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Amyloid precursor protein mediates monocyte adhesion in AD tissue and ApoE^{-/-} mice

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Abstract

Amyloid precursor protein (APP) is a type 1 integral membrane protein which is highly conserved and ubiquitously expressed. Numerous data suggest it functions in cellular adhesion. For example, APP binds components of the extracellular matrix to propagate intracellular signaling responses. In order to investigate adhesion-related changes in inflamed vasculature, brains from apolipoprotein E^{-/-} (apoE^{-/-}) mice were examined for changes related to APP then compared to human Alzheimer's disease (AD) brains. Cerebrovasculature from mouse apoE^{-/-} and human AD brains revealed strong immunoreactivity for APP, APP phosphorylated at tyrosine residue 682 (pAPP) and Aβ. Further, Western blot analyses from mouse apoE^{-/-} and AD brains showed statistically higher protein levels of APP, pAPP and increased APP association with the tyrosine kinase, Src. Lastly, utilizing a modified Stamper-Woodruff adhesion assay, we demonstrated that adhesion of monocytic cells to apoE^{-/-} and AD brain endothelium is partially APP-dependent. These data suggest that endothelial APP function coupled with increased Aβ production are involved in the vascular dysfunction associated with atherosclerosis and AD.

Keywords

Alzheimer's disease; amyloid beta; cardiovascular; endothelial; vascular; adhesion

1. Introduction

Major research efforts continue to focus on the role of Aβ, a cleavage product of amyloid precursor protein, APP, in the progression of disease while the function of the holoprotein, APP is unclear. Numerous data suggest a role in cellular adhesion via cell-cell or cell-matrix interactions. The N-terminal crystalline structure of APP is reminiscent of a cell surface receptor (Rossjohn et al., 1999) and APP contains a well defined 682YENPTY₆₈₇ cytoplasmic motif, characteristic of tyrosine kinase receptors. Furthermore, it has been shown to bind components of the extracellular matrix, including: collagen, laminin, and proteoglycans (Kibbey et al., 1993; Williamson et al., 1995; Behr et al., 1996).

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Disclosure Statement

There are no actual or potential conflicts of interest.

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Adhesion of immune cells to the endothelium is a well characterized response leading to the phenotypic activation of the endothelium and eventual diapedesis of immune cells. APP-dependent adhesion may play a role in the phenotype of both immune and endothelial cells. For example, our previous data derived from using THP-1 monocytic cells demonstrated that monocytic cells utilize an APP-dependent activation response when adhering to collagen. This APP-mediated response involves recruitment and activation of specific non-receptor tyrosine kinases, Syk and Lyn (Sondag and Combs 2004). Interestingly, APP expression is upregulated in both cell types following neuronal injury and focal ischemia and activating stimuli, in microglia and endothelial cells, respectively (Goldgaber et al., 1989; Forloni et al., 1992; Banati et al., 1993, 1994). Furthermore, APP overexpression in endothelial cells is toxic (Jahroudi et al., 1998). Finally, endothelial cells express the secretase enzymes required to generate A β peptides (Davies et al., 1998) and vasculature from AD brains and its mouse models, show increased immunoreactivity for APP and A β (Kalaria et al., 1996; Kalaria 1997). Taken together these data suggest that APP has the ability, at least in some cell types, to mediate a general adhesion activation response that is propagated by specific tyrosine kinase activities.

It is interesting that atherosclerosis and AD share many common risk factors, (Rosamond et al., 2007; Rosendorff et al., 2007) but more importantly, atherosclerosis and other cardiovascular diseases, themselves, are significant risk factors for developing AD. Indeed, there are several reports in the literature linking the biology of APP with apoE, two protein risk factors relevant to AD and atherosclerosis. Numerous data support a role for apoE in the metabolism of APP (Fryer et al 2003, 2005; Van Dooren et al 2006). There is an inverse relationship between apoE levels and A β in some murine models of AD. Decreased expression of the cholesterol transporter, ATP-binding cassette transporter A1 (ABCA1), leads to decreased levels of soluble apoE in the brain and increased parenchymal and vascular amyloid deposition in mouse models of AD (Hirsch-Reinshagen, et al., 2005; Koldamoya et al., 2005). In addition, apoE4 expression in particular, appears to increase APP processing and A β production both *in vitro* and *in vivo* perhaps by interacting with the apolipoprotein E receptor 2 (ApoER2) (Ye et al., 2005; Ho et al., 2006; He et al., 2007). Conversely, APP expression and its gamma secretase-dependent cleavage appear necessary for appropriate expression of lipoprotein receptor, LRP1, and subsequent apoE levels in the brain (Liu et al., 2007). Finally, APP and apoE receptors are proteolytically processed by similar secretase activities (Hoe et al., 2008).

While both diseases involve changes in the biology of these proteins and share some vascular changes and dysfunction, a specific, mechanistic pathophysiology linking these two diseases remains unclear. Taken together, these data and our previous work demonstrating that APP has the ability to activate immune cells in an adhesion-dependent activation, led us to hypothesize that endothelial APP is involved in the vascular inflammation associated with atherosclerosis and AD. Our results demonstrate endothelial changes common to AD and atherosclerotic tissue in brain vasculature. Therefore the pathophysiology of both diseases involves vascular changes in not only APP expression and phosphorylation but likely function. This endothelial phenotype may represent a common target for interventional strategies addressing either disease.

2. Materials and methods

Materials

The anti β -amyloid and anti-cSrc antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-APP antibody was purchased from Zymed Laboratories (San Francisco, CA). The mouse IgG₁ isotype control, anti-von Willebrand factor and anti-APP (22C11) antibodies were purchased from Chemicon (Temecula, CA). Anti- α tubulin was purchased from Sigma (St. Louis, MO). The anti-Thr 668 pAPP antibody was purchased from

Abcam (Cambridge, MA). Anti-pAPP was generated by immunizing rabbits against the phospho-₆₈₂ phosphoYENPTY₆₈₇ sequence of human APP₆₉₅. Affinity purified anti-pTyr₆₈₂APP antibodies were used. Goat anti-rabbit, bovine anti-mouse and bovine anti-goat horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Mice

App^{tm1Dbo/J} homozygous (APP^{-/-}), Apoe^{tm1Unc/J} homozygous (apoE^{-/-}) and wild type mice (C57BL6/J) were purchased from Jackson Laboratory. Mice were provided food and water ad libitum and housed in a 12 hour light/dark cycle. Mice were sacrificed at 12 months for brain analyses.

Human Tissue

AD and age-matched control temporal cortex was obtained from the University of Washington Alzheimer's Disease Research Center supported through NIH P50AG05136. All animal use and human tissue use was approved by the University of North Dakota IACUC and IRB, respectively.

Tissue Culture

THP-1 cells, a monocytic cell line commercially available from the American Type Culture Collection (Manassas, VA.), are derived from the peripheral blood of a human with acute monocytic leukemia. THP-1 cells were grown in RPMI 1640 media (Life Technologies, Inc., Rockville, MD) containing 5mM Hepes, 10% heat inactivated fetal bovine serum (U.S. Biotechnologies, Inc., Parkerford, PA), and 1.5 µg/ml penicillin/streptomycin/neomycin (Gibco (Invitrogen), Rockville, MD).

Cell stimulation

THP-1 cells were removed from growth medium into serum-free RPMI 1640 and incubated at 37°C for 30 minutes prior to stimulation. To increase the amount of APP localized to the surface, cells were stimulated with 25 ng/ml lipopolysaccharide (LPS) (Sigma, St. Louis, MO) for 4 hours at 37° prior for use in the adhesion assay.

Aβ Preparation

In order to generate fibrillar and oligomeric peptides, Aβ 1-42 was purchased from Bachem (Torrance, CA) or American Peptide (Sunnyvale, CA). Oligomers were generated as described in Chromy et al., 2003. Briefly, Aβ1-42 peptide was dissolved in cold hexafluoro-2-propanol (HFIP) to a final concentration of 1mM. The peptide was aliquoted and dried under vacuum. The aliquoted peptides were stored at -80 degree C until use. For use in cell experiments, the peptide was dissolved in DMSO to a final concentration of 5mM then diluted to 100µM in Ham's/F12 media. The peptide oligomers were then incubated 24 hours, 4 degree C then spun 14,000 rpm, 4 degree C, 10 min. The supernatant was collected as the oligomeric Aβ peptide. Fibrils were prepared by dissolving Aβ 1-42 peptides in ddH2O to a final concentration of 250µM then incubated at 37 degree C for 1 week (Floden et al., 2005). Peptides were resolved by 15% SDS-PAGE and Western blotted using anti-Aβ antibody sc-5399 (Santa Cruz).

Cell labeling

To fluorescently label activated THP-1 cells, we incubated previously stimulated THP-1 cells for 10–15 minutes at 37°C with Cyquant DNA-labeling dye from Invitrogen's Cyquant NF cell proliferation assay kit (Invitrogen (Life Technologies), Rockville, MD).

Immunohistochemistry

To perform immunohistochemistry, tissue was collected and fixed in 4% paraformaldehyde. Tissue sections (40 μ m) were immunostained using anti-APP, anti-pAPP, and anti-A β (Vector Blue) or anti-vonWillebrand (DAB) (Vector Laboratories, Burlingame, CA). To double immunostain tissue sections, sections were first immunostained using anti-von Willebrand factor using DAB as the chromagen (Vector Laboratories, Burlingame, CA). Sections were then stripped with 0.2N HCl, 10 min., 25 °C, rinsed and immunostained with anti-APP, anti-pAPP or anti-A β using Vector VIP Blue alkaline phosphatase as the chromagen (Vector Laboratories, Burlingame, CA). As a control for staining due to non-specific binding of secondary antibodies, sections were immunostained in parallel as described in the absence of primary antibodies. To verify the specificity of our anti-A β and anti-phosphoAPP antibodies, antibody binding competition was performed by pre-incubating anti-A β and anti-pAPP antibodies with recombinant A β 1-42 (American Peptide) for the A β antibody and pTyr682NPTY (immunizing peptide) or YNPTY (negative control peptide) for the pAPP antibody for 1 hour at 25°C before immunostaining with the primary antibodies.

Western blotting

To perform Western blot analyses, tissue was collected and lysed in ice cold RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄ 10 mM NaF, 1 mM EDTA, 1mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate). Tissue lysates were sonicated and insoluble material removed by centrifugation (14,000 rpm, 4°C, 10 min). Protein concentrations were quantified by the Bradford method (1976). Proteins were resolved by 10% SDS-PAGE and transferred to PVDF membranes for Western blotting. Western blots were incubated with primary antibodies overnight at 4°C. Blots were probed with affinity purified horseradish peroxidase-conjugated secondary antibodies and antibody binding was visualized by enhanced chemiluminescence. Optical density of protein bands from 6 samples (apoE^{-/-} and wild-type, AD and wild-type) were quantified using Adobe Photoshop software and statistical analysis performed. Data are presented as mean values ($\pm$ SD), and statistical significance was determined by unpaired t test (*p < 0.001). pAPP: APP ratios were calculated by taking the individual pAPP optical densities divided by the corresponding individual APP optical densities (*p < 0.001).

Immunoprecipitation

For co-immunoprecipitations, tissue was collected and lysed in ice-cold Triton lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄ 10 mM NaF, 1 mM EDTA, 1mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1% Triton). Brain homogenates were incubated on ice for 15 minutes, followed by centrifugation to remove insoluble material (14,000 rpm, 4°C, 10 min). Homogenates were incubated with precipitating antibody (anti-APP) (1 μ g of antibody/mg protein lysate) for 2 hours at 4°C, followed by incubation with protein A/G agarose beads for 2 hours at 4°C. Resulting immunoprecipitates were washed three times in Triton buffer and resolved by 10% SDS-PAGE and Western blotted as described. Optical density of Src protein bands from three independent experiments were quantified using Adobe Photoshop software and statistical analysis performed (unpaired t test). Data are represented as mean ($\pm$ SD) (*p < 0.001).

Stamper-Woodruff adhesion assay

We utilized a modified Stamper-Woodruff adhesion assay as previously described by Stamper and Woodruff (1976). ApoE^{-/-}, APP^{+/-}, APP^{-/-}, APP^{+/+} (wild-type/control), AD and age-matched control fixed brain sections (40 μ m) of the temporal cortex were fluorescently immunolabeled with anti-smooth muscle, actin (to indicate arteries versus veins), mounted onto subbed slides and allowed to air dry at room temperature. Once dry, sections were

incubated with or without 22C11 (anti-APP) or mouse IgG₁ (isotype control) antibodies in phosphate buffered saline (PBS) solution (PBS containing 0.5% BSA, 2% FBS) for 1 hour at room temperature. Sections were rinsed with PBS solution before an hour-long incubation with stimulated and labeled THP-1 cells (as described above) at room temperature with gentle agitation (50 rpm). Sections were rinsed three times with PBS solution to rinse off non-adherent cells. Tissue sections and adherent cells were fixed with 4% paraformaldehyde for 20 minutes at 37°C. Images were captured using a Zeiss (Zeiss, Thornwood, NY) LSM 510 Meta confocal microscope.

Quantification of adherent cells

Number of adherent cells was determined in a double blinded fashion by counting the number of cells adherent to smooth muscle actin immunolabeled vessel walls in 8 fields of four to five independent sections from at least three animals from each background. Data are presented as mean values ($\pm$ SD), and statistical significance was determined by unpaired ANOVA with Tukey-Kramer's *post hoc* comparison (* $p < 0.001$).

3. Results

ApoE^{-/-} cerebrovasculature demonstrated increased levels of APP and pAPP accompanied by an increased association with the non-receptor tyrosine kinase, Src

Based upon our interest in comparing vascular changes in AD versus atherosclerotic tissue we chose to focus on cerebrovasculature. We hypothesized that APP can mediate a similar activating response in endothelial cells as we have observed in monocytes/microglia. To begin addressing the role of APP in the cerebrovasculature, we first examined the cerebrovasculature of apoE^{-/-} atherosclerotic and wild-type mice to ascertain APP disease-related changes. ApoE^{-/-} and age-matched wild-type brain tissue was collected and analyzed at 12 months. The cerebrovasculature from apoE^{-/-} mice showed increased immunoreactivity for APP, pAPP and A β (Figure 1) which colocalized with the endothelial marker, vonWillebrand factor. To verify the specificity of our anti-pTyr682 APP antibody we performed control experiments preincubating the antibody with the phosphorylated immunizing peptide to compete away specific immunoreactivity versus the non phosphorylated version of the peptide (Figure 1B). As an additional measure to assure that the antibody recognized a phosphorylated epitope, we also dephosphorylated sections via alkaline phosphatase pretreatment to attenuate specific staining with the anti-pAPP antibody (Figure 1B). Similarly, we preincubated anti-A β antibody with recombinant A β to compete away specific tissue immunoreactivity thus verifying the specificity of the vascular A β staining observed (Figure 1B). Western analysis of fibrillar and oligomeric preparations revealed that the anti-A β antibody employed was able to recognize both fibrillar and soluble/oligomeric forms of the peptide (Figure 1C).

In order to quantify the immunohistochemical results, we next examined protein levels using Western blot analyses. As predicted, apoE^{-/-} mouse brain tissue demonstrated significantly higher levels of APP and pAPP protein compared to wild type controls. Although our pAPP antibody recognized phosphorylation at Tyr682, prior work has demonstrated that changes in APP phosphorylation at Thr668 regulate processing of APP and A β generation (Feyt et al., 2007; Taru et al., 2004). In order to determine whether changes in phosphorylation at Thr668 were also increased in apoE^{-/-} brains compared to wild type controls, we used a commercial antibody to perform Western analysis from the lysates. There was no detectable level of Thr668 phosphorylation in either sample type suggesting a unique type of signaling and biology in the vessels (Figure 2). In addition, when the ratio of Tyr682 phosphorylated APP to total APP was compared, we did not detect a statistically significant difference in the apoE^{-/-} brain versus wild type controls (Figure 2c). The changes in pAPP immunoreactivity and protein levels suggested that APP was involved in an active tyrosine kinase-dependent signaling response.

To further characterize this possibility we determined whether specific tyrosine kinases were recruited to APP in a fashion typically employed by ligand-stimulated receptors. As predicted, co-immunoprecipitations from 12 month old wild type and apoE^{-/-} brains demonstrated that APP from apoE^{-/-} tissue had a statistically higher association with the non-receptor tyrosine kinase, Src (Figure 2d, 2e), compared to the wild-type brains. These data suggest that endothelial APP levels are increased during atherosclerotic disease progression. Furthermore, due to its C-terminal phosphorylation and Src association, it suggests APP is involved in an active tyrosine kinase-based signaling response.

AD cerebrovasculature demonstrated increased levels of APP and pAPP, accompanied by an increased association with the tyrosine kinase, Src

After observing the robust changes in apoE^{-/-} cerebrovasculature we next determined whether similar endothelial APP related changes were recapitulated in AD brain vasculature. To do this we, examined AD and age-matched control brain temporal cortex tissue. Consistent with work by Kalaria et al (Kalaria et al., 1996; Kalaria 1997) AD brain vasculature displayed increased immunoreactivity for APP and A β . However, we also observed increased immunoreactivity for pAPP (Figure 3). Immunoreactivity in the human brains did colocalize with vonWillebrand factor, the endothelial marker, but also extended diffusely beyond the endothelial layer to likely include additional cell types (Figure 3). In order to gain an impression of overall changes in total protein levels, we next examined APP and pAPP by Western blot analyses. AD brain tissue demonstrated significantly higher levels of APP and pAPP protein compared to controls (Figure 4a and 4b). The ratio of pAPP: APP was also significantly higher in AD tissue compared to control brains (Figure 4c). We next performed APP co-immunoprecipitation pull-down assays to determine whether APP had an increased association with Src in AD brains similar to that we had observed in apoE^{-/-} brains. Immunoprecipitations demonstrated statistically increased Src association with APP from AD brains compared to controls (Figure 4d and 4e). These data demonstrate that cerebral vasculature in not only AD brains but also mouse atherosclerotic tissue share common APP-related changes perhaps indicative of a shared pathophysiology.

Adhesion of monocytic cells onto brain endothelium is at least partially APP-dependent

Due to its ability to bind numerous components of the extracellular matrix it has been hypothesized that APP functions in cellular adhesion via cell-cell or cell-matrix interactions (Kibbey et al., 1993; Williamson et al., 1995; Beher et al., 1996; Small et al., 1999; Soba et al., 2005). Soba et al (2005) demonstrated that APP can form homo- and heterodimer complexes via its extracellular domain with other APP molecules or with other APP family members, APP like protein (APLP)-1 and APLP2, and such dimerization promotes cellular adhesion. While our histological and Western data support our hypothesis of a functional role of APP in endothelial dysfunction and plaque progression, these remain correlative. We utilized a modified Stamper-Woodruff adhesion assay to assess a functional role of APP in increased endothelial cell adhesiveness. We examined the adhesion of monocytic lineage THP-1 cells to brain endothelium from apoE^{-/-}, APP^{+/+} (wild-type), APP^{+/-}, and APP^{-/-} mice (Figure 5). We also examined the adhesion of monocytic cells to the brain endothelium of human AD and age-matched control tissue (Figure 6). As expected the apoE^{-/-} and AD brain vasculature displayed a significant increase in the number of monocytic cells adherent to the endothelium. In order to elucidate the importance of endothelial APP in the adhesion of monocytic cells to brain endothelium, we performed an adhesion assay using brain sections from APP^{+/+} and APP^{-/-} animals as well. Adhesion of monocytic cells, demonstrated a gene dose dependence on endothelial APP expression (Figure 5). As further evidence to demonstrate that the monocytic interaction with the vasculature was APP dependent, we pre-incubated tissue sections with an anti-N-terminal anti-APP antibody, 22C11, to bind surface APP on both the apoE^{-/-} and APP^{+/+} (wild-type) mouse tissue along with AD and control human tissue.

Preincubated tissue sections had significantly attenuated adherence of monocytic cells to brain endothelium on the apoE^{-/-} and wild-type tissue to the level seen in APP^{-/-} brain sections (Figure 5). Antibody preincubation also significantly attenuated the number of adherent cells to the endothelium of AD and control tissue (Figure 6). These data suggest that adhesion of monocytic cells to brain endothelium both basally and during pathophysiologic settings is partially endothelial APP-dependent.

4. Discussion

The proteolytic breakdown product of APP, A β , has a hypothesized role in the progression of AD and while research focuses on APP and A β in neurons, the function of APP in other cell types remains less defined. Here, we examined APP expression and function in vascular endothelial cells within the cerebrovasculature of apoE^{-/-} mice and human AD tissue. We observed increased vascular immunoreactivity for APP, pAPP, and A β in both mouse atherosclerotic and human AD cerebrovasculature demonstrating common APP-related changes in both disease entities. Furthermore, we also observed that human monocytic adhesion to cerebrovasculature was dependent upon endothelial APP expression. These data suggest that APP-mediated changes in the vasculature occur early in cardiovascular disease, perhaps contributing directly to monocyte adhesion to the inflamed endothelium and subsequent atherosclerotic plaque deposition. More importantly, these changes are not unique to cardiovascular disease but are also observed in AD brains supporting a hypothesis for a shared mechanistic pathophysiology.

We currently demonstrated that endothelial APP is required for monocyte adhesion but it will be important to know whether APP is also behaving as an extracellular receptor to alter endothelial phenotype. Our current observations of increased APP tyrosine phosphorylation, Src association, and A β immunoreactivity strongly suggest that APP mediates a specific tyrosine kinase-dependent signaling response and is subsequently proteolyzed to A β . While coimmunoprecipitations of APP from AD brain tissue and from the brain tissue of atherosclerotic mice revealed an increased association with the tyrosine kinase, Src, it is not definitively an association between endothelial APP and Src. However, our immunostaining results demonstrate abundant immunoreactivity for pAPP preferentially in the vasculature rather than the brain parenchyma supporting the notion that tyrosine kinase activity is altered in the endothelium. Indeed, numerous data support a role for APP in mediating signaling responses. APP is able to bind numerous proteins via interactions with its C-terminal domain (Borg et al., 1996; Howell et al., 1999; Russo et al., 2002; Scheinfeld et al., 2002; Venezia et al., 2004). Previously, we demonstrated the recruitment and activation of the tyrosine kinases, Syk and Lyn, and proinflammatory activation of both monocytic cells and microglia when cells were treated with an N-terminal anti-APP antibody to mimic ligand binding (Sondag and Combs 2004, 2006). Furthermore, Gianni *et al* (2003) demonstrated in an APP overexpressing cell line that a constitutively active form of Src increased A β production which was diminished when treated with PP2, a Src kinase inhibitor. Trommsdorff et al (1998) demonstrated interaction between APP and mammalian Disabled (mDab1) and demonstrated that upon phosphorylation of mDab1, Src and abl, nonreceptor tyrosine kinases, are recruited to APP. It is intriguing to speculate that the increased Src-APP association in endothelial cells from apoE^{-/-} and AD brains could lead to a phosphorylation-dependent increase in A β generation in the vasculature and blood. Mechanistically, this could lead to specific vascular dysfunction based upon prior observations that A β has a plethora of effects on endothelial activation to alter protein expression, facilitate immune cell adhesion, disrupt blood-brain barrier integrity, and mediate toxicity (Andras et al., 2008; Gonzalez-Velasquez et al., 2008; Marco and Skaper, 2006; Folin et al., 2005). More importantly, this implies that tyrosine kinase function could be therapeutically targeted to modulate APP function and A β generation in the vasculature. This specific mechanistic dissection of the function of endothelial APP will require future effort.

Taken together, these data along with our results presented here, suggest that APP likely mediates a specific tyrosine kinase-dependent signaling response within endothelial cells which may directly contribute to their acquisition of a reactive, pro-atherogenic phenotype.

While we have shown that endothelial APP is essential for monocyte adhesion to brain endothelium the exact nature of the monocytes-endothelial interaction and the role of monocytic APP expression is unknown. It is also unclear if endothelial APP has a specific ligand although membrane APP has been shown to interact with full length APP as well as APP cleavage products. Many groups have shown that fibrillar A β can bind to APP possibly in an autocrine fashion to mediate APP clustering and signal transduction (Chung et al., 1999; Lorenzo et al., 2000; Wagner et al., 2000; Van Nostrand et al., 2002). Further, it has been demonstrated that soluble A β induces APP homodimerization in neuronal cell lines (Lu et al., 2003). Shaked et al (2006) has also demonstrated direct binding of soluble monomeric-oligomeric A β to the extracellular domain of APP. Alternatively, *in vitro* studies have shown that membrane-bound APP can form dimers with another APP molecule in a cis (same cell) or trans (opposing cell) fashion (Rossjohn et al., 1999; Scheuermann et al., 2001; Lu et al., 2003; Wang and Ha 2004; Soba et al., 2005). These results suggest that either an A β -APP interaction or a transdimerization between monocytic APP and endothelial APP may interact with one another to mediate adhesion and/or increased APP-dependent proinflammatory signaling in the endothelium. Defining the possibility of an APP transdimerization event is an intriguing possibility for future effort, particularly in light of our observation that monocyte adhesion to the endothelium was decreased in an APP gene dosage-dependent fashion.

There is well characterized accumulation of A β fibrils in the leptomeningeal and cortical arterial vessels and capillaries of AD brains termed cerebral amyloid angiopathy (CAA) and capillary CAA (CapCAA) characterized by accumulations of, more commonly A β 1-40 or A β 1-42, respectively (Yamaguchi et al., 1992; Attems et al 2004; Attems and Jellinger 2004). Indeed, greater than 85% of AD cases reportedly demonstrate A β accumulation in the cortical vessels upon autopsy and they positively correlate with vascular lesion during disease (Mandybur 1975; Olichney et al., 2000).

It is important to keep in mind that typical CAA is associated with the outer portion of the basement membrane around the smooth muscle cells rather than in subendothelial space (Yamaguchi et al., 1992). Indeed, later stage pathology is associated with progressive muscle cell degeneration and relative sparing of the endothelial layer (Yamaguchi et al., 1992; Zekry et al., 2003). Our observations from apoE^{-/-} tissue were associated with small vessel and capillary endothelial immunoreactivity changes and therefore atypical of classically described CAA. However, the immunoreactivity changes we observed in the AD brains for pAPP, A β , and APP presented in varying degrees of expansion from the vasculature into the parenchyma consistent with a typical CAA presentation and suggesting that other cell types may be involved. Indeed, reactive glia are associated with human CAA and represent a distinct clinical entity from parenchymal A β deposition and gliosis (Kinnecom et al., 2007). Furthermore, mice expressing vasculotropic mutant APP Dutch/Iowa (E693Q/D694N) display robust accumulation of microvascular amyloid and abundant, associated reactive microglia and astrocytes (Xu et al., 2007). It is perhaps not surprising, then, that the tyrosine phosphorylated form of APP detectable by our antibody was statistically increased only in the AD brains and not from the apoE^{-/-} animals. One possibility is that the protein changes we observed in AD brains include additional cells beyond the endothelial layer, consistent with our immunohistological staining. Therefore, we can not exclude that possibility that the APP-related changes observed in the apoE^{-/-} and AD cerebrovasculature may be partially mechanistically distinct.

We have not yet determined a mechanism by which the loss of apoE expression leads to the particular changes in APP we have observed. Based upon prior data, apoE expression should be important for vascular amyloidosis. For example, data from APP transgenic models demonstrate that both endogenous murine apoE as well as exogenous human apoE expression is involved in mediating vascular deposition of A β (Fryer et al 2003, 2005; Van Dooren et al 2006). In addition, data from human studies suggest that the apoE ϵ 4 allele influences cerebrovascular versus parenchymal A β deposition (Chalmers et al., 2003) and perhaps represents an independent risk factor for CAA independent of AD (Premkumar et al., 1996). It has also been suggested that the lower affinity of apoE ϵ 4 for A β as compared to the ϵ 2 and ϵ 3 isoforms is responsible for decreased clearance of A β and thus increased deposition in AD (LaDu et al., 1994; Zhou et al., 1996; Yang et al., 1997). Holtzman et al. (2000) have also demonstrated that crossing ADTg mice (APP^{V717F}) onto an apoE^{-/-} background actually decreased A β load and neurodegeneration again suggesting that apoE is involved in A β fibril deposition. Nevertheless, we observed that mice lacking apoE still demonstrate a robust endothelial vascular amyloid immunoreactivity perhaps independent of previously described deposition events. In support of this possibility, in vitro cell line studies have demonstrated that apoE, when added to cell cultures, can stimulate decreased A β generation suggesting that the role of apoE in modulating A β deposition may be paradigm dependent (Irizarry et al., 2004).

We have demonstrated increased APP, pAPP and A β immunoreactivity in the cerebrovasculature of atherosclerotic tissue as well as cerebrovascular AD tissue and increased APP association with tyrosine kinase, Src. Together with our result of APP-dependent adhesion of monocytes to brain endothelium we have now extended prior reports of APP and A β immunoreactivity in the cerebrovasculature of AD brains and its mouse models (dela Torre and Mussivand 1993; Esiri et al., 1999; Li et al., 2003; Herzig et al., 2004) as well as within advanced human atherosclerotic plaques (De Meyer et al., 2002). Our data support the hypothesis that APP is involved in mediating increased vascular adhesion and extravasation of immune cells but also may mediate a phenotypic change in endothelial cells leading to activation, A β secretion and vascular dysfunction associated with AD and cardiovascular disease. In support of this shared mechanism, an earlier report demonstrated in B6Tg2576 mice expressing the Swedish mutation of human APP that an atherogenic diet induced aortic atherosclerotic changes and increased A β deposition both within the brain and the aortic lesion. In fact, these mice also demonstrated aortic lesions that positively correlated with cerebral A β load even when maintained on a normal diet (Li et al., 2003). Perhaps more importantly, the aortic lesions demonstrated A β immunoreactivity similar to the histological observations we have made. Together, the prior work and our current observations suggest that endothelial APP represents a potential therapeutic target in both cardiovascular disease and AD.

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References

- András IE, Rha G, Huang W, Eum S, Couraud PO, Romero IA, Hennig B, Toborek M. Simvastatin protects against amyloid beta and HIV-1 Tat-induced promoter activities of inflammatory genes in brain endothelial cells. *Mol Pharmacol* 2008;73:1424–1433. [PubMed: 18276775]
- Attens J, Jellinger KA. Only cerebral capillary amyloid angiopathy correlates with Alzheimer pathology--a pilot study. *Acta Neuropathol (Berl)* 2004;107:83–90. [PubMed: 14655019]
- Attens J, Lintner F, Jellinger KA. Amyloid beta peptide 1-42 highly correlates with capillary cerebral amyloid angiopathy and Alzheimer disease pathology. *Acta Neuropathol (Berl)* 2004;107:283–291. [PubMed: 14986026]

- Banati RB, Gehrman J, Kreutzberg GW. Glial beta-amyloid precursor protein: expression in the dentate gyrus after entorhinal cortex lesion. *Neuroreport* 1994;5:1359–1361. [PubMed: 7919199]
- Banati RB, Gehrman J, Czech C, Monning U, Jones LL, Konig G, Beyreuther K, Kreutzberg GW. Early and rapid de novo synthesis of Alzheimer beta A4-amyloid precursor protein (APP) in activated microglia. *Glia* 1993;9:199–210. [PubMed: 7507467]
- Behr D, Hesse L, Masters CL, Multhaup G. Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. *J Biol Chem* 1996;271:1613–1620. [PubMed: 8576160]
- Borg JP, Ooi J, Levy E, Margolis B. The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol Cell Biol* 1996;16:6229–6241. [PubMed: 8887653]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
- Chalmers K, Wilcock GK, Love S. APOE epsilon 4 influences the pathological phenotype of Alzheimer's disease by favouring cerebrovascular over parenchymal accumulation of A beta protein. *Neuropathol Appl Neurobiol* 2003;29:231–238. [PubMed: 12787320]
- Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, Jones BW, Fernandez SJ, Lacor PN, Horowitz P, Finch CE, Krafft GA, Klein WL. Self-assembly of Abeta(1-42) into globular neurotoxins. *Biochemistry* 2003;42:12749–12760. [PubMed: 14596589]
- Chung H, Brazil MI, Soe TT, Maxfield FR. Uptake, degradation, and release of fibrillar and soluble forms of Alzheimer's amyloid beta-peptide by microglial cells. *J Biol Chem* 1999;274:32301–32308. [PubMed: 10542270]
- Combs CK, Coleman PD, O'Banion MK. Developmental regulation and PKC dependence of Alzheimer's-type tau phosphorylations in cultured fetal rat hippocampal neurons. *Brain Res Dev Brain Res* 1998;107:143–158.
- Davies TA, Billingslea AM, Long HJ, Tibbles H, Wells JM, Eisenhauer PB, Smith SJ, Cribbs DH, Fine RE, Simons ER. Brain endothelial cell enzymes cleave platelet-retained amyloid precursor protein. *J Lab Clin Med* 1998;132:341–350. [PubMed: 9794706]
- de la Torre JC, Mussivand T. Can disturbed brain microcirculation cause Alzheimer's disease? *Neurol Res* 1993;15:146–153. [PubMed: 8103579]
- De Meyer GR, De Cleen DM, Cooper S, Knaapen MW, Jans DM, Martinet W, Herman AG, Bult H, Kockx MM. Platelet phagocytosis and processing of beta-amyloid precursor protein as a mechanism of macrophage activation in atherosclerosis. *Circ Res* 2002;90:1197–1204. [PubMed: 12065323]
- Esiri MM, Nagy Z, Smith MZ, Barnetson L, Smith AD. Cerebrovascular disease and threshold for dementia in the early stages of Alzheimer's disease. *Lancet* 1999;354:919–920. [PubMed: 10489957]
- Feyt C, Pierrot N, Tasiaux B, Van Hees J, Kienlen-Campard P, Courttoy PJ, Octave JN. Phosphorylation of APP695 at Thr668 decreases gamma-cleavage and extracellular Abeta. *Biochem Biophys Res Commun* 2007;357:1004–1010. [PubMed: 17459339]
- Floden AM, Li S, Combs CK. Beta-amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor alpha and NMDA receptors. *J Neurosci* 2005;25:2566–2575. [PubMed: 15758166]
- Forloni G, Demicheli F, Giorgi S, Bendotti C, Angeretti N. Expression of amyloid precursor protein mRNAs in endothelial, neuronal and glial cells: modulation by interleukin-1. *Brain Res Mol Brain Res* 1992;16:128–134. [PubMed: 1334190]
- Fryer JD, Taylor JW, DeMattos RB, Bales KR, Paul SM, Parsadanian M, Holtzman DM. Apolipoprotein E markedly facilitates age-dependent cerebral amyloid angiopathy and spontaneous hemorrhage in amyloid precursor protein transgenic mice. *J Neurosci* 2003;23:7889–7896. [PubMed: 12944519]
- Fryer JD, Simmons K, Parsadanian M, Bales KR, Paul SM, Sullivan PM, Holtzman DM. Human apolipoprotein E4 alters the amyloid-beta 40:42 ratio and promotes the formation of cerebral amyloid angiopathy in an amyloid precursor protein transgenic model. *J Neurosci* 2005;25:2803–2810. [PubMed: 15772340]
- Gianni D, Zambrano N, Bimonte M, Minopoli G, Mercken L, Talamo F, Scaloni A, Russo T. Platelet-derived growth factor induces the beta-gamma-secretase-mediated cleavage of Alzheimer's amyloid

- precursor protein through a Src-Rac-dependent pathway. *J Biol Chem* 2003;278:9290–9297. [PubMed: 12645527]
- Goldgaber D, Harris HW, Hla T, Maciag T, Donnelly RJ, Jacobsen JS, Vitek MP, Gajdusek DC. Interleukin 1 regulates synthesis of amyloid beta-protein precursor mRNA in human endothelial cells. *Proc Natl Acad Sci U S A* 1989;86:7606–7610. [PubMed: 2508093]
- Gonzalez-Velasquez FJ, Moss MA. Soluble aggregates of the amyloid-beta protein activate endothelial monolayers for adhesion and subsequent transmigration of monocyte cells. *J Neurochem* 2008;104:500–513. [PubMed: 17953673]
- He X, Cooley K, Chung CH, Dashti N, Tang J. Apolipoprotein receptor 2 and X11 alpha/beta mediate apolipoprotein E-induced endocytosis of amyloid-beta precursor protein and beta-secretase, leading to amyloid-beta production. *J Neurosci* 2007;27:4052–4060. [PubMed: 17428983]
- Herzig MC, Winkler DT, Burgermeister P, Pfeifer M, Kohler E, Schmidt SD, Danner S, Abramowski D, Sturchler-Pierrat C, Burki K, van Duinen SG, Maat-Schieman ML, Staufenbiel M, Mathews PM, Jucker M. Abeta is targeted to the vasculature in a mouse model of hereditary cerebral hemorrhage with amyloidosis. *Nat Neurosci* 2004;7:954–960. [PubMed: 15311281]
- Hirsch-Reinshagen V, Maia LF, Burgess BL, Blain JF, Naus KE, McIsaac SA, Parkinson PF, Chan JY, Tansley GH, Hayden MR, Poirier J, Van Nostrand W, Wellington CL. The absence of ABCA1 decreases soluble ApoE levels but does not diminish amyloid deposition in two murine models of Alzheimer disease. *J Biol Chem* 2005;280:43243–43256. [PubMed: 16207707]
- Hoe HS, Pocivavsek A, Dai H, Chakraborty G, Harris DC, Rebeck GW. Effects of apoE on neuronal signaling and APP processing in rodent brain. *Brain Res* 2006;1112:70–79. [PubMed: 16905123]
- Hoe HS, William Rebeck G. Functional interactions of APP with the apoE receptor family. *J Neurochem*. 2008 Jul 28; [Epub ahead of print].
- Holtzman DM, Bales KR, Tenkova T, Fagan AM, Parsadanian M, Sartorius LJ, Mackey B, Olney J, McKeel D, Wozniak D, Paul SM. Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* 2000;97:2892–2897. [PubMed: 10694577]
- Howell BW, Lanier LM, Frank R, Gertler FB, Cooper JA. The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol Cell Biol* 1999;19:5179–5188. [PubMed: 10373567]
- Irizarry MC, Deng A, Lleo A, Berezovska O, Von Arnim CA, Martin-Rehrmann M, Manelli A, LaDu MJ, Hyman BT, Rebeck GW. Apolipoprotein E modulates gamma-secretase cleavage of the amyloid precursor protein. *J Neurochem* 2004;90:1132–1143. [PubMed: 15312168]
- Jahroudi N, Kitney J, Greenberger JS, Bowser R. Endothelial cell dysfunction in response to intracellular overexpression of amyloid precursor protein. *J Neurosci Res* 1998;54:828–839. [PubMed: 9856867]
- Kalaria RN. Cerebrovascular degeneration is related to amyloid-beta protein deposition in Alzheimer's disease. *Ann N Y Acad Sci* 1997;826:263–271. [PubMed: 9329698]
- Kalaria RN, Premkumar DR, Pax AB, Cohen DL, Lieberburg I. Production and increased detection of amyloid beta protein and amyloidogenic fragments in brain microvessels, meningeal vessels and choroid plexus in Alzheimer's disease. *Brain Res Mol Brain Res* 1996;35:58–68. [PubMed: 8717340]
- Kibbey MC, Jucker M, Weeks BS, Neve RL, Van Nostrand WE, Kleinman HK. beta-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. *Proc Natl Acad Sci U S A* 1993;90:10150–10153. [PubMed: 8234269]
- Kinnecom C, Lev MH, Wendell L, Smith EE, Rosand J, Frosch MP, Greenberg SM. Course of cerebral amyloid angiopathy-related inflammation. *Neurology* 2007;68:1411–1416. [PubMed: 17452586]
- Koldamova R, Staufenbiel M, Lefterov I. Lack of ABCA1 considerably decreases brain ApoE level and increases amyloid deposition in APP23 mice. *J Biol Chem* 2005;280:43224–43235. [PubMed: 16207713]
- LaDu MJ, Falduto MT, Manelli AM, Reardon CA, Getz GS, Frail DE. Isoform-specific binding of apolipoprotein E to beta-amyloid. *J Biol Chem* 1994;269:23403–23406. [PubMed: 8089103]
- Li L, Cao D, Garber DW, Kim H, Fukuchi K. Association of aortic atherosclerosis with cerebral beta-amyloidosis and learning deficits in a mouse model of Alzheimer's disease. *Am J Pathol* 2003;163:2155–2164. [PubMed: 14633589]

- Liu Q, Zerbinatti CV, Zhang J, Hoe HS, Wang B, Cole SL, Herz J, Muglia L, Bu G. Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. *Neuron* 2007;56:66–78. [PubMed: 17920016]
- Lorenzo A, Yuan M, Zhang Z, Paganetti PA, Sturchler-Pierrat C, Staufenbiel M, Mautino J, Vigo FS, Sommer B, Yankner BA. Amyloid beta interacts with the amyloid precursor protein: a potential toxic mechanism in Alzheimer's disease. *Nat Neurosci* 2000;3:460–464. [PubMed: 10769385]
- Lu DC, Shaked GM, Masliah E, Bredesen DE, Koo EH. Amyloid beta protein toxicity mediated by the formation of amyloid-beta protein precursor complexes. *Ann Neurol* 2003;54:781–789. [PubMed: 14681887]
- Mandybur TI. The incidence of cerebral amyloid angiopathy in Alzheimer's disease. *Neurology* 1975;25:120–126. [PubMed: 46597]
- Marco S, Skaper SD. Amyloid beta-peptide1-42 alters tight junction protein distribution and expression in brain microvessel endothelial cells. *Neurosci Lett* 2006;401:219–224. [PubMed: 16644119]
- Olichney JM, Hansen LA, Hofstetter CR, Lee JH, Katzman R, Thal LJ. Association between severe cerebral amyloid angiopathy and cerebrovascular lesions in Alzheimer disease is not a spurious one attributable to apolipoprotein E4. *Arch Neurol* 2000;57:869–874. [PubMed: 10867785]
- Premkumar DR, Cohen DL, Heder P, Friedland RP, Kalaria RN. Apolipoprotein E-epsilon4 alleles in cerebral amyloid angiopathy and cerebrovascular pathology associated with Alzheimer's disease. *Am J Pathol* 1996;148:2083–2095. [PubMed: 8669492]
- Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y. Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007;115:e69–171. [PubMed: 17194875]
- Rosendorff C, Beer MS, Silverman JM. Cardiovascular risk factors for Alzheimer's disease. *Am J Geriatr Cardiol* 2007;16:143–149. [PubMed: 17483665]
- Rosjohn J, Cappai R, Feil SC, Henry A, McKinstry WJ, Galatis D, Hesse L, Multhaup G, Beyreuther K, Masters CL, Parker MW. Crystal structure of the N-terminal, growth factor-like domain of Alzheimer amyloid precursor protein. *Nat Struct Biol* 1999;6:327–331. [PubMed: 10201399]
- Russo C, Dolcini V, Salis S, Venezia V, Violani E, Carlo P, Zambrano N, Russo T, Schettini G. Signal transduction through tyrosine-phosphorylated carboxy-terminal fragments of APP via an enhanced interaction with Shc/Grb2 adaptor proteins in reactive astrocytes of Alzheimer's disease brain. *Ann N Y Acad Sci* 2002;973:323–333. [PubMed: 12485888]
- Scheinfeld MH, Roncarati R, Vito P, Lopez PA, Abdallah M, D'Adamio L. Jun NH2-terminal kinase (JNK) interacting protein 1 (JIP1) binds the cytoplasmic domain of the Alzheimer's beta-amyloid precursor protein (APP). *J Biol Chem* 2002;277:3767–3775. [PubMed: 11724784]
- Scheuermann S, Hamsch B, Hesse L, Stumm J, Schmidt C, Behr D, Bayer TA, Beyreuther K, Multhaup G. Homodimerization of amyloid precursor protein and its implication in the amyloidogenic pathway of Alzheimer's disease. *J Biol Chem* 2001;276:33923–33929. [PubMed: 11438549]
- Shaked GM, Kummer MP, Lu DC, Galvan V, Bredesen DE, Koo EH. Abeta induces cell death by direct interaction with its cognate extracellular domain on APP (APP 597–624). *Faseb J* 2006;20:1254–1256. [PubMed: 16636103]
- Small DH, Clarris HL, Williamson TG, Reed G, Key B, Mok SS, Beyreuther K, Masters CL, Nurcombe V. Neurite-outgrowth regulating functions of the amyloid protein precursor of Alzheimer's disease. *J Alzheimers Dis* 1999;1:275–285. [PubMed: 12214125]
- Soba P, Eggert S, Wagner K, Zentgraf H, Siehl K, Kreger S, Lower A, Langer A, Merdes G, Paro R, Masters CL, Muller U, Kins S, Beyreuther K. Homo- and heterodimerization of APP family members promotes intercellular adhesion. *Embo J* 2005;24:3624–3634. [PubMed: 16193067]
- Sondag CM, Combs CK. Amyloid precursor protein mediates proinflammatory activation of monocytic lineage cells. *J Biol Chem* 2004;279:14456–14463. [PubMed: 14732706]
- Sondag CM, Combs CK. Amyloid precursor protein cross-linking stimulates beta amyloid production and pro-inflammatory cytokine release in monocytic lineage cells. *J Neurochem* 2006;97:449–461. [PubMed: 16539666]

- Stamper HB Jr, Woodruff JJ. Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. *J Exp Med* 1976;144:828–833. [PubMed: 956727]
- Taru H, Yoshikawa K, Suzuki T. Suppression of the caspase cleavage of beta-amyloid precursor protein by its cytoplasmic phosphorylation. *FEBS Lett* 2004;567:248–252. [PubMed: 15178331]
- Trommsdorff M, Borg JP, Margolis B, Herz J. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J Biol Chem* 1998;273:33556–33560. [PubMed: 9837937]
- Van Dooren T, Muyllaert D, Borghgraef P, Cresens A, Devijver H, Van der Auwera I, Wera S, Dewachter I, Van Leuven F. Neuronal or glial expression of human apolipoprotein e4 affects parenchymal and vascular amyloid pathology differentially in different brain regions of double- and triple-transgenic mice. *Am J Pathol* 2006;168:245–260. [PubMed: 16400027]
- Van Nostrand WE, Melchor JP, Keane DM, Saporito-Irwin SM, Romanov G, Davis J, Xu F. Localization of a fibrillar amyloid beta-protein binding domain on its precursor. *J Biol Chem* 2002;277:36392–36398. [PubMed: 12107175]
- Venezia V, Russo C, Repetto E, Salis S, Dolcini V, Genova F, Nizzari M, Mueller U, Schettini G. Apoptotic cell death influences the signaling activity of the amyloid precursor protein through ShcA and Grb2 adaptor proteins in neuroblastoma SH-SY5Y cells. *J Neurochem* 2004;90:1359–1370. [PubMed: 15341520]
- Wagner MR, Keane DM, Melchor JP, Auspaker KR, Van Nostrand WE. Fibrillar amyloid beta-protein binds protease nexin-2/amyloid beta-protein precursor: stimulation of its inhibition of coagulation factor XIa. *Biochemistry* 2000;39:7420–7427. [PubMed: 10858290]
- Wang Y, Ha Y. The X-ray structure of an antiparallel dimer of the human amyloid precursor protein E2 domain. *Mol Cell* 2004;15:343–353. [PubMed: 15304215]
- Williamson TG, Nurcombe V, Beyreuther K, Masters CL, Small DH. Affinity purification of proteoglycans that bind to the amyloid protein precursor of Alzheimer's disease. *J Neurochem* 1995;65:2201–2208. [PubMed: 7595508]
- Xu F, Grande AM, Robinson JK, Previti ML, Vasek M, Davis J, Van Nostrand WE. Early-onset subicular microvascular amyloid and neuroinflammation correlate with behavioral deficits in vasculotropic mutant amyloid beta-protein precursor transgenic mice. *Neuroscience* 2007;146:98–107. [PubMed: 17331655]
- Yamaguchi H, Yamazaki T, Lemere CA, Frosch MP, Selkoe DJ. Beta amyloid is focally deposited within the outer basement membrane in the amyloid angiopathy of Alzheimer's disease. An immunoelectron microscopic study. *Am J Pathol* 1992;141:249–259. [PubMed: 1632466]
- Yang DS, Smith JD, Zhou Z, Gandy SE, Martins RN. Characterization of the binding of amyloid-beta peptide to cell culture-derived native apolipoprotein E2, E3, and E4 isoforms and to isoforms from human plasma. *J Neurochem* 1997;68:721–725. [PubMed: 9003062]
- Ye S, Huang Y, Müllendorff K, Dong L, Giedt G, Meng EC, Cohen FE, Kuntz ID, Weisgraber KH, Mahley RW. Apolipoprotein (apo) E4 enhances amyloid beta peptide production in cultured neuronal cells: apoE structure as a potential therapeutic target. *Proc Natl Acad Sci U S A* 2005;102:18700–18705. [PubMed: 16344478]
- Zekry D, Duyckaerts C, Belmin J, Geoffre C, Moulis R, Hauw JJ. Cerebral amyloid angiopathy in the elderly: vessel walls changes and relationship with dementia. *Acta Neuropathol (Berl)* 2003;106:367–373. [PubMed: 12898153]
- Zhou Z, Smith JD, Greengard P, Gandy S. Alzheimer amyloid-beta peptide forms denaturant-resistant complex with type epsilon 3 but not type epsilon 4 isoform of native apolipoprotein E. *Mol Med* 1996;2:175–180. [PubMed: 8726460]

Figure 1. Cortical apoE^{-/-} cerebrovasculature demonstrated increased immunoreactivity for APP, pAPP, and Aβ which colocalized with vonWillebrand factor, an endothelial marker

A) Fixed tissue sections (40μm) from apoE^{-/-} and age-matched wild type (control) mice (12 months old) were A) single and double-labeled with anti-APP, pAPP, or Aβ antibodies (Vector Blue) and vonWillebrand factor (DAB). Sections are representative from 4–5 animals per background. **B)** Sections were immunostained with anti-pAPP or Aβ antibodies and visualized using Vector Blue as the chromagen. Dephosphorylation of APP in fixed sections was performed by incubating sections in PBS containing 0.1% Triton X-100 for 15 mins followed by treatment with 40 units/ml alkaline phosphatase (phosphatase tx.) overnight at 37°C. Antibody binding competition was performed by pre-incubating anti-Aβ and anti-pAPP antibodies with their immunizing peptides, Aβ1-42, pYNPTY (p-peptide), or YNPTY (negative control, non-p-peptide), for 1 hour at 25°C before immunostaining. **C)** Oligomeric (Aβ_o) and fibrillar (Aβ_f) preparations of human recombinant Aβ1-42 peptide were separated by 15% SDS-PAGE and Western blotted using anti-Aβ antibody.

Figure 2. APP and pAPP protein levels were increased in brains of apoE^{-/-} mice compared to age-matched controls and associated with the tyrosine kinase, Src

A) Brain tissue from 12 month old apoE^{-/-} and wild type control mice was lysed, quantified, and the proteins resolved by 10% SDS-PAGE and Western blotted using anti-APP, pAPP, Thr 668 pAPP, and α -tubulin (loading control) antibodies. **B)** Optical density of APP and pAPP protein bands from 6 animal samples were normalized against respective α -tubulin levels and averaged ($\pm$ SD) (*p<0.001 from respective control) **C)** The optical density of pAPP bands from 6 animals were also normalized against their respective APP levels (ratio of pAPP:APP) and averaged ($\pm$ SD). **D)** Co-precipitations were performed by immunoprecipitating APP in 1% Triton buffer from apoE^{-/-} and control brains. The immunoprecipitates were resolved by 10% SDS-PAGE and Western blotted using anti-c-Src, APP, and pAPP antibodies. Immunoprecipitations are representative of 3 independent experiments. **E)** The optical density of Src protein bands from 3 immunoprecipitation experiments were averaged ($\pm$ SD) (*p<0.001 from control).

Figure 3. AD cerebrovasculature displayed increased immunoreactivity for APP, pAPP, and A β which colocalized with the endothelial marker, vonWillebrand factor
Temporal cortex tissue from AD and age-matched control cases were sectioned (40 μ m) and single and double-labeled with anti-APP, pAPP, or A β antibodies (Vector Blue) and vonWillebrand factor (DAB). Sections are representative from 3 AD subjects and 3 age-matched control subjects.

Figure 4. AD brain lysates have increased protein levels of APP and pAPP compared to age-matched control tissue and associated with the tyrosine kinase, Src

A) Temporal cortex samples from AD and control cases were lysed and proteins quantified and resolved by 10% SDS-PAGE and transferred to PVDF membranes for Western blotting using anti-APP, pAPP, or α -tubulin, (loading control) antibodies. **B)** Optical density of APP and pAPP protein bands from 6 AD and 6 control samples were normalized against respective α -tubulin levels and averaged ($\pm$ SD) (* p <0.001 from respective control) **C)** The optical density of pAPP bands from the 6 AD and 6 control samples were also normalized against their respective APP levels (ratio of pAPP: APP) and averaged ($\pm$ SD) (* p <0.001 AD versus age matched control brains). **D)** Co-precipitations were performed by immunoprecipitating APP in 1% Triton buffer from AD and control brains. The immunoprecipitates were resolved by 10% SDS-PAGE and Western blotted using anti-c-Src, APP, and pAPP antibodies. Immunoprecipitations are representative of 3 independent experiments. **E)** Optical density of Src protein bands in 3 immunoprecipitation experiments were averaged ($\pm$ SD) (* p <0.001 AD versus age matched control brains)

Figure 5. Adhesion of monocytic cells to brain arterial endothelium is partially APP-dependent

A) Tissue sections were immunolabeled with an anti-smooth muscle actin antibody (Texas Red) prior to use in the adhesion assay. THP-1 cells were pre-treated for 4 hours with 25ng/ml LPS, incubated with Cyquant dye reagent (green) for 15 mins before being used in a modified Stamper-Woodruff adhesion assay. Briefly, mounted apoE^{-/-}, APP^{+/+} (wild-type/control), APP^{+/-}, or APP^{-/-} brain tissue sections (40μm) of the temporal cortex were incubated with or without 22C11 (anti-APP) or mouse IgG₁ (isotype control) for one hour at room temperature before the addition of THP-1 cell suspensions. Cells were allowed to adhere to brain endothelium for one hour with gentle agitation, non-adherent cells were rinsed away, and adherent cells were fixed with 4% paraformaldehyde and slides prepared for viewing. Representative images are shown. **B)** The mean number of adherent cells (±SD) per condition was calculated and graphed from 8 random fields on four to five independent sections from 3 animals per background (*p<0.001, **p<0.01).

Figure 6. Adhesion of monocytic cells to human AD and age-matched control brain endothelium is partially APP-dependent

A) Prior to use in the adhesion assay, tissue sections were immunolabeled with anti-smooth muscle actin antibody (Texas Red). Monocytic cells (THP-1) were pre-treated for 4 hours with 25ng/ml LPS and incubated with Cyquant dye reagent (green) for 15 mins before being used in a modified Stamper-Woodruff adhesion assay as previously described. Mounted AD and age-matched control brain tissue sections (40μm) from the temporal cortex were pre-incubated with or without 22C11 (anti-APP) or mouse IgG₁ (isotype control) for one hour at room temperature prior to the addition of THP-1 cell suspensions. **B)** The mean number of adherent cells ($\pm$ SD) per condition was calculated and graphed from counting 8 random fields on four to five independent sections from 3 animals per background (*p<0.001, ***p<0.05).