terminal portion of the molecule for this modification to occur. However, expression of the Gal4-CTFγ protein in the absence of any detectable transactivation is in marked contrast to previous reports using the cytosolic portion of CTFγ for the Gal4 assay. This may suggest that there is divergent transactivation capacities germane to different carboxy-terminal cleavage products.

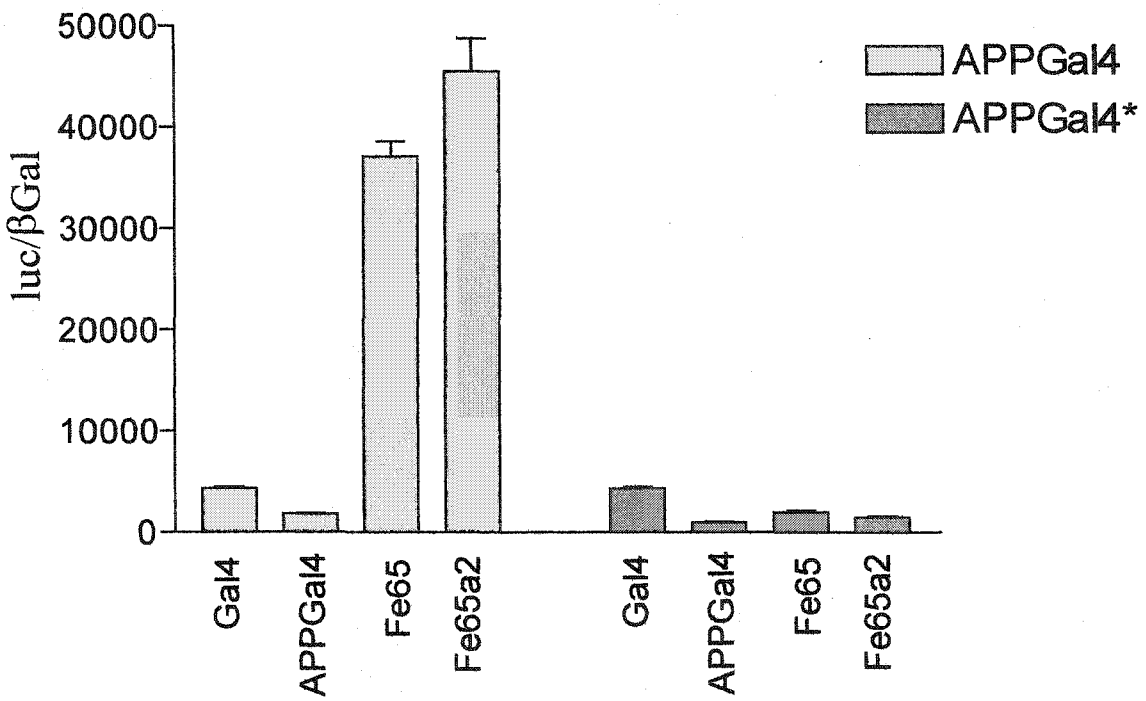
Fe65 and Fe65a2 in endogenous transactivation by APP-Gal4-CTFγ

To test whether the two isoforms of Fe65, neuronal and a2, act differently upon endogenous transactivation via CTFγ, these two forms of Fe65 were transfected into both Cos7 cells and HT22 cells. As a negative control, a version of APP-Gal4-CTFγ bearing a mutation at the Fe65 binding site was employed. In both the Cos7 cells and the HT22 cells, it was observed that both forms of Fe65 promote high levels of endogenous transactivation (Fig. 14). However, neither promoted any activation in the mutant form of APP-Gal4-CTFγ. This suggests that the affects observed are due to direct interaction of the two isoforms of Fe65 and CTFγ. The lack of any difference observed between the

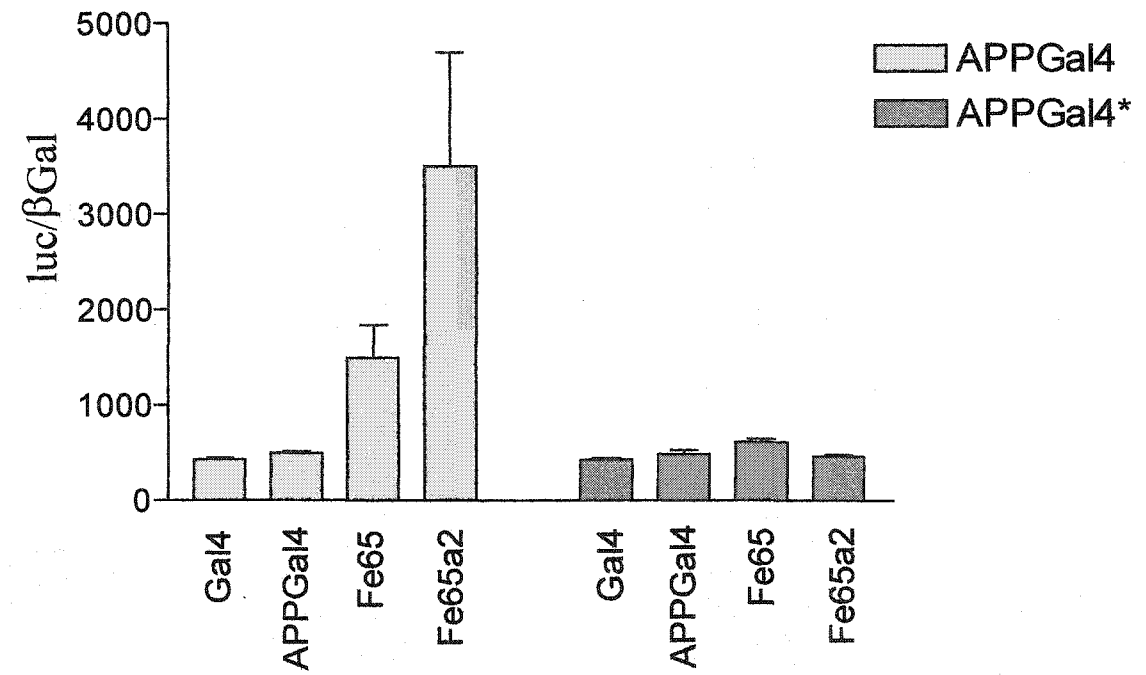
two in signaling may suggest a distinct role for these isoforms in the promotion of cleavage versus nuclear signaling.

Figure 14. Promotion of endogenous transactivation by Fe65 neuronal and a2 isoforms. Cos7 cells (A) and HT22 mouse hippocampal blastoma cells (B) were transiently transfected with either Gal4, APP-Gal4-CTFγ or APP-Gal4-CTFγ* (contains mutation at the Fe65 binding site NPTY→NATA) in the presence or absence of the two Fe65 isoforms.

A.



B.



Chapter IV: Discussion

FAD mutation in APP and Presenilin-1 are loss of function mutations

The central tenet of the amyloid hypothesis of Alzheimer's Disease is that FAD mutations in APP, Presenilin-1 and Presenilin-2 are gain of function mutations resulting in the elevated production of the pathogenic form of β-amyloid(Hardy and Selkoe 2002). While the work presented in this thesis in no way refutes the role of β-amyloid in the pathogenesis of AD, it does strongly suggest that the gain of function notion attributed to FAD mutants in APP and Presenilin-1 is wrong. Gain of function mutations are generally characterized as mutations that result in elevated enzymatic activity or signaling attributable to a disruption in some component of the regulatory process associated with a pathway or interaction. The primary observation made in the body of this work is that the global effect of FAD mutations in both APP and Presenilin-1 result in decreases in the total cleavage of the APP γ-secretase substrate.

In all of the APP FAD mutations belonging to what is herein referred to as Class II mutations, namely those mutations clustering to the transmembrane domain of APP adjacent to the γ-secretase cleavage site, produce a net decrease in liberation of the carboxy-terminal moiety of APP. The one exception observed to this phenomenon occurs with the Swedish mutation, which resides proximal to the BACE cleavage site (see Fig 1). In this particular instance, no consistent difference between the mutant and wild-type APPGV16 was observed. This is particularly surprising given the countless reports, many of which have already been discussed in the introduction section on

biochemical characterization of FAD mutations, of the Swedish mutation leading to dramatically increased levels of β-amyloid. Consequently, it is surprising that a mutation that results in elevated β-amyloid production would not have a greater impact on intracellular liberation of CTFγ. Given the that the Swedish mutation accompanies increases in the BACE cleaved APP CTF (in this case, CTF refers to the α-secretase and β-secretase cleaved APP products)(McPhie et al. 2001), these results suggest that perhaps the BACE cleaved substrate is proteolytically processed by the γ-secretase complex to a lesser extent; this could be due to differential substrate affinities or differential subcellular compartmentation. However, the reconciliation between the apparent lack of difference with the Swedish mutation and the decreases observed with the Class II FAD APP mutants suggests four possible explanations, each of which will be explored in some detail in the following segment of this discussion section.

Interestingly, the Class II APP mutations which consistently result in decreased levels of γ-secretase mediated CTFγ liberation span the transmembrane domain, with the most carboxy-terminal mutation residing proximal to the cytosolic face of the membrane. The Australian mutation (L648P) resides two residues carboxy-terminal to the recently characterized Notch-like APP cleavage site(Sastre et al. 2001; Weidemann et al. 2002; Yu et al. 2001a). While it is not yet clear whether cleavage at this position occurs sequentially with cleavage at more N-terminal positions oralternatively to cleavage at those positions, it is intriguing to note that this mutation produced profoundly deleterious consequences across the spectrum of APPGV16 concentrations used. This mutation may

not be the most profoundly impacted, yet it does provide support for the notion that APP cleavage at this site plays an important role in the proteolytic processing of APP. This is a point that will be revisited in the discussion of integrative models.

Concordant with the observations with Class II FAD APP mutations, all of the Presenilin-1 mutations examined consistently resulted in decreased APPGV16 cleavage. This observation directly rebuffs the notion of FAD presenilin mutations as gain of function aberrations. These observations are not inconsistent with isolated observation made by other groups. Recent characterization of mutations at a specific residue in Presenilin-1 demonstrated by in vitro γ-secretase assays that while β-amyloid production was undisturbed, the CTFγ moiety liberated was significantly decreased(Moehlmann et al. 2002). The Presenilin-1 L166R mutation also results in decrements in CTFγ-GV16 activity within the results presented here. Consequently, the conception of FAD Presenilin-1 mutations as loss of function mutations does have some precedent. Further, in early work using the human FAD Presenilin-1 mutants in genetic screens to attempt rescue of egg laying behavior (dependent on Notch signaling) in *C. elegans*, it was also observed that the FAD Presenilin-1 mutant genes failed to provide rescue while the wild-type human gene did(Baumeister et al. 1997). Interestingly, the single point mutations that were observed to be insufficient to rescue egg-laying behavior (C410Y and A246E) were implemented in the work presented here and demonstrated marked attenuation of γ-secretase mediated cleavage of APPGV16.

While some precedent exists for the biochemical observation that Presenilin-1 mutations may belong to the loss of function classification, the question remains as to whether there is any evidence to support a plausible connection between a loss of γ-secretase function and aspects of the neuropathology associated with AD. Little information can be obtained on this specific topic from the majority of mouse models generated as over-expression of the FAD mutant APP transgenes does not offer a direct correlation with loss of functionality, as most of these models exhibit elevated β-amyloid levels (discussed in the introduction in the section on mouse models). Further, the Presenilin-1 null mutant animals are homozygous lethal(Shen et al. 1997). However, within the brain-specific conditional Presenilin-1 knockout mouse model, the animals did exhibit learning impairments in the complete absence of elevated β-amyloid(Yu et al. 2001b). These data provide at least mild support for the notion that impairments in γ-secretase activity could be directly related to some of the cognitive dementia associated with AD pathogenesis. Further, within Familial Frontotemporal Dementia, a form of cognitive neuropathology resembling Alzheimer's Disease, a single loss of function Presenilin-1 mutation was genetically linked to the disorder(Amtul et al. 2002). Interestingly, the loss of function was determined by demonstrating that the insR352 Presenilin-1 mutation associated with Familial Frontotemporal Dementia results in repressed levels of β-amyloid production. Consequently, there is support for the notion that loss of γ-secretase activity can be correlated with dementia-like symptoms, the only countervailing argument is that FAD mutations result in elevated β-amyloid levels—

thereby, if one can reconcile the elevation in β-amyloid with the loss of functionality within the presenilin genes a holistic etiology of AD pathogenesis may begin to be formulated.

Models to reconcile decreased CTFγ and increased β-amyloid

The primary task in making sense of FAD mutations as loss of function perturbations is to reconcile how decreased γ-secretase cleavage can result in elevated β-amyloid production. There are several models that could explain how these two sets of observations could be unified, leading to a cohesive view of the etiological basis of AD pathogenesis. These models shall be proposed in summary and then a discussion of each shall ensue. The first plausible explanation is that there are multiple routes to the development of AD pathology that can arise from either the elevation in β-amyloid levels, particularly the pathogenic form, or from a disruption in normal γ-secretase function. The second model is that loss of function attributable to γ-secretase activity within the APP FAD Class II mutations is the biochemical mechanism leading to the development of elevated levels of β-amyloid, and that the decreased enzymatic function of γ-secretase does not play a fundamental role per se. The third model is that the elevation of β-amyloid directly impacts the intracellular signaling pathway associated with the γ-secretase generated APP CTFγ fragment. The fourth model, which is really a combination of the second and third models, suggests that AD pathogenesis is due to disruption of APP signaling via the CTFγ fragment either by direct decreases in γ-secretase activity or by disruption of CTFγ signaling promoted by elevations in β-

amyloid levels. The fifth model, which is admittedly highly unlikely, is that β-amyloid has little to due with AD pathogenesis and that the real etiological causes are attributable to modifications in γ-secretase processing of APP leading to a decrement in total γ-secretase mediated cleavage of alternative γ-secretase substrates.

The first model proposed is that β-amyloid and γ-secretase disruption are two separate pathways to the same pathogenesis. Such a model would appear to be the least cohesive as it suggests a redundancy of pathogenic etiology that requires a common result from very dissimilar events. The proposal of this model is really put forward as a potential resolution to how mutations that lead to elevated β-amyloid production in the absence of repressed γ-secretase function can be explained, such as the Swedish mutations. The crux of this idea centers around the notion that APP may play a critical physiological role in maintenance of neuronal viability and that disruption of this role could underlie AD progression. If elevation in β-amyloid results in a disruption of this function, which normally involves γ-secretase processing, then either repression of γ-secretase activity or elevation of β-amyloid could result in a similar physiological effect. While there is no direct evidence for such a mechanism, one could postulate that normative function of APP entails complex formation with another signaling co-factor, such as the speculative APP ligand. If this interaction promotes γ-secretase mediated cleavage, as with Notch, and subsequent signaling, then one could envision a mechanism whereby lumenal or extracellular aggregation of the pathogenic form of β-amyloid could perturb this interaction. Consequently, β-amyloid could disrupt γ-secretase function over

time as β-amyloid deposition or aggregation or protofibrillar assemblages are promoted, resulting in a redundancy of pathogenic etiology. For example, the LDL receptor-related protein (LRP) is known to associate with members of the ApoE family(Beisiegel et al. 1989), forming a ligand-receptor complex. LRP has been shown to be involved in the binding and regulation of both the APP holoprotein as well as the secreted extracellular domain(Kinoshita et al. 2001; Kounnas et al. 1995; Pietrzik et al. 2002). The LRP ligand ApoE also binds to extracellular β-amyloid, potentially facilitating plaque formation, in an isoform specific manner(Schmechel et al. 1993; Strittmatter et al. 1993a; Strittmatter et al. 1993b). Consequently, one can envision a model wherein ApoE bound LRP stimulates γ-secretase mediated APP signaling and that perturbations to the level of γ-secretase activity or ApoE sequestration with pathogenic β-amyloid could both result in decreased APP signaling.

The second model is really just a re-iteration of the amyloid hypothesis with a slight variation, gain of function with respect to β-amyloid generation is promoted by loss of function mutations to γ-secretase. The primary motivating force for this model comes from the observation that there are multiple γ-secretase cleavage sites within the transmembrane domain of APP. Recent work using mass spectroscopic peptide sequencing to characterize the CTFγ fragment have all demonstrated, counter to expectation, that the only observable cytosolic fragment corresponds to a cleavage product yielding a fifty amino acid polypeptide referred to as C50(Sastre et al. 2001; Weidemann et al. 2002; Yu et al. 2001a). The C50 cleavage site bears similarity to both

the sequence and transmembrane position of the S3 γ-secretase cleavage site in Notch. The classic γ-secretase cleavage site that results in the liberation of β-amyloid Aβ40 and Aβ42 would yield cytosolic CTFγ fragments of fifty-nine and fifty-seven amino acids respectively. The theoretical carboxy-terminal fragments associated with the β-amyloid cleavage positions are known as C59 and C57. These observations have promoted two lines of speculation about the γ-secretase processing of APP, suggesting that γ-secretase cleavage is either a multi-step proteolytic process involving both the classic β-amyloid and S3 Notch-like cleavage sites or that these are independent and alternative γ-secretase cleavage sites. Given the multiplicity of β-amyloid species, suggesting the lack of a definitive proteolytic end-product, the most parsimonious conclusion is that the β-amyloid and S3 Notch-like site are alternative cleavage points for γ-secretase.

Irrespective of which model of γ-secretase mediated cleavage turns out to be correct, if one postulates that the perturbation of γ-secretase cleavage leaves the Aβ42 cleavage position relatively unencumbered, one can reconcile elevated Aβ42 production with a net decrease in overall γ-secretase cleavage of APP. Within the alternative cleavage sites model, one would speculate that the S3 Notch-like cleavage site is the predominant cleavage position. By this account, one would predict that the S3 cleavage site is hampered by the introduction of FAD mutations to either APP or presenilin. If cleavage at the predominant site is impaired, this could reasonably be expected to result in elevation in cleavage at the normally less utilized cleavage positions (corresponding to the Aβ40 and Aβ42 cleavage sites). Consequently, the FAD mutations would result in a

shift in cleavage position from the S3 site to the pathogenic Aβ42 site. Such an explanation could easily account for decreases in total γ-secretase APP cleavage and elevation in Aβ42 production, as a fractional increase in the atypical cleavage position may still result in less overall γ-secretase cleavage.

However, if the sequential cleavage model of γ-secretase proteolytic processing turns out to be correct, the same explanation could be put forward with the minor caveat that the Aβ42 cleavage position is relatively less impaired. The sequential cleavage model is really two distinct models suggesting that either cleavage occurs first at the β-amyloid sites followed by γ-secretase cleavage at the S3 site, or that cleavage occurs first at the S3 site followed by cleavage at the β-amyloid cleavage site. The mechanics of how each mechanism could occur shall be discussed now in turn. First, the sequential model holding that γ-secretase cleavage occurs initially at the β-amyloid position necessitates the idea that the carboxy-terminal fragment remains associated with γ-secretase until cleavage occurs at the S3 site—this is formally required to explain how C50 is still the only observable C-terminal product. Consequently, if the FAD mutations bias the initial cleavage event to the Aβ42 site then one would anticipate that an impairment in completion of the proteolysis would result in elevated Aβ42 and a decrease in liberated C50 CTFγ. Mechanistically, if one posits that the efficiency of cleavage at the Aβ42 site is lower than the Aβ40 site then the issue is resolved and no further speculation is necessary. Simply, FAD mutations would decrease Aβ40 cleavage and increase the less efficient cleavage at the Aβ42 site resulting in decreased γ-secretase

processing overall and increased Aβ42 production. If one does not posit that the two β-amyloid sites have differential cleavage efficiencies, then one can again reconcile the elevation of Aβ42 and decreased γ-secretase processing overall by positing that FAD mutations bias towards cleavage at the Aβ42 site but deleterious impact the completion of the proteolysis at the S3 Notch-like site. Consequently, there are two distinct models for how to reconcile elevated Aβ42 production and decreased γ-secretase activity within the sequential β-amyloid-cleavage-first explanation.

Alternatively, if one adopts the sequential S3-cleavage-first model, one can still reconcile elevated Aβ42 production and decreased γ-secretase activity. The crux of this explanation again makes the supposition that the FAD mutations will leave cleavage at the Aβ42 site relatively (relative to Aβ40) unimpaired while simultaneously inhibiting cleavage at the S3 site. Consequently, if cleavage at the S3 site decreases by some fractional percentage while the bias towards cleavage at the Aβ42 site increases by a greater fraction, the overall effect will be a net increase in Aβ42 production and a net decrease in overall γ-secretase activity. Given that Aβ40 is known to be the predominant β-amyloid product in non-pathogenic conditions, it is not a huge leap to suggest that a relatively small shift in the overall percentage of cleavage from the Aβ40 site to the Aβ42 cleavage site could result in a dramatic increase in the observed levels of Aβ42. Such an account does make the presumption that FAD mutations will result in a net decrease in the observed levels of Aβ40 in pathogenic conditions. Observations concordant with that prediction have been made(De Jonghe et al. 2001).

Consequently, irrespective of the model of γ-secretase proteolytic processing that is adopted, one can reconcile the observation that increases in Aβ42 accompany a net decrease in overall γ-secretase activity. The relevance of this resolution to the second model is that it suggests one plausible explanation for how a common pathology arises from a decrease in γ-secretase activity. Namely, AD pathology is attributable to elevations in Aβ42 release, and the observed impairment in γ-secretase activity is merely the biochemical mechanism that promotes this event. While somewhat uninteresting, it would explain how mutations, such as the Swedish mutation, which increases β-amyloid in the absence of deleterious impact to γ-secretase function can be etiologically integrated with the observation that Class II APP mutations and all FAD Presenilin-1 mutations tested lead to a common pathology. Further, such an explanation could address how gene dosage effects observed in Down's syndrome produce a similar neuropathological outcome to specific point mutations in APP and presenilin.

The third model proposed suggests that the common etiological cause of AD is a disruption of CTFγ signaling resulting from the elevation in β-amyloid levels. Again, in many ways, this model conserves a great deal of the attributes associated with the classic amyloid hypothesis, yet suggests that the critical target of β-amyloid action is negative regulation of the signaling capacities associated with CTFγ rather than the plaque forming amyloidogenesis directly. The work demonstrating APP as a nuclear signaling complex suggests that post-proteolytic processing by γ-secretase, the CTFγ moiety moves to the nucleus, in association with Fe65 and Tip60, resulting in the activation of specific

forms of transcription(Cao and Sudhof 2001). As discussed earlier, APP has essentially two different binding regions, one N-terminal and at the C-terminus containing the YENPTY motif. Since the APP CTFγ was found to be a critical component of the transcriptional activation complex, which is not due solely to its Fe65 binding properties, it seems reasonable to assume that it is involved in binding another component of the transactivation complex with its N-terminal region. Otherwise, Fe65 ought to be sufficient to stimulate the transactivation of the Gal4-Tip60 construct as Fe65 binds directly to Tip60—yet, it was not. Accordingly, it seems likely that interruption of the two binding regions of the APP CTFγ would disrupts its nuclear signaling capacity.

Proteolytic degradation of the CTFγ moiety is still not fully understood. It appears that the insulin degrading enzyme (IDE) may play a role(Edbauer et al. 2002a). However, there is also a critical caspase cleavage site directly in the center of the CTFγ. (Gervais et al. 1999; Weidemann et al. 1999)The physiological consequence of caspase mediated cleavage at this site within APP have been suggested to either promote β-amyloid formation(Gervais et al. 1999) or to generate a second pathological peptide that promotes neuronal apoptosis(Lu et al. 2000). Which member of the caspase family performs this critical role is disputed, with suggestions that either caspase-3(Gervais et al. 1999) or caspase-8(Lu et al. 2000) may be the predominant cleaving protease. However, despite the ambiguities around the exact mechanistic induction of cleavage, caspase mediated APP proteolysis does target APP resulting in a truncated CTFγ known as C31. Since this region of the peptide retains the YENPTY motif involved in Fe65 complex

formation, it is likely that the C31 peptide would function as a dominant negative transcriptional repressor by binding Fe65 but lacking a domain for the association of other components of the transcriptional activation complex. Such a mechanism of caspase mediated transcriptional regulation to promote apoptosis has been observed within the NF-kappaB system, wherein caspase-3 cleaves the transactivation domain from p65 resulting in a protein which retains DNA binding capacity but possesses no mechanism for transactivation(Levkau et al. 1999).

The relevance of this event for the model proposed here is that by multiple accounts, increases in β-amyloid result in the activation of numerous members of the caspase family. Elevated β-amyloid has been specifically associated with the activation of both caspase-3 and caspase-8(Ivins et al. 1999; Reilly 2000; Harada and Sugimoto 1999), the two caspases implicated in the proteogenesis of C31. Consequently, irrespective of ontological origin, increases in β-amyloid may be directly coupled to activation of caspase cleavage of CTFγ. While, one can argue that activation of effector caspases, such as caspase-3, may lead to such profoundly deleterious consequences for a cell, that it is unlikely that any one specific proteolytic event is going to turn the tide away from the rocky shores of almost certain apoptosis. Yet, proto-initiator caspases, such as caspase-8, have been more directly implicated in the proteolytic generation of C31. Therefore, abrogation of CTFγ signaling could still play a critical role in determining neuronal survival. Obviously, the assumption being made here is that CTFγ transcriptional activation has some trophic effect. While there is no incontrovertible

evidence to attest to this idea, it seems the most consistent with the observation that FAD mutations result in decreases in the generation of CTFγ and that caspase mediated proteolytic disruption of CTFγ promotes neuronal apoptosis.

The fourth model is identical to the third, with the expectation that disruption of CTFγ signaling by either decreased γ-secretase activity or β-amyloid induced caspase cleavage of CTFγ is the primary etiological cause. The real distinction between model three and four is that within the fourth model, either a decrease in γ-secretase activity or elevation in β-amyloid are sufficient independently to promote AD pathogenesis. However, in situations in which both are present, the severity of the impact may be compounded. Such a model may explain why FAD mutations that robustly stimulate β-amyloid formation, as is the case with the Swedish mutation, do not promote the earliest form of AD pathogenesis. This may also contribute to explaining why Presenilin-1 mutations often produce an earlier-onset and more severe form of AD than do the APP mutations, the earliest on-set occurring with the L166P mutation resulting in an age of onset of 24 years (see the AD Mutation Database), which has been reported to have profoundly comprised γ-secretase activity(Moehlmann et al. 2002).

The fifth model, the most divergent hypothetical explanation of AD pathogenesis, suggests that neither β-amyloid nor CTFγ signaling play a fundamental role in AD, but rather the decrement in γ-secretase mediated cleavage of alternative substrates is the true etiological foundation of the AD pathology. The work here, and some work described by others, strongly supports the idea that Presenilin-1 FAD mutations are loss of function

mutations which result in a decrement in global γ-secretase enzymatic function. Consequently, it would be difficult to postulate a mechanism whereby the decrement in γ-secretase mediated cleavage of APP was not equivalently maintained across the growing set of γ-secretase substrates. Herein, it seems plausible that FAD mutations in Presenilin-1 and -2 may impact a whole set of signaling molecules whose signaling is controlled by regulated intramembrane proteolysis. If one couples this notion with aspects of the models proposed above which suggest that the enzymatic impairment of γ-secretase is the biochemical mechanism leading to the elevation in Aβ42, then the same arguments that apply to how CTFγ could be a key mediator of the etiology of AD apply equally well to other γ-secretase substrates.

While this model makes some intuitive sense in the context of presenilin mutations, it may be less obvious how mutations in APP can lead to a decrement in γ-secretase mediated processing of alternative substrates. Yet, if APP mutations result in the formation of a less efficient substrate, then one could imagine that they behave like weak transition state inhibitors. Namely, if the γ-secretase processing of APP is impaired, it seems logical to assume that the γ-secretase enzyme-substrate complex will persist for longer periods of time, potentially blocking other γ-secretase substrates. Speculation of this sort is supported by the current two step model of γ-secretase substrate processing(Esler et al. 2002; Kornilova et al. 2003). First, a recognition step occurs in which the substrate is docked and moved into what is thought to be the catalytic pore formed by the eight transmembrane segments of heterodimeric presenilin complex.

The second step entails the proteolytic cleavage of the substrate. Further support for such this model comes from the observation that Notch and APP, two γ-secretase substrates, can reciprocally inhibit the processing of the other(Kimberly et al. 2003). Consequently, if native forms of a γ-secretase substrate can inhibit processing of alternative targets, an impaired substrate that is still recognized by the γ-secretase complex is likely to impair alternative substrates to an even greater degree. Further, such a model could also account for the impact of both the Swedish mutation and the elevation in gene dosage associated with Down's syndrome, as both would lead to increased quantities of γ-secretase substrate, leading to a greater competition for processing among the other γ-secretase substrates. The list of current γ-secretase substrates includes: APP, Notch, ErbB4, nectin-I alpha, potentially the Notch ligands Delta1 and Jagged2, as well as p75 and related homologues NRH1 and NRH2(Ikeuchi and Sisodia 2003; Kim et al. 2002; Kimberly et al. 2003; Lee et al. 2002a; Rochette and Murphy 2002). Clearly, the list of substrates will grow with time, and with it the possibility that alternative γ-secretase substrates could play a role in the etiology of AD.

All of the above models are put forward with the goal of reconciling elevated β-amyloid and decreased γ-secretase activity. However, it should at least briefly be mentioned that not all APP FAD mutations lead to increased β-amyloid levels. Within studies exploring the Arctic mutation (E693G), there was noted a net decrease in the levels of both Aβ40 and Aβ42 in the plasma of those afflicted with the disorder and in cell culture models(Nilsberth et al. 2001). This study did go on to find that this mutation

in the β-amyloid peptide sequence did lead to elevated protofibrillar formation, suggesting that this mutation may increase either the effects of the amyloid protofibrils or result in elevated sedimentation despite decreases in total levels of β-amyloid. Yet, strictly speaking, it is important to note that not all APP FAD mutations result in elevations in β-amyloid. Further, studies looking at β-amyloid levels resulting from neuronal expression of APP Class II FAD mutants showed that the increased Aβ42 / Aβ40 ratio did not necessarily represent an overall increase in secreted β-amyloid(De Jonghe et al. 2001). Further, it could be argued within this same work, that a comparison of Aβ42 generated from mutant APP expression was not necessarily higher than observed in the wild-type, but rather it was the ratio alone that was consistently elevated. However, this is a weaker argument, so it shall not be pursued any further. These points, coupled with the earlier discussion about the ambiguity of AD pathological hallmarks, does suggest that while the role of β-amyloid is undoubted important, one need not necessarily constrain etiological models of AD with the presumption of β-amyloid primacy in the pathology.

APP Signaling: the role of CTFγ and Fe65

The discovery that APP possesses transcriptional signaling capacity opens the door to a whole new arena of exploration in the quest for the ultimate theory of AD pathogenesis. Drawing analogy to Notch, where γ-secretase processing plays a clear role in developmental neurogenesis and survival(Huppert et al. 2000; Xue et al. 1999; Zhang et al. 2000), it is tempting to speculate that homologous processing of APP may have a

similar level of importance in neuronal physiology. While APP may bind the Notch repressor Numb, thereby inhibiting Notch signaling following γ-secretase mediated APP cleavage(Roncarati et al. 2002), this is clearly not a complete characterization of the role of APP nuclear signaling. Identification of specific gene targets regulated by the CTFγ/Fe65/Tip60 complex will invariably provide insight into the physiological role of this transcriptional pathway.

The work presented here demonstrates that one effect of the FAD APP and Presenilin-1 mutations is to globally decrease the level of γ-secretase mediated cleavage of APP, which will lead to a decrement in liberated CTFγ and subsequent nuclear signaling. From this observation alone, it seems likely that the nuclear signaling events associated with the CTFγ/Fe65/Tip60 complex will play a trophic countervailing role to AD pathogenesis. However, this interpretation is not without peril, as the over-expression of CTFγ and Tip60 has been shown to foster apoptosis in H4 neuroglioma cells(Kinoshita et al. 2002a). While this would seem to offer a strong argument against the 'CTFγ neurotrophic' hypothesis, it must be noted that in this work the C58 CTFγ fragment was used, corresponding to the carboxy-terminal fragment generated by cleavage at the β-amyloid site. Work presented here (Fig. 13A) suggests that the C59 CTFγ does not possess the transactivation potential previously described using only the intracellular extent of the CTFγ. Consequently, one intriguing model is that CTFγ products resulting from the different cleavage positions have distinctly different levels of transactivation potential. If this model is correct, then CTFγ products resulting from γ-

secretase mediated cleavage at the β-amyloid site would function as repressors of the CTFγ/Fe65/Tip60 signaling pathway.

Multiple models were discussed in the previous section in an attempt to reconcile decreases in γ-secretase activity and increases in β-amyloid production. The model that is favored here is that the cleavage positions described represent alternative cleavage sites. Since all the work done characterizing the CTFγ fragment have shown that the observed intracellular product corresponds to cleavage at the Notch-like site, this is the most likely dominant (physiologically normal) cleavage position. Cleavage at this position would result in the C50 CTFγ, which is the closest product to that which was originally demonstrated to possess transactivation potential. The C44 fragment used in the initial work does not correspond to any known cleavage position. Given that the transmembrane portion of CTFγ is highly hydrophobic, it seems highly likely that inclusion of the transmembrane polypeptide region may have deleterious consequences for association of requisite binding partners in an aqueous environment such as the cytosol. This possibility is particularly significant given that the N-terminal region of the APP CTFγ is likely to be involved in a second binding interaction that facilitates transactivation, unless it merely functions as an allosteric regulator of Fe65.

The role of Fe65 in the APP signaling pathway has previously been characterized as a necessary co-factor for transcriptional activation, yet the work presented here suggests an additional role for Fe65 in the stimulation of γ-secretase mediated APP cleavage (Fig 8). The observation that Fe65 promotes dramatic increases in the level of

APPGV16 activity that cannot be ascribed to its previously demonstrated role in CTFγ stabilization(Kinoshita et al. 2002b), suggests that Fe65 binding to the intracellular domain of APP stimulates its processing by the γ-secretase complex. The topological characterization of presenilin suggests that both the presenilin NTF and CTF fragments have large intracellular extents fosters the notion of intracellular regulation of γ-secretase activity. The notion that Fe65 may directly regulate γ-secretase processing of APP is supported by previous work showing that Fe65 directly modulates the level of β-amyloid secretion(Sabo et al. 1999). The plausible relevance of this observation to AD pathogenesis is supported by the finding that a specific allele of Fe65 that confers resistance to late-onset AD(Hu et al. 2002a), more effectively stimulates increases in APPGV16 activity than does the non-protective neuronal allele. Collectively, this suggests that Fe65 may be a dual regulatory of APP mediated signaling, playing an initial role in the stimulation of γ-secretase mediated cleavage of APP followed by co-migration to the nucleus with the CTFγ and Tip60 to activate transcription.

While the exact mechanistic account of how this transactivation is accomplished is still unknown, some multiplicity of effect may be anticipated. As previously mentioned, the CTFγ/Fe65/Tip60 complex plays an activating role in the NF-kappaB system with respect to displacement of the p50 homodimer bound repressor complex(Baek et al. 2002). Herein, coordinated regulation of the KAI1 gene was accomplished by the CTFγ/Fe65/Tip60 heterotrimeric complex in association with MEKK1 stimulated dissociation of the heterotetrameric repressor. While the primary

function associated with KAI1 is suppression of tumor metastasis(Bienstock and Barrett 2001), it is also interacts with numerous cell-surface receptors, among which are integrins and cadherins, to promoting CD82 induced cytoskeletal rearrangements(Lagaudriere-Gesbert et al. 1998). Whether this newly identified transcriptional target plays any role in AD pathogenesis is yet to be determined. However, it does confirm the role of the APP CTFγ/Fe65/Tip60 complex in nuclear signaling.

However, while CTFγ nuclear signaling is a compelling model as an etiological explanation of AD pathogenesis, it is important to also note that a decrement in γ-secretase mediated cleavage of APP may play a much broader role in neuronal physiology. The earlier discussion of APP binding partners (in the introduction section on APP signaling) briefly touched upon the literature suggesting that APP may be involved in vesicle targeting to the synapse. In this context it is interesting to note that another APP binding factor, Mint1/X11, has also been shown to play a role in vesicular exocytosis(Van Gassen et al. 2000). Mint1/X11 forms a complex with members of the Munc18-1 family, which are requisite regulators of synaptic vesicle exocytosis(Biederer and Sudhof 2000). Genetic ablation of the Mint1 gene in mice leads to abrogated GABAergic synaptic transmission(Ho et al. 2003). While the role of APP in regulating the X11/Mint1 promotion of synaptic vesicle exocytosis is not clear, it does suggest an alluring model for APP in synaptic function. Further, while the physiological consequences of APP association with X11/Mint1 to synaptic vesicle exocytosis is not clear, X11/Mint1 has been shown to play a role in the regulation of APP proteolysis by γ-

secretase. The PDZ domain of X11/Mint1 bind directly to Presenilin-1(Lau et al. 2000). The APP mediation of Presenilin synaptic localization through its association with kinesin light chain (KLC) of the microtubule motor complex kinesin-1 has already been discussed(Kamal et al. 2001). Yet, in this context, it is important to note that the primary point of speculation was that APP interaction with KLC may promote vesicle targeting to the axon terminal. Interestingly, the association between APP and X11, as well as Munc-18-1 family members, decreases the level of APP processing by γ-secretase and its subsequent generation of β-amyloid(Borg et al. 1998; Ho et al. 2002). Consequently, synaptic APP may play a role in the regulation of vesicle targeting to the synapse through its interaction with KLC and exocytosis via the interaction with X11. Further, the possibility that Fe65 and X11 are involved in opposed regulation of γ-secretase mediated cleavage of APP suggests an intriguing role for APP in synaptic signaling. While the implications of decreased γ-secretase activity associated with the FAD mutations awaits a mechanistic account of the role of APP in exocytosis, it seems highly plausible that some perturbation of synaptic transmitter release may be anticipated.

Regulated subcellular localization and post-translational modification of APP CTFγ

The subcellular localization of the CTFγ-GFP, both in Cos7 and PC12M cells, demonstrated a perinuclear localization (Fig. 9). While passage of APP through the ER and Golgi complex is known to be involved in its glycosylation, maturation and potentially a site of γ-secretase processing, no evidence suggests post-proteolytic targeting to this subcellular domain. One highly speculative model accounting for CTFγ

subcellular localization is that the N-terminal region maintains its association with KLC following cleavage by γ-secretase. Since KLC has been localized to the ER and Golgi system(Fullerton et al. 1998; Lippincott-Schwartz et al. 1995), this would provide a potential mechanism accounting for the observation that CTFγ is targeted to the perinuclear region. Since it was observed that co-transfection with Fe65 resulted in the shift in localization to the nucleus (Fig. 10), KLC may function as a docking site for the APP CTFγ which is alternatively targeted to the nucleus by Fe65. The observation that mutations introduced to the KKK stop-transfer sequence in CTFγ resulted in ubiquitous cytoplasmic localization of CTFγ-GFP, may suggest that the N-terminal portion of CTFγ mediates the interaction with KLC. However, it is not currently known what CTFγ-GFP binds which targets it to the perinuclear region. Yet, clearly whatever the binding partner is, the interaction depends upon the presence of the KKK motif.

In an attempt to stabilize the CTFγ fragment from the rapid proteolytic degradation that accompanies its liberation from the membrane, the N-terminal lysine residues were targeted. The original rationale for making this mutation was the stabilization observed with CTFγ-GFP following the addition of proteasome inhibitors, strongly suggesting a proteasome mediated mechanism of proteolytic degradation. Given that N-terminal lysine targeted substrate ubiquitination by the E3 ubiquitin ligase is the known mechanism of proteasome targeting(Varshavsky 1997), the N-terminal tripartite lysine in CTFγ seemed the most obvious conjugation site. Surprisingly, substitution of these residues did not stabilize the CTFγ-GFP_{TSA} protein. In an attempt to determine if

an alternative lysine residue mediated the phenomenon, proteasome inhibitor stabilized CTFγ-GFP and CTFγ-GFP_{TSA} were examined for the classic poly-ubiquitin ladder. Again, surprisingly, it was observed that neither protein appeared to be ubiquitinated. However, the CTFγ-GFP protein ran as a doublet while CTFγ-GFP_{TSA} did not. While this provided little insight into the mechanism of proteolysis of CTFγ, it did suggest that the amino-terminal KKK sequence may be the site of a post-translational modification.

Given that phosphorylation at Thr668 is known to create a trans to cis conformational shift in CTFγ, it was speculated that the post-translation modification observed was due to phosphorylation at this site, the recognition of which was indirectly controlled by the presence of the KKK motif. Such a notion was supported by the fact that one of the phosphorylating kinases that targets this residue, cdk5, is regulated by a proteasome targeted co-factor(Patrick et al. 1998). However, cdk5 inhibitors did not prevent this modification and the phospho-Thr668 carboxy-terminal antibody recognized both bands of the doublet, negating the possibility that the observed post-translational modification was due to phosphorylation at this site.

While the nature of the post-translational modification and the modifying enzyme both remain unknown, one tantalizing possibility is that the lysine-directed modification is due to the acetylation of CTFγ by Tip60. Tip60, a histone acetyltransferase, is known to complex with APP through Fe65. Further, histone acetyltransferases, specifically target lysine residues for modification(Marmorstein 2001). Given that the KKK site is clearly involved in perinuclear localization,, it is possible that lysine-directed

modification of CTFγ at this site may facilitate dissociation of the CTFγ/Fe65/Tip60 complex and its subsequent migration to the nucleus. Alternatively, given the decrement in endogenous transactivation from APP-Gal4CTFγ with the addition of Tip60 (Fig 13A), it is also plausible that modification at this site plays an inhibitory role in CTFγ/Fe65/Tip60 transactivation. Whatever the modification and the modifier, it remains an interesting question how the modification observed at this site effects the physiological role of CTFγ signaling.

Overview

The primary point of this work is that FAD mutations result in a decrement in γ-secretase mediated cleavage of APP, yet what that means for neuronal physiology remains to be determined. One brief and highly speculative model is that APP functions as a 'cargo molecule' targeting Presenilin-1 containing vesicles to the axon terminal via its association with the KLC portion of kinesin-1. Once there, complex countervailing regulation by Fe65 and X11 determines the γ-secretase mediated cleavage of APP. Following cleavage, the CTFγ/Fe65/Tip60 complex may migrates back to the nucleus initiating transcriptional events requisite to the well-being of hippocampal and tertiary cortical neurons. Alternatively, the role of APP in vesicle targeting may be completely distinct from its role in nuclear signaling. Either way, the decrement in γ-secretase activity associated with early-onset FAD mutations in APP and Presenilin-1 will invariably have a broad spectrum of consequences for neuronal physiology resulting in the complex pathology of Alzheimer's Disease.

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