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Mutations in amyloid precursor protein affect its interactions with presenilin/ γ -secretase

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Abstract

Alzheimer's disease is characterized by accumulation of toxic β -amyloid ($A\beta$) in the brain and neuronal death. Several mutations in presenilin (PS1) and β -amyloid precursor protein (APP) associate with an increased $A\beta_{42/40}$ ratio. $A\beta_{42}$, a highly fibrillogenic species, is believed to drive $A\beta$ aggregation. Factors shifting γ -secretase cleavage of APP to produce $A\beta_{42}$ are unclear. We investigate the molecular mechanism underlying altered $A\beta_{42/40}$ ratios associated with APP mutations at codon 716 and 717. Using FRET-based fluorescence lifetime imaging to monitor APP-PS1 interactions, we show that I716F and V717I APP mutations increase the proportion of interacting molecules earlier in the secretory pathway, resulting in an increase in $A\beta$ generation. A PS1 conformation assay reveals that, in the presence of mutant APP, PS1 adopts a conformation reminiscent of FAD-associated PS1 mutations, thus influencing APP binding to PS1/ γ -secretase. Mutant APP affects both intracellular location and efficiency of APP-PS1 interactions, thereby changing the $A\beta_{42/40}$ ratio.

Introduction

β -amyloid ($A\beta$) is the toxic species known to aggregate and form the extracellular plaques found in the brain of Alzheimer's disease (AD) patients. $A\beta$ is generated through the processing of the β -amyloid precursor protein (APP) by β - and γ -secretases, at the ecto and transmembrane domains respectively. Processing of APP occurs as it traffics through the constitutive secretory pathway. The exact compartments where $A\beta$ is generated are not clearly defined. BACE1, the predominant β -secretase, is mainly localized to the Golgi/TGN and endosomes where following BACE cleavage the transmembrane and C-terminal domains of the APP protein remain forming the C99 fragment (Vetrivel and Thinakaran, 2006). The activity of γ -secretase localizes to several compartments: ER, Golgi/TGN, endosomes, and plasma membrane (Kaether et al., 2002; Vetrivel et al., 2004; Chyung et al., 2005). Therefore, processing may be dependent, to some extent, on the subcellular localization of the APP- γ -secretase complex.

The precision of γ -secretase transmembrane cleavage of APP determines the length of the $A\beta$ species generated. $A\beta$ toxicity correlates with fragment length, where smaller fragments have little to no toxicity, while the 42 amino acid fragment ($A\beta_{42}$) is highly fibrillogenic (Hilbich et al., 1991; Bitan et al., 2003; Chen et al., 2006; Vetrivel and Thinakaran, 2006). It has been suggested that early generation of $A\beta$ in the TGN permits intraneuronal oligomerization, allowing $A\beta$ to possess pathogenic potential prior to plaque formation (Walsh et al., 2000; Cataldo et al., 2004).

All pathogenic APP mutations associated with familial AD (FAD) cluster around the α -, β -, or γ -secretase cleavage sites and cause early onset AD. While many APP mutations increase total A β production, mutations located near the γ -secretase cleavage site have been shown to either selectively increase A β ₄₂ or increase A β _{42/40} or A β _{42/total} ratio (Goate et al., 1991; Jarrett et al., 1993; Suzuki et al., 1994; De Jonghe et al., 2001; Bergman et al., 2003; Roher et al., 2004; Tamaoka et al., 1994). We chose to focus on two APP mutations, V717I (London mutation) and I716F, located near the γ -secretase cleavage site and increasing the A β _{42/40} ratio. The V717I mutation may increase the hydrophobicity of the APP transmembrane domain to stably anchor the protein within the membrane (Goate et al., 1991). I716F, a synthetic mutation derived from determination of the γ -secretase cleavage specificity, shows a dramatic increase in the A β _{42/40} (Lichtenthaler et al., 1999).

Presenilin 1 (PS1) is a nine-transmembrane domain protein that contains the catalytic site of γ -secretase. We have previously shown that several FAD mutations in PS1, which increase A β _{42/40} ratio consistently change PS1 conformation by decreasing the proximity between the N- and C-termini ("closed") and altering alignment of the APP-PS1 interaction (Berezovska et al., 2005). We propose that active PS1/ γ -secretase exists in equilibrium of conformational states (e.g., "open" and "closed"), permitting changes in APP-PS1 interactions and accounting for γ -cleavage at A β ₄₀ or A β ₄₂, respectively. Thus, FAD PS1 mutations may manifest their pathogenicity by shifting the equilibrium to favor the "closed" PS1 conformation, leading to an increased A β _{42/40} ratio. A subset of A β ₄₂-lowering non-steroidal anti-inflammatory drugs (Weggen et al., 2001) had an opposite effect on PS1 conformation ("opening") (Leo et al., 2004). A recent report by Kukar et al. (2008) showed that some γ -secretase modulators (GSMs) change the A β _{42/40} ratio by directly binding to the A β region of APP. Although the precise mechanism of GSMs effect on the A β _{42/40} ratio is unknown, this suggests that the binding of a small molecule to APP can allosterically modulate the APP/ γ -secretase interaction.

Our goal is to investigate the mechanism of APP-related AD pathology to determine whether an APP mutation-related increase in the A β _{42/40} ratio is associated with altered APP-PS1 interactions and/ or PS1 conformation. We show that both V717I and I716F APP mutations elevate the A β _{42/40} ratio and increase intracellular A β levels, which correlate with an increased proportion of APP-PS1 interacting molecules and altered conformation and subcellular distribution of the APP-PS1 interaction. We propose that APP mutations located near the γ -cleavage site may alter the membrane positioning of APP to affect alignment with PS1/ γ -secretase, thereby stabilizing PS1 in a "close" conformation favoring A β ₄₂ cleavage. Moreover, mutant APP seems to associate with PS1/ γ -secretase earlier in the secretory pathway leading to increased intracellular A β .

Results

ELISA experiments show differential A β production from wild type and mutant APP C99

To analyze the effect of APP mutations on A β generation a human specific ELISA was used to measure A β levels. CHO cells were transiently transfected with wild type and mutant human APP C99 constructs, the membrane bound C-terminal fragment of the human APP, which is the product of APP ectodomain shedding by β -secretase. C99 represents a direct substrate for the γ -secretase. Consistent with the previously published data, we found that the amount of secreted A β was significantly different in APP mutants compared to that of the wild type APP expressing cells. Both V717I and I716F mutations caused an increase in the A β _{42/40} ratio (Table 1). In addition, cells expressing either V717I or I716F mutant APP contained significantly increased amounts of intracellular A β ₄₀ (Table 1).

Increased proportion of APP-PS1 interacting molecules in APP mutant cells

Since A β generation was altered in APP mutants, we analyzed whether mutations in APP would affect its interaction with PS1/ γ -secretase. First, we confirmed that the levels of wild type and mutant APP C99 expression were comparable in transiently transfected CHO cells. Western blot analysis showed that there was no statistically significant difference in APP C99 and PS1 expression in cells transfected with wild type, I716F, or V717I APP C99 constructs (Supplemental Fig. 1). Next, we used the Tecan FLT Ultraevolution plate reader with fluorescent lifetime capabilities (Jones et al., 2006) to monitor APP-PS1 interactions. Jones et al. have shown that in cells stably overexpressing FAD mutant PS1 it is possible to detect small changes in the average donor fluorophore lifetime (FRET efficiency) per well and changes in FRET strength as a representation of the proximity and of the proportion of interacting molecules, respectively (Jones et al., 2006). In this study, we used transiently transfected CHO cells in 96-well plates to analyze the differences between APP and PS1 interactions in the presence of wild type or mutant APP C99 constructs in a high-throughput screen format. The average lifetime of Alexa 430 donor labeling of the PS1 loop domain in the absence of an acceptor fluorophore is ~3000 ps. When a FRET acceptor fluorophore (Cy3) was added to the C-terminus of APP, Alexa 430 lifetime was significantly shortened, thereby indicating FRET (Table 2A). The lifetime results obtained using the Tecan plate reader showed that APP mutations cause an increase in the donor fluorophore lifetime shortening reflecting closer APP-PS1 proximity and/or a stronger interaction (Table 2A). The Tecan analysis only permits a whole cell analysis of the APP-PS1 interaction as it averages the donor fluorophore lifetime of each well of a 96-well plate. However, the Tecan can also provide FRET strength data by comparing the amplitudes of the populations that display or do not display FRET in each well, which is indicative of the number of interacting molecules (Jones et al., 2006). The analysis of the proportion of the donor fluorophores that exhibit FRET revealed that the basal number of interacting wild type APP and PS1 molecules per cell is ~11%. Both APP mutations significantly increased the number of interacting APP and PS1 molecules (Table 2A). These data provide added evidence that APP mutants appear to have a closer proximity to PS1, and are more likely to interact stably with PS1 within the cell compared to wild type APP.

APP mutations bring APP and PS1 into closer proximity within the proximal area of the cell

To investigate whether I716F and V717I APP mutations affect subcellular location of the APP-PS1 interaction we performed a FLIM assay on a cell-by-cell basis as previously described (Berezovska et al. 2003). One of the advantages of the FLIM assay is that it allows not only the determination of whether two molecules or epitopes interact with each other (lifetime changes), but can also detect the subcellular location and closeness of the interaction. By monitoring average pixel lifetimes in selected regions of the cell (i.e. proximal, closer to the nucleus, or peripheral, closer to the plasma membrane), we can determine the average proximity between PS1 loop domain and APP C-terminus (CT) in various subcellular compartments. The lifetime of Alexa 488 donor fluorophore labeling PS1 loop in the absence of an acceptor fluorophore was ~2400 ps. As expected, an average donor fluorophore lifetime per cell was significantly shortened when an APP C-terminal acceptor fluorophore was added indicating FRET (Table 2B). Both APP mutations, which increase the A β _{42/40} ratio, led to a significant increase in the shortening of the donor fluorophore lifetime compared to that of the wild type APP C99 expressing cells, thus confirming the data obtained by using Tecan (Tables 2A, B). This suggests that APP mutations cause an increased interaction between APP and PS1.

The closest proximity between APP and PS1 molecules (as indicated by red pixels) was detected at the cell periphery and near the plasma membrane (Fig. 1). It is of note, that in the presence of either V717I (Fig. 1F), or I716F (Fig. 1H) APP mutations, there is an increase in the number of yellow-to-red pixels (FRET present) inside the cell compared to that in wild

type APP expressing cells (Fig. 1D). By monitoring average per pixel donor fluorophore lifetimes in selected regions of the cell (i.e. proximal or peripheral), we found that near the plasma membrane APP and PS1 molecules come into close proximity and cause similar shortening of the donor fluorophore lifetime in cells expressing wild type and mutant APP constructs (~1400 ps) (Table 2B). However, in cells containing either V717I or I716F mutant APP, donor fluorophore lifetime in the proximal region of the cell was significantly shorter than that in the wild type APP expressing cells (~1700 ps versus 2100 ps, Table 2B). These data indicate an increased interaction between APP and PS1 in the area further away from the cell surface in cells expressing V717I and I716F APP mutations, which suggests a more stable interaction between APP and PS1 in the early secretory compartments prior to reaching the plasma membrane/periphery of the cell.

APP mutations alter PS1 conformation

We have previously shown that several FAD PS1 mutations that increase the $A\beta_{42/40}$ ratio change the proximity between the N- and C-termini of the PS1 molecule/heterodimer as detected by FLIM (Berezovska et al., 2005). Recent data suggest that small molecules that bind the juxta-membranous region of APP can also impact $A\beta_{42/40}$ ratios (Kukar et al., 2008). We therefore tested whether PS1 conformation would also be affected in cells expressing APP mutations that cause an increase in the $A\beta_{42/40}$ ratio. The lifetime of Alexa 488 donor fluorophore labeling PS1 NT in the absence of an acceptor fluorophore was ~2400 ps. When an acceptor fluorophore (Cy3) was added to label the C-terminus of PS1, a shortening of the donor lifetime occurred in the presence of both wild type and mutant C99 proteins indicating the presence of FRET (close proximity) between the PS1 NT and CT in intact fixed cells.

The donor fluorophore lifetime averaged over the entire cell was not statistically different between wild type and mutant APP expressing cells (Table 3). However, using a pixel-by-pixel FLIM analysis in the proximal versus peripheral regions of the cell, we found that in cells expressing the C99 APP mutants the PS1 conformation in the proximal area was altered compared to that in the C99 APP wild type expressing cells. The degree of the donor fluorophore lifetime shortening was significantly greater in the interior of the cells expressing V717I and I716F APP mutants, reflecting closer PS1 NT- CT proximity within the early secretory pathway, compared to that in the wild type APP cells (Table 3).

Fig. 2 shows pseudo-colored FLIM images for the PS1 conformation assay demonstrating the subcellular distribution of the molecules that do not (in blue) and do (closer to red) display FRET in intact cells expressing wild type, V717I, or I716F mutant APP. The V717I (Fig. 2C) and I716F (Fig. 2D) have an increase in yellow and red (FRET present) pixels inside the cell, compared to that in wild type APP expressing cells (Fig. 2B). These data suggest that the close PS1 N- and C-termini proximity in the interior region of the APP mutant cells correlates with an increase in the $A\beta_{42/40}$ ratio.

APP trafficking in cells expressing wild type or mutant APP

The change in the localization of interactions APP-PS1 populations due to APP mutations as shown by FLIM (Fig. 1) may indicate an alteration in APP trafficking to the plasma membrane. To determine whether the APP mutations affect APP trafficking/location within the cell we employed two approaches: 1) utilizing a photoactivatable-GFP (PAGFP) fusion protein to monitor APP trafficking from ER/Golgi towards the plasma membrane; and 2) measuring cell surface/early endosomal APP by using APP constructs (HA-BAP-APP) containing HA and Biotin-Acceptor peptide sequences.

First, a PAGFP was tagged to the C-terminus of the wild type or V717I APP695 to monitor the trafficking of APP out of the perinuclear region of the cell. To minimize variability due to

different mitotic states of the CHO cells, the cells were synchronized by reduced serum, co-transfected with APP-PAGFP and RFP-tagged PS1 constructs, and imaged live using a Zeiss LSM 510 confocal microscope (Fig. 3A). The PS1 RFP was used to identify cells transfected with APP-PAGFP as the efficiency of co-transfection is >90%. APP-PAGFP was activated in a selected perinuclear region of the cell (region of interest, ROI, in Fig. 3A) using a pulse-laser for 10 s. The cell was then imaged over the course of an hour in order to monitor the mean intensity of the photoactivated APP-PAGFP in the ROI over time to determine the movement of the activated APP-PAGFP out of the photoactivated area (Supplemental data, Figure S2). Following data collection, the cells were fixed and immunostained with ER and Golgi markers in order to ensure that the ROI activation area co-localized with these organelles (Fig. 3A). We found that the decrease in APP-PAGFP intensity in the initially activated area was comparable in wild type and mutant APP expressing cells indicating that the rate of trafficking of the activated APP-PAGFP molecules out of the perinuclear region was not altered in the presence of the V717I FAD APP mutation (Fig. 3B).

Next, we analyzed whether the amount of APP at/near the cell surface differs between the wild type and mutant APP expressing cells. CHO or HEK cells were transfected with HA-BAP-APP constructs (wild type, I716F, or V717I), which have a Biotin acceptor peptide that ensures specific biotinylation of the transfected wild type or mutant APP at the cell surface, and an HA tag, which we used to monitor the level of total cellular HA-BAP-APP expression. Mock-transfected cells (no DNA) were used as a control. Twenty-four hours after transfection the cells were biotinylated in the presence of BirA. The *Escherichia coli* enzyme biotin ligase (BirA, Avidity LLC., Aurora CO) specifically ligates biotin to the single lysine residue of the BAP, thus selectively labeling transfected HA-BAP-APP. The total levels of expression of the wild type and mutant APP were comparable, as detected by probing for the HA tag (Fig. 3C). Blotting with SA-IR700 detected only that portion of the APP molecules that were present at the cell surface during the biotinylation (Fig. 3C). Since the cells were incubated with biotin for 60 min at 30 °C, the detected SA signal may represent both cell surface and early endocytosed APP. First, the ratio of SA to HA (SA/HA) was calculated for wild type, I716F, and V717I APP expressing cells. The SA/HA ratio for APP mutants was expressed as a percent of that in wild type APP expressing cells. We found that the percent of APP molecules at the cell surface was slightly increased in APP mutants (106±4.5 in I716F and 108±5 in V717I, respectively), however the difference did not reach statistical significance. Thus, we conclude that I716F and V717I APP mutations do not significantly affect APP trafficking.

Discussion

Several missense mutations in APP have been implicated in Alzheimer's disease pathogenesis (<http://www.alzforum.org>), and are associated with altered A β generation and/or propensity of A β to form aggregates. The APP mutations that cluster near the APP γ -secretase cleavage site have been shown to shift the balance of γ -secretase cleavage products towards the A β ₄₂ over A β ₄₀ species, which seems to be sufficient to cause the disease. In this study we investigate the molecular mechanism of pathology associated with two APP mutations located near the γ -secretase cleavage site at codon 717 and 716 using advanced microscopy techniques in combination with biochemical approaches. The I716F and V717I APP mutations have previously been shown to elevate the A β _{42/40} ratio or A β _{42/total} by affecting the proportion of longer A β species generated (Goate et al., 1991; Lichtenthaler et al., 1999; Suzuki et al., 1994; Tamaoka et al., 1994). Our data using a human specific ELISA, which measures secreted A β , concurs with the previous findings by showing that V717I and I716F APP mutations cause a significant increase in the A β _{42/40} ratio. Moreover, we detect an increase in the accumulation of the intracellular A β ₄₀ in cells expressing either one of the APP mutations tested, compared to that in the wild type APP cells (Table 1). Intracellular A β ₄₂ levels could not be reported as they were below the sensitivity of the BAN50 ELISA in the transiently transfected cells.

To assess whether mutations in APP affect its interactions with PS1 in intact cells we employed a previously described FRET-based APP-PS1 interaction assay. The FLIM assay monitoring APP-PS1 interactions revealed that in cells expressing V717I or I716F APP mutants there was a significant shortening in the average Alexa 488 donor lifetime per cell, compared to that in the wild type APP expressing cells. Interestingly, this average per cell shortening of the donor fluorophore lifetime was mainly due to increased interactions between PS1 and mutant APP molecules in more proximal areas of the mutant APP cells. This may indicate a change in the alignment of APP and PS1 and/or an increase in the fraction of interacting APP and PS1 molecules earlier in the secretory compartments.

Several lines of evidence suggest that intraneuronal accumulation of A β precedes the accumulation and deposition of extracellular A β , and thus is likely an early cause of synaptic dysfunction and neuronal cell death (Gouras et al., 2000; Cuello, 2005; Oddo et al., 2006; Li et al., 2007; Bayer et al., 2008). However, the mechanism behind this early intracellular A β accumulation is not known. The enhanced interaction of the mutant APP with PS1/ γ -secretase early in the secretory pathway, which we observed in our FLIM assay, may cause an earlier production of A β leading to increased intraneuronal A β accumulation. It has been shown that A β oligomerization is concentration dependent (Harper and Lansbury, 1997), thus elevated levels of intracellular A β associated with APP mutations (A β ₄₀ and likely A β ₄₂ with its high propensity to form protofibrils) may lead to increased intracellular oligomeric A β species and cause cell toxicity.

Based on our findings it is tempting to speculate that the mutant APP molecules may have an increased propensity to be cleaved by γ -secretase earlier, in the ER/Golgi, compared to wild type APP. The earlier mutant APP-PS1/ γ -secretase association within the cell, coupled with normal trafficking rate out of the ER/Golgi, as demonstrated by using photoactivatable-GFP tagged APP, provides an increased amount of time for the APP-PS1 interaction to occur. We propose that this results in a greater number of mutant APP and PS1 interacting molecules, therefore leading to earlier APP processing and a subsequent increase in intracellular A β . The analysis of the FRET strength using the Tecan plate-reader (Jones et al., 2006) showed that APP mutations cause an increase in the proportion of interacting APP and PS1 molecules, when amplitudes of the populations that do and do not display FRET were compared in wild type and mutant APP expressing cells. The relatively low baseline proportion of PS1 molecules interacting with APP within the cell could be an indication that only a fraction of PS1 molecules comprise functionally active γ -secretase and/or that there is a vast number of other PS1/ γ -secretase substrates within the cell (Lleo, 2008).

A β ₄₂ represents a pathogenic A β species due to its high propensity to oligomerize (Jarrett and Lansbury, 1993) and is believed to be a driving force in A β aggregation and deposition in the AD brain. The generation of different A β species has been extensively studied, however, it remains unclear what cellular and molecular factors/events underlie the shift in the production of A β ₄₂ over A β ₄₀. It has been suggested that generation of distinct A β species may occur in different subcellular compartments. The activity of γ -secretase appears to be localized to several subcellular compartments (Kaether et al., 2002; Vetrivel et al., 2004; Chyung et al., 2005), and may be dependent, to some extent, on this localization with data suggesting that APP which is cleaved in the Golgi or ER has an elevated A β _{42/40} ratio compared to A β generated at or near the cell surface (Annaert et al., 1999; Greenfield et al., 1999; Iwata et al., 2001). Other studies suggest that A β ₄₂ is produced in the trans-Golgi and endosomal compartments (Takeda et al., 2004). All of these observations suggest that assembly and maturation of γ -secretase leads to the formation of a catalytically active complex whose activity (i.e. preferential A β species cleavage) can be modulated through allosteric modifiers, interactions with its substrates, or changes in the microenvironment, as in its subcellular localization.

Previously, we have shown that a change in the A $\beta_{42/40}$ ratio due to FAD PS1 mutations or treatment with A β_{42} -lowering NSAIDs correlates well with the change in conformation of the PS1 molecule (PS1 NT-CT proximity) and interaction between the PS1 loop domain and APP CT (Lleo et al., 2004; Berezovska et al., 2005). This suggests that PS1/ γ -secretase may exist as a mixture of meta-stable conformations with the PS1 NT and CT domains either somewhat closer together or further apart, corresponding to changes in APP-PS1 alignment and accounting for γ -cleavage at A β_{42} and A β_{40} , respectively. This hypothesis is supported by Behr et al (2004) demonstrating in cell-free systems that selected NSAIDs and their derivatives at certain concentrations could non-competitively inhibit A β_{42} production via allosteric modulation of the γ -secretase conformation. Furthermore, Isoo et al (2007) used a cysteine scanning approach to demonstrate that an elevated A $\beta_{42/40}$ ratio due to manipulations of one of the γ -secretase complex members, Pen2, is a result of the γ -secretase conformational change. Since both V717I and I716F APP mutants result in an increased A $\beta_{42/40}$ ratio, we used the FLIM assay to determine whether a change in the A $\beta_{42/40}$ ratio was associated with a change in PS1 conformation. PS1 has been suggested to form a ring structure, a catalytic pore embedded within the lipid bilayer, with PS1 NT and CT close together when in active form (Annaert et al., 2001; Esler et al., 2002; Sato et al., 2006). Recently, a nine-transmembrane topology of the PS1 molecule has been reported with the CT in the extracellular space (Henricson et al., 2005; Laudon et al., 2005; Oh and Turner, 2005; Spasic et al., 2006), thus placing PS1 NT and CT on the opposite site of the membrane. Since FRET occurs at distances <10 nm, our FLIM assay in fixed and detergent permeabilized cells is consistently able to detect FRET between PS1 NT and CT, and is sensitive to even a slight alteration in PS1 conformation and/or alignment between PS1 and APP. Indeed, we found that in cells expressing the APP mutants, PS1 had a “closer” NT-CT proximity compared to that in cells expressing wild type APP. Of note, the changed PS1 conformation was only detected in more proximal areas of the APP mutant cells. This finding may suggest that in cells expressing mutant APP i) PS1 adopts a mature conformation earlier in the secretory pathway, possibly due to its impaired trafficking, and/or ii) that the interaction with mutant APP earlier in the secretory pathway stabilizes PS1 in the “close” NT-CT conformation.

Taken together, our current data indicate that V717I and I716F APP mutations lead to several interrelated changes in APP and PS1 that could be responsible for their associated pathogenic outcome. There is a subcellular redistribution of the APP-PS1 interacting molecules in APP mutants with a higher proportion of the interaction in the ER/Golgi area. Mutant APP molecules seem to interact earlier in the secretory pathway with the PS1/ γ -secretase, which combined with the normal exit rate out of the ER/Golgi, results in an increased proportion of APP-PS1 interacting molecules in APP mutant cells, and therefore increased intracellular A β . Moreover, both APP mutations studied are located within the APP transmembrane domain and may affect how APP is positioned within the membrane, thus influencing the way APP binds to PS1/ γ -secretase, which then leads to a change in the γ -secretase cleavage site on APP (increased A $\beta_{42/40}$ ratio). We have previously shown that V715F and L720P mutations in APP modulate A β production and alter PS1 conformation in a similar way as A β_{42} -lowering NSAIDs (Tesco et al., 2005). We suggested that it is conceivable that NSAIDs, which shift γ -secretase cleavage away from A β_{42} production may target the transmembrane portion of APP and alter its helical conformation, which, in turn, alters the conformation of the PS1/ γ -secretase complex (Tesco et al., 2005). Indeed, Kukar et al (2008) have recently shown that small molecule γ -secretase modulators that affect the A $\beta_{42/40}$ ratio bind directly to the residues 28-36 of A β located within the APP transmembrane domain. This finding further confirms that alterations in the APP TMD via genetic (mutations) or pharmacological manipulations can affect the way APP substrate is presented to γ -secretase, which causes indirect conformational changes in the latter and results in predominant cleavage at the A β_{38} , A β_{40} , or A β_{42} position.

Thus, we propose that alteration in PS1 conformation and/or its alignment with APP may be responsible for a change in the A β _{42/40} ratio, a major factor leading to AD pathogenesis in cases with APP V717I mutation. Accordingly, development of drugs that can allosterically modulate γ -secretase or APP-PS1 binding interactions, in contrast to inhibiting the proteolytic activity, may represent attractive therapeutic strategies to prevent A β ₄₂-mediated neurotoxicity.

Experimental methods

Cell culture conditions and transient transfection

Chinese Hamster Ovary (CHO) and HEK cells were maintained in OPTI-MEM media (Gibco, Gaithersburg, MD) with 5% FBS in an incubator at 37 °C containing 5% CO₂. Cells were plated on four-well glass slides (Nalge Nunc International, Naperville, IL; FLIM experiments), poly-L-lysine coated glass-bottom 96-well plates (Nalge Nunc International, Naperville, IL; TECAN experiments), or either 12 well or 100 mm cell culture dishes (ELISA experiments). Twenty-four hours after passaging of cells, transient transfection was performed using Superfect (Qiagen, Valencia, CA), Lipofectamine 2000 (Invitrogen, Gaithersburg, MD), or FuGene (Roche) according to the instructions of the manufacturer. The following expression constructs were transiently overexpressed in a 1:1 ratio in cells: wild type PS1 (a generous gift from Drs. Andrew Nyborg and Todd Golde, Jacksonville, FL) and wild type C99, or mutant V717I, or I716F C99, that contain an IgK signal peptide and are tagged with V5 at the C-terminus (generous gifts from Dr. Dora Kovacs, Boston, MA).

For protein trafficking studies, a photoactivatable version of EGFP (PAGFP) fused to the C-terminus of wild type APP695 (APP-PAGFP) was generated by mutating the appropriate amino acids of wild type EGFP in order to shift its minor absorption peak (Patterson and Lippincott-Schwartz, 2002). The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to create PAGFP constructs of mutant V717I or I716F APP. PS1 RFP loop was co-transfected with APP-PAGFP as a transfection control.

A β -Enzyme-Linked ImmunoSorbent Assay, ELISA

Forty-eight hours after transient transfection of CHO cells with the respective pairs of PS1 and C99 constructs, cells were lysed in 70% formic acid for intracellular A β detection. Aliquots of conditioned media were collected in parallel to measure secreted A β . Levels of intracellular and secreted A β ₄₀ and A β ₄₂ were assessed by A β -ELISA as previously described (Fukumoto et al., 2003). Briefly, the capture antibody was BAN50 (anti-A β 1-16 aa) and detection antibodies were HRP-conjugated BA-27 (anti-A β ₄₀) and BC05 (anti-A β ₄₂).

Immunocytochemistry

Twenty-four hours after transient transfection, cells were washed in 1×Tris-buffered saline (TBS), fixed in 2% paraformaldehyde for 15 min and washed in TBS again. To permeabilize the cells and minimize non-specific staining, cells were incubated in 1.5% normal donkey serum (NDS) containing 0.01% Triton-X 100 for 45 min. Primary antibodies gtNT (Sigma-Aldrich, MO) and RbS182 (Sigma-Aldrich) were used to tag the N- and C-termini of PS1, respectively, in the PS1 NT-CT conformation assay. Mouse anti PS1 loop (Chemicon, Temecula, CA) and rabbit anti APP CT (c8, Selkoe et al., 1988) were used in the APP-PS1 proximity assay. Both sets of antibodies were applied in NDS for 1 h at room temperature (FLIM experiments) or overnight at 4 °C (TECAN experiments). After three washing steps in TBS, secondary antibodies Alexa Fluor 488 (Invitrogen, Gaithersburg, MD; FLIM experiments) or Alexa Fluor 430 (TECAN experiments) and Cy3 (Jackson Immunoresearch, West Grove, PA) were applied for 1 h at room temperature. Glass slides were enclosed with

coverslips using GVA Mounting Solution (Zymed, South San Francisco, CA) and cells on 96-well plates were maintained in PBS prior to analysis.

Detection of Fluorescence Resonance Energy Transfer (FRET) using Fluorescence Lifetime Imaging Microscopy (FLIM)

To assess the PS1 conformation and interaction between PS1 and wild type or mutant C99 we employed a validated FLIM assay (Berezovska et al., 2003; Lleo et al., 2004; Berezovska et al., 2005). In the APP-PS1 assay, the proximity of the APP C-terminus to the PS1 loop region, which is close to the active site of the γ -secretase complex, was assessed in intact cells. In the PS1 NT-CT conformation assay, the proximity between the N- and C-termini was monitored.

The epitopes of interest were labeled with Alexa Fluor 488 as donor fluorophore (PS1 NT or PS1 loop) and Cy3 as acceptor fluorophore (PS1 CT or APP CT), respectively. If the fluorophores are in close proximity (<10 nm) of each other, the donor fluorophore lifetime decreases due to a non-radiative transfer of part of its emission energy to the acceptor fluorophore. As a negative control, the donor fluorophore lifetime is measured in the absence of an acceptor fluorophore. A multiphoton microscope (Radiance 2000, Bio-Rad, Hercules, CA) using a femtosecond mode-locked Ti:Sapphire Laser (Mai Tai; Spectra-Physics, Mountain View, CA) at 800 nm was used to perform lifetime imaging measurements. Data was acquired on a time correlated single-photon counting (TCSPC) acquisition board (SPC 830; Becker and Hickl, Berlin, Germany) and a high-speed photomultiplier tube (MCP R3809; Hamamatsu, Hamamatsu City, Japan). Data analysis was performed using SPC Image (Becker and Hickl, Berlin, Germany), in which donor fluorophore lifetimes are determined by fitting the data to one (negative control) or two (experimental conditions) exponential decay curves. A 128×128 pixel matrix was created to display donor fluorophore lifetimes on a pixel-by-pixel basis in pseudo-colored images, in which red pixels represent molecules in close proximity (FRET present), whereas blue pixels represent molecules that are greater than 10 nm apart from each other (FRET absent).

Detection of FRET using a high-throughput, true time resolved fluorescent plate reader

Transiently transfected cells on 96-well plates were immunostained for an epitope on the PS1 loop, which was labeled by Alexa Fluor 430 as donor fluorophore and an epitope on the APP CT, which was labeled by Cy3 as the acceptor fluorophore. A TECAN FLT Ultraevolution system (Tecan Trading AG, Switzerland) was employed to compare the interaction of PS1 and wild type or mutant C99, respectively. In this system, excitation of the donor fluorophore is carried out by a 440 nm laser head with a repetition rate of 20 million pulses per second and the donor fluorophore decay curve is reconstructed by TCSPC. After data acquisition using XFluor Software (Tecan Trading AG, Switzerland), data was analyzed using a recently developed method for fitting fluorescence lifetime (Jones et al., 2006). In this model, the decay curve is assumed to follow the equation $A = A_1 \exp [(-t/\tau_1)\beta] + A_1 \exp (-t/\tau_1) + A_2 \exp (-t/\tau_2)$, in which A_1 , A_1 and A_2 represent the intensity of the instrument's background autofluorescence, the donor fluorophores that do not, and do display FRET, respectively. The characteristic constants of each of the fluorophore decay components are represented by τ_1 , β , τ_1 and τ_2 . Control experiments, which are performed to avoid cross-talk between decay components, include individual fits of the background and donor fluorophore lifetimes (Jones et al., 2006).

APP trafficking

Trafficking of APP out of ER/Golgi—To monitor APP trafficking in live cells we cloned human APP into photoactivatable-GFP (PAGFP) vector, which was a generous gift from Dr. Lippincott-Schwartz (Patterson and Lippincott-Schwartz, 2002). CHO cells were synchronized using serum starvation (Biosource protocol) for ~36 h prior to transient co-transfection with wild type or mutant APP-PAGFP constructs and PS1 with an RFP tag cloned into the loop

region between transmembrane domains 6 and 7. Twenty-four hours after transfection, quantification of protein trafficking was performed on a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) in live cells using a warming chamber at 37 °C supplemented with 5% CO₂. Transfected cells were identified using the 543 nm laser line to visualize PS1-RFP, and a 10 s laser-pulse at 750 nm was used to convert non-visible PAGFP constructs to green in the defined region of interest (ROI) near the nucleus. After photoactivation in perinuclear regions, z-stack images were acquired at 488 and 543 nm at 0, 5, 10, 15, 20, 25, 30, 45, and 60 min to assess anterograde transport of APP constructs out of the photoactivated area into more distal compartments. The GFP-intensity in the initially activated area was quantified using Adobe Photoshop 7.0.

Cell surface APP

To measure APP (wild type or mutant) at the cell surface we employed site-specific biotin ligase biophysical probes as described in Chen et al. (2005). The HA-BAP-APP constructs (wild type, I716F and V717I) were generated by inserting an HA tag followed by a 15-amino acid biotin acceptor peptide (BAP) at the N-terminus of APP 695 after the signal peptide sequence. The cells were transfected with wild type or mutant HA-BAP-APP using Eugene 6 reagent according to the manufacturer's instructions (Roche, Indianapolis, IN). The APP at the cell surface was biotinylated using 0.3 μM BirA and 10 μM biotin-AMP for 60 min at 30 °C. The cells were washed in PBS with 5 mM MgCl₂ (pH 7.4), lysed in PBS containing 1% Triton-X-100, 0.1% SDS and protease inhibitors. An equal amount of total protein from lysed cells expressing wild type or mutant APP were resolved on a 4-20% Tris-Glycine gel (Invitrogen), transferred onto PVDF membrane, and probed with streptavidin (SA)-IRDye 700 (Li-Cor, Lincoln, Nebraska) to detect biotin and with IRDye 800 labeled antibody against HA tag. The membrane was scanned using Odyssey Infrared Imaging System (Li-Cor Biosciences), which allows simultaneous detection of the two different antigens on the same blot by visualizing the IR dyes in different fluorescence channels (700 and 800 nm). The quantitative analysis of the western blot bands was performed using NIH Image J processing program (<http://rsb.info.nih.gov/ij/docs/intro.html>), and the cell surface HA-BAP-APP was normalized to the total expressed HA-BAP-APP.

Statistical analysis

StatView for Windows, Version 5.0.1 (SAS Institute, Inc) was employed to perform statistical analysis using Fisher's PSLD analysis of variance (ANOVA). Samples were considered significantly different if $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.mcn.2009.02.008.

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Fig. 1.

APP mutations alter the subcellular localization of the APP-PS1 interaction. Cells were immunostained with antibodies against PS1 loop domain and APP C-terminus, and labeled with Alexa 488 and Cy3, respectively. A, C, E, G - The intensity images of the Alexa 488-PS1 immunoreactivity in cells co-transfected with wild type and mutant APP C99 constructs. B, D, F, H - FLIM pseudocolor images of the donor fluorophore (Alexa488) lifetime distribution indicating where in the cell APP and PS1 molecules are interacting. The distance between the two molecules is depicted using a pseudocolor scale where blue pixels indicate molecules that do not display FRET and red pixels indicate APP-PS1 molecules coming into closest proximity. In the negative control (A, B) acceptor fluorophore was omitted. Pseudocolor images were analyzed on a pixel by pixel basis, thereby separating proximal and peripheral regions (outlined). The continuous colorimetric scale shows color-coded fluorescence lifetime in picoseconds.

Fig. 2.

APP mutations alter PS1 conformation preferentially in the proximal region of the cell. CHO cells were co-transfected with wild type PS1 and wild type APP C99 (A, B), V717I APP C99 (C) or I716F APP C99 (D). PS1 NT and CT were labeled with Alexa 488 and Cy3, respectively. In the negative control (A) acceptor fluorophore was omitted. FLIM pseudocolor images indicate Alexa 488 donor fluorophore lifetime distribution within the cell. The donor fluorophore lifetimes are depicted using a pseudocolor scale where blue pixels indicate molecules that do not display FRET and yellow-to-red pixels indicate where in the cell the N- and C-termini of PS1 molecules are coming into close proximity. The white outline in the pseudocolor FLIM image indicates perinuclear and peripheral regions of the cell where average donor lifetimes were analyzed on a pixel-by-pixel basis. The continuous colorimetric scale shows color-coded fluorescence lifetime in picoseconds.

Fig. 3.

V717I and I716F APP mutations do not affect APP trafficking. (A, B) CHO cells were co-transfected with PS1 RFP and either wild type or V717I APP 695 PAGFP following serum starvation for synchronization. Just prior to photoactivation, media was replaced with Opti-MEM without phenol red supplemented with 5% FBS. During acquisition cells were placed in a 37 °C chamber supplemented with 5% CO₂. PS1-RFP loop was used to identify PAGFP transfected cells. (A) Qualitative analysis of the ROI (outlined) where APP-PAGFP was activated and followed for the selected periods of time. Following data collection the cells were fixed and immunostained with ER and Golgi markers to confirm the area of activation. (B) Quantitative analysis of the PAGFP-APP trafficking out of the ER/Golgi. The z-stack images

were merged and analyzed for changes in mean fluorescence intensity in the activated area. Approximately 15 cells were measured for each mutation. The data was evaluated using Fisher's PLSD ANOVA. (C) Western blot analysis of APP at/near the cell surface. The cells were transfected with wild type, I716F or V717I HA-BAP-APP construct. Mock-transfected cells (no DNA) were used as a negative control. The transfected cells were incubated for 60 min at 30 °C with BirA in the presence or absence of Biotin-ATP. The cells were lysed and resolved by electrophoresis on 4-20% Tris-Glycine gel. The biotin (SA, cell surface APP) and HA signal (total transfected APP) were detected by double-labeling of the PVDF membrane with IR700 and IR800 labeled antibodies, respectively, and visualized using Odyssey IR Imaging System. *-non-specific band. “-” mock transfected cells.

Table 1

APP mutations alter the secreted A $\beta_{42/40}$ ratio and intracellular A β 40 levels in CHO cells.

APP	Secreted A β_{40} (normalized to WT)	Secreted A β_{42} (normalized to WT)	Secreted A $\beta_{42/40}$ (normalized to WT)	Intracellular A β_{40} (normalized to WT)
WT APP	1 $\pm$ 0.44	1 $\pm$ 0.08	1 $\pm$ 0.4	1 $\pm$ 0.01
V717I APP	0.58 $\pm$ 0.09 [*]	0.99 $\pm$ 0.4	1.8 $\pm$ 0.57 [*]	1.7 $\pm$ 0.03 [*]
I716F APP	0.15 $\pm$ 0.05 [*]	2.37 $\pm$ 0.4 [*]	18 $\pm$ 3.6 [*]	1.24 $\pm$ 0.02 [*]

CHO cells were transiently co-transfected with β -gal (as a transfection efficiency control) and wild type, V717I, or I716F APP C99 constructs. Media for secreted A β ELISA analysis was collected 48 h post-transfection. The level of A β for each condition was adjusted to β -gal. Cells for intracellular A β ELISA analysis were lysed 48 h post-transfection in 70% formic acid. Samples were neutralized with PBS (pH 11) to reach pH 7.2 prior to analysis with the ELISA BAN50 detection antibody.

^{*} <0.01 Fisher's PLSD ANOVA compared to WT APP, $n=3$ experiments.

Table 2A

Effect of the APP mutations on APP-PS1 interactions: Tecan plate reader analysis of the APP-PS1 interactions on a high-throughput screen format.

WT PS1 + APP	Alexa 430 Lifetime (mean±SD, ps)	FRET Strength Normalized to WT APP
WT APP	1231±101	100±25
V717I APP	1127±110 [*]	133±26 [*]
I716F APP	1150±101 [*]	120±31 [*]

CHO cells growing in 96-well plates were transfected with wild type PS1 and wild type, V717I, or I716F mutant APP C99 constructs. Alexa 430 lifetime shortening reflects proximity between PS1 loop and APP CT domains. The FRET strength in APP mutants was normalized to wild type APP. This data is the summation of three individual experiments.

* <0.01 Fisher's PLSD ANOVA compared to WT APP.

Table 2B

Effect of the APP mutations on APP-PS1 interactions: FLIM analysis of the APP-PS1 interaction.

WT PS1+APP	Alexa 488 Lifetime (mean $\pm$ SD, ps)		
	Average Per Cell	Cell Periphery	Cell Interior
WT APP ($n=37$)	1888 $\pm$ 259	1421 $\pm$ 213	2089 $\pm$ 139
V717I APP ($n=43$)	1567 $\pm$ 215 [*]	1419 $\pm$ 174	1688 $\pm$ 179 [*]
I716F APP ($n=40$)	1762 $\pm$ 268 [*]	1395 $\pm$ 286	1644 $\pm$ 130 [*]

CHO cells were co-transfected with wild type PS1 and wild type, V717I, or I716F mutant APP C99 constructs. Alexa 488 lifetime in the absence of a Cy3-acceptor was 2368 $\pm$ 94 ps ($n=35$). n =cell number.

^{*} <0.01 Fisher's PLSD ANOVA compared to WT APP.

Table 3

Effect of APP mutations on PS1 NT-CT conformation.

WT PS1 + APP	Alexa 488 Lifetime (mean±SD, ps)		
	Average per cell	Cell periphery	Cell interior
WT APP (<i>n</i> =68)	1982±182	1381±138	1750±99
V717I APP (<i>n</i> =80)	2009±111	1382±117	1673±73 *
I716F APP (<i>n</i> =76)	1896±180	1360±131	1673±53 *

FLIM analysis was performed to monitor PS1 NT-CT proximity in intact cells. CHO cells were co-transfected with wild type PS1 and wild type, V717I, or I716F mutant C99 constructs. The N- and C-termini of PS1 were labeled with Alexa 488 and Cy3, respectively. Alexa 488 (donor fluorophore) lifetime in the absence of a Cy3-acceptor was ~2400 ps (*n*=35). Shortening of the Alexa 488 (labeling PS1 NT) lifetime in the presence of Cy-3 acceptor on the PS1 CT indicates FRET, and is proportional to the distance between PS1 NT and CT. The average donor fluorophore lifetimes per cell, on the cell periphery and in the proximal area (cell interior) are shown in ps for cells expressing wild type, V717I, or I716F APP. This data is the summation of three individual experiments. The data was evaluated using Fisher's PLSD ANOVA. *n*=total cell number

*
 <0.01 Fisher's PLSD ANOVA compared to WT APP.