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Promoting and preventing alzheimer's disease in a transgenic mouse model: Apolipoprotein e and environmental enrichment

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Promoting and Preventing Alzheimer's Disease in a Transgenic Mouse Model:

Apolipoprotein E and Environmental Enrichment

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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To my father, for all his love and countless sacrifices.

and

To my wife, Heather, for her endless moral support and a reluctant understanding that I will most likely be a student forever.
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Besides age, inheritance of the apoE-ε4 allele is the main risk factor for late-onset AD. To determine the role of apoE in amyloid deposition, we studied mice expressing both mutant human amyloid β-protein precursor (APP) and presenilin 1 (PS1) that were either normal or knocked-out for apoE. By 7 months, amorphous Aβ deposition developed equally in both lines, indicating that Aβ alone is sufficient for deposition to occur. In contrast, filamentous amyloid deposition was catalyzed at least 3000 fold by apoE. Electron micrographs further illustrate the filamentous nature of these plaques. These results and other, behavioral, data indicate that the primary function of apoE in AD is to promote the polymerization of Aβ into mature, neurotoxic, amyloid.

ApoE is also synthesized in the liver and is crucial in cholesterol metabolism, for mice lacking apoE exhibit hypercholesterolemia. We investigated neuropathology in mice using an uncommon technique, parabiosis,
to determine whether apoE in the peripheral circulation influences brain amyloid formation. This surgical procedure allows exchange of proteins via peripheral circulation. We show that plasma apoE is found in parabiosed PS/APP/apoE-KO mice, rescuing their hypercholesterolemia. Unexpectedly, amyloid deposition is reduced in parabiosed PS/APP/apoE-KO mice compared to PS/APP controls. ApoE in the periphery seems to slightly reduce amyloid burden, by likely promoting efflux of Aβ from the brain. These findings reinforce that the mechanisms whereby apoE affects Aβ metabolism are complex, and the modulation of peripheral apoE metabolism is not likely to impact AD neuropathology.

Since cognitive stimulation is associated with lower risk of AD, we sought to investigate the preventative potential of environmental enrichment (EE) using our mouse model. At weaning, mice were placed into either enriched or standard housing (SH). Behavioral testing at 4½-6 months showed that EE-PS1/APP mice outperformed mice in SH, and were behaviorally indistinguishable from nontransgenic mice. PS1/APP mice given both EE and behavioral testing had 50% less brain β-amyloid (Aβ), but did not exhibit changes in dendritic morphology. Microarray analysis of hippocampal RNA revealed large EE-induced changes in the expression of genes/proteins related to memory, neuroprotection, and Aβ sequestration. Inhibition of one such protein, PDE4, a cAMP-phosphodiesterase, by Rolipram, mimicked the cognitive benefits of EE.
INTRODUCTION

ALZHEIMER’S DISEASE

Alzheimer’s disease (AD) is the most prevalent form of dementia in people over the age of 65, and it is estimated that for every five years a person lives over the age of 65, the chance of developing AD doubles. In fact, approximately 58% of those living at the age of 95 develop AD (Ebly et al., 1994). Using data from the year 2000 United States census, it was estimated that there were roughly 4.5 million Americans living with the disease, and that, due to the rapidly aging population, this figure should triple by the year 2050 (Hebert et al., 2003). At a current estimated cost of $100 billion per year to the US economy, AD is a burden to more than patients, families, and health care providers.

The main overt symptoms of AD, initially described by Alois Alzheimer in 1907, are progressive memory loss and dementia. From a pathological perspective, AD results in two distinct brain lesions, neuritic plaques and neurofibrillary tangles, with concomitant neuronal loss, occurring primarily in the hippocampus and cerebral cortex, two brain regions intimately associated with memory and higher cognitive functions.

Neurofibrillary tangles are highly insoluble cytosolic aggregates made of paired helical filaments (PHF) of an abnormally phosphorylated (hyperphosphorylated) microtubule associated protein, tau. Tau normally
stabilizes and promotes the assembly of microtubules, but when phosphorylated at either serine 262 or 214, it detaches from the microtubule and is made available for polymerization (Mandelkow and Mandelkow, 1998). Tau is necessary for the outgrowth of neurites, so it is likely that alterations in microtubule binding could contribute to a breakdown in intracellular transport and the resultant dying back of neurons indicative of AD. It has also been shown that cyclin dependant kinase 5 (cdk5) can be constitutively active in patients with AD. Cdk5 can hyperphosphorylate tau, therefore inducing NFT formation, and has been shown to accumulate in tangle-bearing neurons, adding yet another potential mechanism for the development of AD pathology (Patrick et al., 1999). Since the mouse models employed in our research do not develop neurofibrillary tangles, our efforts focus specifically on the other main pathological hallmark of AD, neuritic plaques.

Neuritic, or amyloid, plaques are extracellular protein aggregates typically surrounded by both dystrophic neurites and reactive astrocytes. The primary constituent of these lesions is a short peptide called amyloid-beta (Aβ). It was the 1984 isolation and characterization of the Aβ peptide by Glenner and Wong that ushered in the modern era of AD research (Glenner and Wong, 1984). Later, it was discovered that Aβ is really the proteolytic cleavage product of a larger, membrane bound, protein termed the amyloid precursor protein (APP) (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). APP, a protein whose function is still not known, is ubiquitously expressed, and its cleavage, resulting in Aβ production, seems to be a normal
cellular event. Discerning the mechanism whereby cellular homeostasis is altered to confer a disease state has been challenging for the field. One known change that does occur in many AD patients, especially those with FAD, is an altered ratio between the two major species of Aβ, the shorter form ending in amino acid (AA) 40, and the longer 42 AA form. Though still far less abundant than Aβ₁₋₄₀, a higher percentage of Aβ₁₋₄₂ is found in diseased brains. This is of particular interest since Aβ₁₋₄₂ seems to have a greater propensity for aggregation (Jarrett et al., 1993). Furthermore, it has been suggested that pre-amyloid, or diffuse, plaques which are immunoreactive, but not classified as amyloid due to a lack of birefringence, are made predominantly of Aβ₁₋₄₂, and serve as the foundation for later Aβ₁₋₄₀ accumulation and eventual amyloid formation (Lemere et al., 1996).

Greater than 97% of all AD cases are sporadic, or of unknown etiology. The remaining cases are attributable to genetic defects. Most of what is known about the cellular events of sporadic AD comes from research on the relatively rare inherited forms of the disease. The majority of familial AD cases are early onset and due to autosomal dominant mutations in the APP, presenilin 1 (PS1), or presenilin 2 (PS2) genes. Another inherited variety of AD takes the form of trisomy 21, or Down’s syndrome (DS). Phenotypically, autosomal dominantly inherited AD is indistinguishable from sporadic AD with the exception of an earlier onset age.

Mutations in the APP gene, which is on chromosome 21, are thought to alter its proteolytic processing. The most common mutations are found as point
mutations adjacent to the two proteolytic cleavage sites utilized to excise the Aβ fragment from the APP holoprotein. Both N-terminal β-site (AA 670/671) and C-terminal γ-site (AA717) mutations increase the amounts of extracellular Aβ1-42, thereby promoting the initial deposition of this amyloidogenic species. Similarly, mutated presenilin proteins, which are also inherited in an autosomal dominant manner, shift the ratio of Aβ1-40/Aβ1-42 towards Aβ1-42.

Presenilin is an intramembrane protease which is thought to be the γ-secretase, or at least an inseparable cofactor in the APP cleavage pathway (Iwatsubo, 2004). Since a genetic ablation of this gene is lethal by birth (Qian et al., 1998), inherited mutations most likely invoke pathogenesis through a toxic gain of function, which is further supported by the fact that, with two exceptions, all of these mutations cause amino acid substitutions (i.e. no frameshifts, stops, etc…). Just increasing the bulk amount of APP also confers a disease state. By having one extra copy of chromosome 21, such as in DS, these individuals develop large amounts of Aβ deposits at an early age, and eventually the memory deficits associated with AD.

Insight into the role of APP and Aβ in AD may be provided by genetic and biochemical studies that have explored the roles of other proteins thought to be involved in the disease process. For example, Aβ amyloid deposits also contain α1-antichymotrypsin (ACT) and apolipoprotein E (ApoE), which are over-expressed in affected regions of the AD brain as part of an inflammatory process (Abraham et al., 1988; Xu et al., 1999). ACT levels are also increased in AD serum and CSF (see Licastro et al., 1995 for data and discussion). It was
proposed at the time of their discoveries that ApoE and ACT might function as amyloid promoters or “Pathological Chaperones”, and both in vitro and in vivo studies support this model.

As with APP, the importance of inflammation and specifically ApoE and ACT in AD is supported by genetic studies. Inheritance of an ApoE ε4 allele is the strongest risk factor for AD besides age, and an ACT/A signal peptide variant that increases mature glycosylated ACT available for secretion also increases AD susceptibility and pathology (Kamboh, 1995; Kamboh et al., 1995). Similarly, polymorphisms in the IL-1 promoter greatly increase the risk of AD. These genetic variations increase IL-1 expression during inflammation and therefore promote amyloid formation by increasing the production of both APP and ACT.

Genetic and biochemical studies have identified key proteins in the AD pathogenic pathway and much research has sought to ascertain the role that Aβ, ApoE, and ACT play in the formation of amyloid and the resultant cognitive dysfunction of AD. To begin to answer these questions, we performed cognitive studies with AD mouse models in which the inflammatory proteins and the Aβ deposition they influence could be regulated without changing the level of monomeric Aβ peptide. To this end, we generated and analyzed a number of ACT and/or apoE-expressing/non-expressing mouse models of AD and found that: 1) ApoE and ACT independently and synergistically promote Aβ immunoreactive and mature amyloid deposition without initially affecting Aβ levels and 2) cognitive impairment in aged AD mice depends on the amyloid promoting effect of ApoE and/or ACT (Nilsson et al., 2004, APPENDIX B).
The compilation of manuscripts presented in this volume seeks, using transgenic mouse models of AD, to more clearly define the role of apoE in amyloidogenesis, and to ascertain whether the systemic circulating pool of apoE is able to exert an affect on pathogenic Aβ lesions localized in the brain. Furthermore, we explore the beneficial pathological and genetic effects of cognitive stimulation on the Alzheimer brain. Both avenues of study, apoE and environmental enrichment, are discussed in detail below.

APOLIPOPROTEIN E

Besides age, the apolipoprotein E gene is the greatest known genetic susceptibility factor for AD. Three apoE alleles exist, \( \varepsilon_2 \), \( \varepsilon_3 \), and \( \varepsilon_4 \), each conferring a different relative risk of developing the disease (\( \varepsilon_4 > \varepsilon_3 > \varepsilon_2 \)). There exists an allelic dose response for the apoE \( \varepsilon_4 \) allele in particular which is exemplified by the 20 year acceleration of the mean age of onset for those people with two copies of apoE \( \varepsilon_4 \) (average onset age = 70) vs. those with a \( \varepsilon_2/\varepsilon_3 \) combination (average onset age = 90) (Strittmatter and Roses, 1996). Another particularly interesting example of the affect of apoE \( \varepsilon_4 \) comes from studies of the Japanese population as a whole, where the allelic frequency of individuals homozygous for apoE \( \varepsilon_4 \) is lower than that of the United states, which results in an overall prevalence of the disease that is therefore lower, and an observed population age of onset of sporadic AD that is shown to be higher, furthing implicating apoE \( \varepsilon_4 \) as a risk factor in AD (Ueki et al., 1993). Though not
known with any great deal of certainty, there are a number of putative roles that apoE plays in relation to AD: ApoE, 1) forms stable complexes with the Aβ peptide; 2) in an isoform-specific manner, alters the aggregation properties of Aβ; 3) inhibits Aβ-induced neurotoxicity; and, 4) promotes the clearance of Aβ in a receptor mediated fashion.

ApoE is a 34 kDa (299 amino acids in length) lipoprotein produced predominately in the liver whose primary function is to transport lipids via either low density lipoproteins (LDL), very low density lipoproteins (VLDL), or high density lipoproteins (HDL). The brain has the second highest amount of ApoE in the body (after the liver) where it is predominantly produced by astrocytes (Driscoll and Getz, 1984). In the course of AD, both the levels of apoE mRNA and protein are increased in astrocytes, and though neurons do not produce apoE, for no apoE mRNA is detectable, apoE protein is present in neurons of patients with AD.

Besides genetic linkage studies implicating apoE’s role in AD, there are a number of reasons that apoE is thought to be directly involved in the process of amyloidogenesis. Several apoE promoter mutations exist whereby the gross levels of apoE are increased which is correlated with an increased risk of AD (Strittmatter and Roses, 1996). Furthermore, apoE has been shown to bind to Aβ, and lastly, apoE accumulates in extracellular amyloid plaques. In vitro, both apoE ε3 and ε4 bind, in an irreversible manner, to the Aβ peptide (Strittmatter et al., 1993). Both isoforms catalyze the formation of fibrils, but the ε4 isoform promotes Aβ polymerization roughly 25-times faster than apoE ε2 or ε3 (Ma et
al., 1994; Wisniewski et al., 1994). Furthermore, the character of the fibrils formed in the presence of apoE adopt a simple 10nm fiber shape, as compared to a non-apoE catalyzed aggregate which can best be described as a twisted ribbon-like conformation. Interestingly, it has been shown that apoE confers a greater degree of protease resistance to Aβ upon binding, which could be responsible for the irreversible nature of this interaction (Zhou et al., 1996), and may help explain the propensity for apoE-mediated fibril accumulation.

The vast majority of existing literature would suggest that ApoE does not affect APP production. Transcription or translation of APP does not change in the presence of apoE, and this has been demonstrated with both mouse and the ε3 and ε4 human isoforms (Bales et al., 1999; Koistinaho et al., 2004). Even though there is a reported gene dose effect of mouse apoE on Aβ immunoreactivity and Thioflavine S staining (apoE+/+ > apoE+/− > apoE−/−), at two months of age there is no difference in Aβ levels, as shown by enzyme linked immunosorbent assay (ELISA) (Bales et al., 1997; Nilsson et al., 2004, APPENDIX B). The immunohistochemical staining patterns of transgenic AD mouse models suggests that Aβ1-42 deposition precedes ApoE deposition, while Ab1-40 follows (Terai et al., 2001), further indicating that it is likely that apoE plays an important role in the maturation of plaques, but not necessarily initial APP processing. Other authors have conversely reported that hippocampal levels of Aβ are suppressed in APP mice expressing human apoE ε3 or ε4 (Fagan et al., 2002). These results, combined with the findings that apoE can complex with both soluble and fibrillar Aβ (Permanne et al., 1997; Russo et al., 1998), has led
to an isoform-dependant dual-role hypothesis for apoE. First, as previously mentioned, the ε4 isoform of apoE promotes the fibrillarization of deposited pre-amyloid Aβ1-42 aggregates into mature amyloid. Secondly, since one manuscript has reported that more soluble Aβ is found in mice with human apoE4 than those expressing apoE3 (Bales et al., 1997), it has been proposed that apoE ε3 inhibits Aβ oligomer deposition (short oligomers of Aβ are thought to be neurotoxic and the precursors to longer fibrils) and that pre-fibrillar Aβ/apoE3 interactions could foster apoE-mediated clearance of Aβ. This precedent has been set by other Aβ-associated molecules such as transthyretin, which have been shown to sequester Aβ (Goldgaber et al., 1993).

The classical amyloid cascade hypothesis describes a process whereby APP is processed into Aβ, which in turn leads to plaque deposition and ultimately neuronal cell dysfunction and death. Unfortunately, the temporal and spatial distribution of brain amyloid deposition does not correlate well with the clinical progression of the disease (Ma et al., 1996; Naslund et al., 2000). There is a large body of research implicating the soluble Aβ oligomer, rather than mature, deposited, amyloid as the causative agent in AD. It has been shown in vitro that neuronal viability is adversely affected by oligomeric Aβ 10-fold more than the fibrillar form, and roughly 40-fold more than the monomeric peptide (Dahlgren et al., 2002). Furthermore, Aβ oligomers cause an increase in inflammatory markers such as interleukin-1 (IL-1) or inducible nitric oxide synthase (iNOS), while fibrillar Aβ does not (Manelli et al., 2004). It therefore seems that oligomeric Aβ is far more neurotoxic than unaggregated or fibrillar Aβ.
Interestingly, ApoE3 has been shown to prevent Aβ-induced neurotoxicity, while ApoE4 has not (Jordan et al., 1998). Specifically, apoE3 attenuates oligomer toxicity (Manelli et al., 2004). This is not a general anti-inflammatory mechanism of apoE, for other inflammatory agents such as LPS are not attenuated in the same manner as Aβ (Hu et al., 1998). Furthermore, Since APP/ApoE-KO mice perform poorly in behavioral tasks designed to measure memory deficits, even with a lack of ApoE-catalyzed plaque formation, a detrimental effect on memory independent of fibrillarization events exists (Dodart et al., 2000). This supports the beneficial role of apoE ε3 in the inhibition of oligomer-induced neurotoxicity and inflammation while not discounting apoE ε4’s catalytic role in promoting Aβ fibrillarization.

ApoE’s role in the brain inflammatory response and neuritic pathology is well characterized, but not widely agreed upon. It is known that apoE levels rise due to brain injury (Poirier et al., 1993), and that that there is a marked reduction in gliosis (as measured by GFAP immunostaining) in apoE knockout mice (Bales et al., 1999). Since Aβ is known to increase apoE production, it has been hypothesized that apoE negatively feeds back to limit further inflammation caused by this molecule. It has been noted by more than one author that apoE-KO mice do not develop dendritic or long term potentiation deficits (Dodart et al., 2000; Holtzman et al., 2000). The neuritic pathology of APP mice (i.e. – swollen and distorted neurites) is absent when apoE is selectively ablated, and dystrophic neurites re-appear when either mApoE, hApoE3, or hApoE4 is expressed, indicating that apoE-induced fibrillar plaque formation is most likely
responsible for neuritic pathology (Holtzman et al., 2000). Opposing research has reported that apoE-KO mice present a 15 to 40% loss in synaptophysin staining and a decrease in tubulin immunoreactivity. This lends credence to the idea that apoE might play an important role in maintaining dendritic and synaptic stability and plasticity, and any alterations could be responsible for typical AD-related synaptic and cytoskeletal deficits (Masliah et al., 1995). Since apoE3, but not apoE4, delays age-dependant decline of synaptophysin levels in the presynaptic terminals of APP mice, the fact that the cognitive decline in AD correlates better with synaptophysin reactivity than plaque load (DeKosky and Scheff, 1990) could be relevant to apoE-conferred risk of AD and an apoE-driven early onset. Furthermore, apoE might even bind to tau and prevent its phosphorylation (Strittmatter et al., 1994) in an isoform specific manner, for apoE ε3 forms an irreversible complex with tau, but ApoE ε4 does not. The same holds true for another microtubule associated protein, MAP2c. Therefore, apoE may play a role in Aβ fibrillarization, inflammation, synaptic health, and neurofibrillary tangle formation.

The apoE molecule contains two functionally important domains: an LDL receptor (LDLr) binding domain, and a lipoprotein binding domain. Neurons express several apoE receptors, including the LDLr, the LDLr-related protein (LRP), and the VLDL receptor (Terai et al., 2001). Neurons are thought to uptake apoE and their associated lipid particles in a receptor mediated fashion, for In vitro, apoE is taken up from the growth media by neurites and neuronal growth cones (Strittmatter and Roses, 1996). After neuronal injury, cholesterol is
endocytosed via apoE and the LDLr in order to support the growth of neurons undergoing reinnervation (Poirier et al., 1993), indicating yet another role for apoE in brain maintenance – lipid transport due to CNS injury. It is likely that apoE’s role in the brain is, like in the periphery, to regulate plasma lipid transport and clearance as the ligand for lipoprotein receptors. ApoE isoform differences emerge yet again, for ApoE ε3 and ε4 bind, with high affinity, to the LDL receptor, while apoE ε2 does not (Weisgraber et al., 1982). This readily explains the atherosclerotic deposition found in apoE ε2/ε2 and apoE-KO mice, due to respectively decreased or abolished receptor mediated apoE endocytosis. A reason that apoE ε4 may be detrimental in AD, aside from its fibrillogenic properties, is that it is thought to be a less efficient cholesterol and lipid mobilizer and transporter during CNS injury (Mahley et al., 1989). Furthermore, apoE-enriched VLDL particles have been shown to modulate neurite outgrowth in an LRP mediated and isoform specific manner, with apoE ε3 stimulating outgrowth to a larger extent than ε4 (Holtzman et al., 1995). Aβ has been found to be preferentially internalized by astrocytes, but ApoE-KO astrocytes do not degrade Aβ, and blocking LRP also blocks the degradation. It has therefore been concluded that apoE is essential for astrocytes to associate with, internalize, and degrade soluble, but not fibrillar Aβ (Koistinaho et al., 2004), once again pointing to the dual role that apoE plays in AD.
Given the importance of apoE, we have decided to further examine the role of endogenous mouse apoE in a transgenic mouse model of AD. To determine which part or product of the deposition process is controlled by apoE, we examined a mouse model in which the production of Aβ is not rate limiting, thus allowing the unique effect of apoE to be determined. We used animals expressing mutant forms of both the human APP and presenilin-1 transgenes which express high ratios of Aβ1-42/Aβ1-40 and develop large amounts of both amorphous and filamentous Aβ (amyloid) deposits. Specifically, we crossed PS1<sup>M146L+/-</sup>, APP<sup>V717F+/-</sup> doubly transgenic animals with an apoE knockout mouse (Bales et al., 1999) in order to explore the effect(s) of apoE in a mouse model that produces large amounts of Aβ1-42 and develops early and severe AD-like pathology. We predicted two likely alternative results: 1) If apoE affects Aβ availability or clearance, it should have little positive or negative effect in our model due to the already large amounts of PS1-enhanced Aβ production in these doubly transgenic mice, or 2) If apoE promotes Aβ fibrillarization, then its expression should elevate the amount, and/or change the character of the brain Aβ deposition.

Mouse brains were analyzed for AD pathology at seven months of age, and we observed that large amounts of amorphous, non-fibrillar, Aβ had been generated in both sets of animals in statistically similar amounts, reflecting the presence of large levels of Aβ1-42. The physical appearance and distribution of the immunoreactive deposits was quite different. We found that mature plaque deposition was strikingly dependent on the presence of apoE. In particular, apoE
expression induced a more than 3000-fold increase in the formation of mature, fibrillar, amyloid (to our knowledge the largest reported effect of any molecule on amyloid formation other than the mutant APP itself). In the absence of apoE, the area occupied by the few thioflavine S-positive Aβ deposits was not sufficiently large to be statistically significantly different from the background thioflavine S staining in a completely non-transgenic mouse. Once apoE’s effect on Aβ maturation was determined, we wished to determine the source of apoE most responsible for this occurrence.

It is a curious fact that most (if not all) of the proteins involved in Alzheimer amyloid formation are also present in the circulation. It is therefore important to know whether the amyloid deposits of the brain are derived in part from the circulating proteins or only from local proteins produced in the brain. The evidence that brain expression of Aβ, apoE, and ACT can lead to local amyloid deposition is strongly supported in the transgenic mouse models of amyloid formation. However, in human, these proteins are made in equal or larger amounts in the rest of the body and, as mentioned above, are present in large amounts in the blood. Certain facts point to the possibility that blood-derived proteins may contribute significantly to the brain deposits. For example, Aβ levels, particularly Aβ1-42 are elevated in the blood of FAD and many SAD patients (Scheuner et al., 1996). Serum ACT and apoE levels are also generally increased in AD patients (Licastro et al., 1995; Lieberman et al., 1995; Blain et al., 1997) and apoE4 has been shown to shuttle Aβ across the blood brain barrier (Martel et al., 1997). It is therefore reasonable to hypothesize that apoE
from the circulation may contribute importantly to the amyloid deposits in the brain.

We attempted to ascertain whether circulating apoE could contribute to Alzheimer amyloid formation using a not often used surgical technique called parabiosis whereby mice are sutured together early in life as to allow the sharing of their blood circulation (Martinez et al., 1959). In practice, any circulating protein produced in one mouse will be passed to the other and vice versa. By parabiosing an endogenously apoE-expressing mouse to an apoE-KO mouse, we sought to determine whether the pathological chaperone, apoE, must be synthesized in the brain or can be derived from the blood. The significance of this experiment for drug development is derived from the fact that circulating proteins are far easier to administer versus those given intra-cortically.

ENVIRONMENTAL ENRICHMENT

The development of drugs to effectively treat and/or prevent AD has proven a most difficult task for the research community. Therefore, significant effort has been placed in studying non-pharmacological means of delaying or preventing AD. One such avenue of study is that of preventative and therapeutic cognitive stimulation regimens. The correlation between basic cognitive abilities in later life and early performance measures such as IQ and educational status have been studied by a number of research groups. For example, Plassman et al. (1995) established that performance on the Army General Classification Test directly correlated with late-life (i.e. 50 years later) cognitive fitness. Similarly,
low levels of education have also been associated with later cognitive deficits and an increased risk of AD (Letenneur et al., 1999). One such study even proposed that each year of education reduces the risk of generating AD by 17% (Evans et al., 1997). Though these results are maybe optimistic, a similar study found that high occupational attainment is also protective against AD (Stern et al., 1994). No doubt, these reports led to one of the most famous psychological inquiries in the AD community; the "nun study." Here, Snowdon et al. (1996) examined early life linguistic ability, in a population of nuns, as a marker for later cognitive decline. The content of autobiographies for admission into their convent written by 93 nuns before the year of 1917 (Average age = 22) were studied. It was found that the scores from cognitive testing (Mini Mental State Exam) administered roughly 50-60 years later were highly correlated with both idea density and grammatical complexity as well as years of education. Furthermore, of those nuns who died during the course of the study (n=14), 100% of those diagnosed with AD had low idea density in their essays. Idea density was also correlated with the severity of AD pathology (Snowdon et al., 1996). Besides education and job attainment, overall low activity levels (physical and especially mental) in mid life have also been shown to increase the risk of AD (Friedland et al., 2001).

Recently, several cognitive stimulation/rehabilitation programs have been developed in an attempt to slow or reverse the cognitive decline in AD (Davis et al., 2001; Wenisch et al., 2005). These programs have generally involved a relatively short 4–12 week period of cognitive training classes and/or daily
caregiver-directed mental activities, resulting in modest degrees of improvement. One such study showed that specific cognitive rehabilitation techniques given to patients with mild AD over a 12-week period improved a variety of cognitive skills with benefits seen even 3 months following CS (Loewenstein et al., 2004). Nonetheless, the therapeutic potential of intensive, longterm CS has not yet been evaluated in AD patients. Moreover, the biochemical and potential pathological changes due to CS cannot be easily studied in humans. Furthermore, for the aforementioned risk factors, early-life education, activity, and cognition, the cause and affect relationship between AD and poor early-life performance markers has not been determined. The fact that AD exerts its affects on the brain at a young age is exemplified by the finding that patients with Down's syndrome can have Aβ_{1-42} accumulation in the brain as early as 12 years old. Do education and mental activity buffer against AD, or does AD predispose one, from an early age, to have a low propensity for participation in mentally stimulating activities? Since retrospective studies like those above cannot sufficiently answer this question, and long-term prospective studies are not practical, the use of rodents, specifically mouse models of AD, must be employed for this direction of study.

Rodents undergoing cognitive stimulation (CS) by being placed in an enriched environment (e.g. socially housed in large cages containing toys, tunnels, running wheels, etc.) are shown to exhibit improved cognitive performance in behavioral testing regimens (e.g. – Morris water maze, Radial arm water maze, etc…) (Kobayashi et al., 2002). EE protects rats against kainic
acid-induced seizures (Young et al., 1999), and can even reverse the cognitive
deficits in rats subjected to long-term lead exposure (Guilarte et al., 2003). In
addition, a number of structural and biochemical changes have been noted in
rodents due to an enriched environment. For example, the overall thickness of
the cerebral cortex has been shown to be increased (Diamond et al., 1972).
There are increases in hippocampal dentate gyrus neurogenesis (Kempermann
et al., 1997; Brown et al., 2003), increases in the density of synapses
(Greenough and Volkmar, 1973; Ramirez-Amaya et al., 1999), and the extent of
dendritic branching (Globus et al., 1973). EE is also protective, in that Young et
al (1999) observed a 45% decrease in spontaneous brain cell death as
measured by TUNEL assay.

Besides physical and structural changes in brain architecture, a large
number of genetic and neurochemical measures have been described as being
altered as a result of exposure to a cognitively stimulating environment. For
example, mRNA and protein levels of glial-derived neurotrophic factor and brain-
derived neurotrophic factor, both of which have been associated with neuronal
plasticity and survival, are increased in the rat hippocampus after exposure to an
enriched environment (Fischer et al., 1992). In addition, the levels of another
growth-related molecule, nerve growth factor (NGF) are increased due to EE.
When injected into the rat forebrain, NGF improves spatial learning and memory
(Fischer et al., 1991). NGF is thought to be important for cholinergic neuron
maintenance, and has been shown to increase levels of choline
acetyltransferase, an enzyme whose levels decrease in the AD brain (Gnahn et
Various receptor and receptor-related proteins also undergo changes in expression due to EE. For example, NMDA receptors, which are known to play a pivotal role in hippocampal synaptic plasticity (Singer, 1990; Gu, 1995), are essential for triggering long-term potentiation and increasing the expression of memory related molecules such as phospholipase A2 (PLA2) and the immediate early gene (IEG) nerve growth factor induced-A (NGFI-A) (Diamond et al., 1972). PLA2 expression is further linked to synaptic plasticity via Ca$^{2+}$-dependant and AMPA receptor-dependant mechanisms (Fischer and Bjorklund, 1991), for GluR1-4 subunits (i.e. AMPA receptor subunits) are increased due to EE. Additionally, the IEG, NGFI-A is thought to be involved in the early stages of memory formation, for its expression is rapidly induced after neuronal activity in brain regions with the most pronounced EE-induced structural changes (Wallace et al., 1995). ARC, or activity regulated cytoskeletal protein, is another IEG up-regulated due to EE and known to be important for learning, for inhibiting it blocks both LTP and long-term memory. These are just a few of the described neurochemical changes induced by cognitive stimulation. Our current study of environmental enrichment hopes to elucidate some of the genetic and biochemical changes most important for preserving memory and reducing pathology in a mouse model of AD.

A previous study from our laboratory sought to elucidate the cognition-enhancing potential of long-term environmental enrichment (EE) as a therapeutic intervention in aged transgenic mouse model of AD, which overexpresses the Swedish doubly mutant (K670N,M671L) amyloid precursor protein and bears
moderate cortical/hippocampal Aβ deposition within mature AD-like neuritic plaques (Arendash et al., 2001a, APPENDIX B). We found that environmental enrichment begun relatively late during aging in APPsw transgenic mice provided cognitive benefit across multiple cognitive domains (reference learning/memory, working memory, recognition, strategy switching – For detailed descriptions of behavioral tasks, see (King and Arendash, 2002)) through mechanisms that do not seem to involve a reduction in brain Aβ deposition. Those results suggested that long-term, intensive cognitive stimulation could be therapeutic in stabilizing or improving cognitive function in Alzheimer’s disease.

One aim of this dissertation sought to answer the key question regarding whether pre-emptive EE, as a means of prophylaxis, can protect against the pathology of AD, or its associated mental decline, and to further characterize the resultant molecular changes. At weaning, doubly transgenic mouse models of AD (hAPPV717F / hPS1M146L) were placed into either standard housing or an enriched environment. These mice were then behaviorally tested between the ages of 4½-6 months. We found that AD mice raised in EE outperformed those raised in SH across a variety of behavioral/memory tasks, in which they were statistically indistinguishable from non-transgenic (NT) mice. We found that, brain Aβ deposition in Tg+ mice was not affected by EE alone, but only when in combination with the behavioral testing paradigm which is in and of itself a form of cognitive stimulation. When combined with behavioral testing, EE resulted in large reductions in brain Aβ deposition. Golgi staining revealed that the extent of dendritic branching and dendritic spine number in the brain were unchanged by
enrichment, indicating that one effect of EE is to overcome the dendritic deficits present in transgenic AD mice. Microarray analysis of hippocampal tissue revealed that transgenic mice exposed to EE exhibited increased expression of multiple memory-related genes, a number of neuroprotective genes involved in anti-apoptotic BAD phosphorylation such as IGF2, IGFBP2, and PRLR, as well as increased expression of a known Aβ sequestering molecule, transthyretin.
Paper I:

Apolipoprotein is required for the formation of filamentous amyloid, but not for amorphous Aβ deposition, in an APP/PS double transgenic mouse model of Alzheimer’s disease

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ABSTRACT

To determine the role of apolipoprotein E (apoE) in the deposition of different forms of Alzheimer amyloid deposit, we studied mice expressing both mutant human amyloid β-protein precursor (AβPP) and presenilin 1 (PS1) that, in addition, were either normal or knocked-out for apoE. By 7 months of age, extensive deposits of amorphous amyloid β (Aβ) had developed equally in both lines, indicating that, when present in high amounts, Aβ alone is sufficient for such deposition to occur. In contrast, filamentous, thioflavine S-positive amyloid deposition in AβPP/PS mice was catalyzed at least 3000 fold by apoE. Electron micrographs further illustrated the filamentous nature of Aβ deposits in mice expressing apoE. These and other behavior data indicate that the primary function of apoE in Alzheimer’s disease is to promote the polymerization of Aβ into mature, beta pleated sheet filaments, a process that is necessary for inducing cognitive decline. Thus, preventing apoE from binding to Aβ may prove to be an effective means of therapeutic intervention.

Key Words: Alzheimer’s Disease, Amyloid β, Amyloid β–Protein Precursor Apolipoprotein E, Transgenic Mouse Model
INTRODUCTION

Several lines of investigation indicate that the deposition of Aß peptide into amyloid underlies the neurodegeneration and cognitive decline that characterize Alzheimer's disease (AD) (Hardy and Selkoe, 2002). Using experimental animal models of AD, two proteins have been shown to be essential for both the formation of such deposits and for the cognitive decline in these animals (Games et al., 1995; Bales et al., 1999; Holtzman et al., 2000; DeMattos et al., 2004; Nilsson et al., 2004). The first is the Aß-peptide itself, particularly the Aß_{1-42} isoform. The second protein is apolipoprotein E (apoE) which, when produced from the ε4 allele, is the strongest known risk factor for developing sporadic AD besides age itself (Wisniewski and Frangione, 1992; Strittmatter and Roses, 1995). Increases in both Aß and apoE expression promote Aß deposition in a dose dependent manner (Games et al., 1995; Bales et al., 1999; Holtzman et al., 2000; Fryer et al., 2003).

Because cognitive deficits in several models of memory/learning observed in mutant AßPP-expressing mouse models of AD are dependent upon pathological chaperones such as apoE (and the Aß deposition that they promote) (Nilsson et al., 2004), it is important to know which part or product of the deposition process is controlled by apoE. We therefore examined a mouse
model of AD in which the production of Aβ (and in particular, C-terminally extended and highly fibrillogenic Aβ species such as Aβ1-42) is not rate limiting, thus allowing the unique effect of apoE to be determined. Specifically, we took advantage of the fact that animals expressing mutant forms of both human AβPP and presenilin-1 transgenes from families carrying inherited Alzheimer’s disease have a high ratio of Aβ1-42/Aβ1-40 and develop large amounts of both amorphous and filamentous Aβ (amyloid) deposits, resembling that of end-stage human AD (Duff et al., 1996; Scheuner et al., 1996; Citron et al., 1997; Holcomb et al., 1998). We then crossed PS1^{M146L+/-}, AβPP^{V717F+/+} doubly transgenic animals with an apoE knockout mouse (Bales et al., 1999) to explore the effect(s) of apoE in a mouse model that develops early and severe AD-like pathology due to the relatively high levels of brain Aβ1-42. Current hypotheses predict two alternative outcomes: 1) If apoE affects Aβ availability or clearance, it should have little positive or negative effect in our model due to the already large amounts of PS1-enhanced Aβ production in PS1^{M146L+/-}, APP^{V717F+/+} mice, or 2) If apoE promotes Aβ polymerization, then its expression should increase the amount, and/or change the character of the Aβ deposits in the brains of these mice.

At seven months of age, both the apoE^{+/−} and apoE^{−/−} animals were euthanized, and the brains analyzed for AD-related pathology. Very large amounts of amorphous, non-fibrillar, Aβ were generated equally in both sets of animals, reflecting the presence of large amounts of Aβ1-42 in the brain. However, the quality of the deposits was strikingly dependent on the presence of apoE. Specifically, apoE expression induced a more than 3000-fold increase in
to our knowledge the largest reported effect of any molecule on amyloid formation other than the mutant AβPP transgene itself. Indeed, in the absence of apoE, the area occupied by the few thioflavine S-positive Aβ deposits was not sufficiently high to be statistically significantly different from the background thioflavine S staining in a completely non-transgenic mouse.

MATERIAL AND METHODS

Construction of transgenic mice. All animals were housed in shoe box cages with static microisolator tops under climate-controlled conditions on a 12 hour light/12 hour dark cycle, fed Harlan Teklad Global Diet #2018 and provided with tap water ad libitum. Heterozygous PDGF-hAβPP(V717F) mice [Swiss-Webster x C57BL/6 x DBA/2] were crossed with PDGF-hPS1(M146L) heterozygotes to generate mice with an AβPP*+/-,PS1*+/− genotype. These mice were subsequently crossed with ApoE−/− mice [Swiss-Webster x C57BL/6 xDBA/2] to generate mice with genotype AβPP*+/-,PS1*+/−ApoE+/−. These mice were bred to generate either AβPP*+/−,PS1*+/−,ApoE+/−, or AβPP*−/−,PS1*+/−,ApoE+/− mice. All offspring were screened by PCR for identity the PDGF-hAPP gene(Games et al., 1995), the PDGF-hPS1 gene(Duff et al., 1996), as well as the mouse ApoE gene and neo gene (Bales et al., 1997).

Immunohistochemical Procedures. Mice that have been fasted over night were anesthetized with Nembutal (0.1mg/g). The animals were then be intracardially
perfused with 0.9% NaCl (25ml) followed by 50ml 4% paraformaldehyde in 1x Sorenson’s phosphate buffer. The mounted sections were processed through antigen retrieval in prewarmed 25mM citrate buffer (pH 7.3) at +82°C for 5 min and further processed as previously described (Nilsson et al., 2001). The sections were incubated with primary antibodies against Aβ (6E10, dil 1:5000) overnight at +4°C. Secondary antibody was anti-mouse IgG developed with NovaRED substrate kit (Vector). Thioflavine S staining was performed as previously described (Nilsson et al., 2001).

Image Analysis. Data were collected from three equally spaced coronal tissue sections for both dorsal hippocampus and overlying parietal cortex (Bregma -1.30 to -2.30 mm) for each mouse. The sections were examined with a Nikon Eclipse E1000 microscope using either 4X or 10X Plan Fluor objective lenses. A Retiga 1300 CCD (Qimaging) with a Qimaging RGB LCD-slider was used to capture images. For thioflavin S, a Nikon BV-2B fluorescence filter cube was used. Customized software written in Visual Basic 6.0 (Microsoft) utilizing Auto-Pro function calls (Image Pro Plus, Media Cybernetics) was used to segment and quantify images. Aβ deposition was calculated as percent area of interest (=Area Stained\_tot/Area Measured\_tot). Results were analyzed using a two-tailed, unpaired student’s t test with Welch’s correction.

Electron Microscopy. Tissue was fixed in 2.5% phosphate buffered glutaraldehyde (Electron Microscopy Sciences) overnight at 4 degrees C. The cells were post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences) in
the above buffer for 1 hour at 4 degrees C. Following buffer rinsing, the cells were dehydrated in a graded series of acetone, then infiltrated and embedded in LX112 plastic (Ladd Research Industries, Inc). Blocks containing tissue were polymerized overnight at 70 degrees C. Cell blocks were sectioned on a Reichert Ultracut E ultramicrotome (Leica, Inc), and sections were stained with 6 % uranyl acetate and Reynold's lead citrate. Sections were observed and photographed with a Philips CM10 electron microscope (FEI, Inc) at 60kV.

RESULTS

Total Aß deposition was measured in both hippocampus and parietal cortex as the percentage of 6E10 antibody immunoreactive staining. Figures 1a-b show that overall Aß deposition was not much affected by the presence or absence of apoE in these PS/AßPP mice. No significant difference was found in either the hippocampus (apoE+, 5.5%±1.3 vs. apoE KO, 8.3%±3.2) or parietal cortex (apoE+, 3.0%±1.3 vs. apoE KO, 1.5%±0.3) (Figure 1c).

Although total Aß deposition was statistically similar in the apoE+/− and apoE+/- mice, there was a clear difference in the regional distribution and morphology of the Aß deposits. The presence of apoE promoted the formation of more compact-type plaque structures throughout the hippocampus and parietal cortex (Figure 1a), while mice without apoE contained few, if any, compact-like plaques anywhere in the brain, instead manifesting a rather diffuse pattern of deposition. Furthermore, mice lacking apoE developed almost no Aß in the
granular and molecular layers of the dentate gyrus of the hippocampus (Figure 1b).

To confirm the distinction between amorphous Aβ deposition and dense core plaques, sections were stained with thioflavine S, which detects β-pleated sheet structures characteristic of mature amyloid plaques. Thioflavine S staining was elevated in PS1<sup>M146L</sup>+/−, AβPP<sup>V717F</sup>+/− mice containing one copy of endogenous apoE by ≥ 3200 fold in both hippocampal and cortical regions as compared to mice with the apoE<sup>−/−</sup> genotype (Figure 2). The increase was statistically significant in both the hippocampus (apoE<sup>+/−</sup>, 0.64%±0.18 vs. apoE<sup>−/−</sup>, 0.0002%±0.0001, p=0.0004) and the parietal cortex (apoE<sup>+/−</sup>, 0.35%±0.04 vs. apoE<sup>−/−</sup>, 0.0001%±0.00005, p=0.0001). Because there was a difference in staining between apoE<sup>+/−</sup> and apoE<sup>−/−</sup> mice of three orders of magnitude, a logarithmic scale was necessary to resolve the low levels of thioflavine S staining in the apoE<sup>−/−</sup> animals (Figure 2c-d). Electron microscopy confirmed the presence of extensive filamentous amyloid deposits in the PS1/AβPP/apoE<sup>+/−</sup> animals and only amorphous deposits in the PS1/AβPP/apoE<sup>−/−</sup> animals (Figure 2).

**DISCUSSION**

In sum, we report that doubly transgenic mice with both human PS1<sup>M146L</sup> and AβPP<sup>V717F</sup> mutations do not develop significant thioflavine S-positive amyloid plaque deposits by 7 months in the absence of apoE expression, despite the accumulation of large amounts of immunoreactive Aβ deposition. Only the presence of apoE allows mature filamentous amyloid deposits to form. These
data contrast with previous reports utilizing mice expressing only a human AβPP mutation (PDAPP mice) in which apoE has been shown to promote both total Aβ deposition and amyloid formation. Evidently, because the AβPP/PS1 double transgenic mice express very high levels of Aβ_{1-42} in the brain, perhaps overriding any effect of apoE on Aβ production or clearance, they provide a clear proof that apolipoprotein E directly catalyzes the polymerization of Aβ into β-sheet fibrils and its consequent aggregation to form mature amyloid plaques.

*In vitro* (Ma et al., 1994) and *in vivo* (Nilsson et al., 2001) studies show that both of the major pathogenic chaperones found in amyloid β plaques, apoE and ACT, promote Aβ filament formation, with apoE showing the higher catalytic activity. Since ACT and apoE act synergistically with regard to Aβ deposition, future studies with ACT expressing mice could show even more drastic amyloid β deposition. Furthermore, since endogenous mouse-apoE is most related to the human ε4 allele of apoE, the human isotype-specific effects of the human apoE alleles, specifically the protective properties of the ε2 allele should also be further explored in this mouse model of AD.

Inasmuch as the presence of amyloid plaques is pathonomic for Alzheimer’s disease, preventing apoE from binding to Aβ and stimulating its polymerization into β-sheet filaments may prove to be a promising therapeutic approach for AD drug development. An indication that such an approach may be effective has been provided by *in vitro* experiments in which small fragments of the Aβ peptide were shown to completely inhibit the apoE-catalyzed
polymerization of Aβ1-42 into neurotoxic amyloid filaments (Ma et al., 1994; Ma et al., 1996).
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Fig. 1. Little or no effect of ApoE on total Aβ deposition - Total Aβ deposition, diffuse and compact, in 7-month-old PS1<sup>M146L+/−</sup>, APP<sup>V717F+/+</sup>, ApoE<sup>+/−</sup> mice (a) and 7-month-old PS1<sup>M146L+/−</sup>, APP<sup>V717F+/+</sup>, ApoE<sup>/−</sup> mice (b), as measured by 6E10 immunostaining. (c) Quantitative image analysis of total Aβ deposition from all investigated animals. Hippocampal amyloid load (left) or cortical amyloid load (right). Solid bar, PS1<sup>M146L+/−</sup>, APP<sup>V717F+/+</sup>, ApoE<sup>+/−</sup> (n=3); open bar, PS1<sup>M146L+/−</sup>, APP<sup>V717F+/+</sup>, ApoE<sup>/−</sup> (n=3)
Fig. 2.  Strong catalytic effect of ApoE on mature amyloid deposition -
Thioflavine S plaque staining is increased over 3200 fold in 7-month-old
PS1$^{M146L+/-}$, APP$^{V717F+/+}$, ApoE$^{+/-}$ mice (a) compared to 7-month-old PS1$^{M146L+/-}$,
APP$^{V717F+/+}$, ApoE$^{-/-}$ mice (b). Quantitative image analysis of compact amyloid
deposition from all investigated animals is represented on a linear y-axis (c) or
logarithmic y-axis (d) measuring hippocampal amyloid load (left) or cortical
amyloid load (right). Solid bar, PS1$^{M146L+/-}$, APP$^{V717F+/+}$, ApoE$^{+/-}$ (n=3); open bar,
PS1$^{M146L+/-}$, APP$^{V717F+/+}$, ApoE$^{-/-}$ (n=3); ***, p<0.001. Transmission electron
microscopy reveals the filamentous nature of plaques in the apoE+/- mice (E,
6300X and G 44,000X) as compared to the more amorphous deposits found in
the apoE-/- mice (F and H).
ApoE  ApoE KO  ApoE  ApoE KO

0.00  0.25  0.50  0.75  1.00

Hippocampus  Cortex

% Area Thioflavin S

***

Hippocampus  Cortex

% Area Thioflavin S

***  ***
REFERENCES


Paper II:

Parabiosis as means to investigate the role of peripherally derived proteins in Alzheimer's disease pathology

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ABSTRACT

Apolipoprotein E (apoE) is expressed in the brain and present in Alzheimer’s disease (AD) amyloid plaques. The apoE-ε4 allele, the main genetic risk factor for late-onset AD, increases amyloid-beta peptide fibrillization and amyloid plaques density. ApoE is also synthesized in the liver and plays a crucial role in cholesterol metabolism, for transgenic mice devoid of apoE show hypercholesterolemia and cardiovascular lipid deposition. Here we investigate the neuropathology of transgenic PS1+/-,APP+/- Alzheimer’s disease mouse models, either with or without the apoE gene, using parabiosis to determine if apoE in the peripheral circulation influences amyloid formation. This surgical procedure allows free exchange of proteins via peripheral circulation. We show that apoE is found in the plasma of parabiosed PS1+/-,APP+/-,apoE-KO mice, resulting in a reversion of hypercholesterolemia. Unexpectedly, amyloid deposition is reduced in parabiosed PS1+/-,APP+/-,apoE-KO mice as compared to unoperated controls. Our findings demonstrate that apoE in the peripheral circulation slightly reduces amyloid burden, most clearly in transgenic mice devoid of apoE, by likely promoting efflux of amyloid-beta peptides from the brain. These findings further reinforce that the mechanisms whereby apoE affects amyloid-beta metabolism are complex, and that the modulation of peripheral apoE metabolism is not likely to profoundly impact Alzheimer’s disease neuropathology.
INTRODUCTION

Amyloid deposits, which are primarily composed of the amyloid beta peptide (Aβ), are a key feature of Alzheimer’s disease (AD) neuropathology, and their formation is likely to contribute to the neuronal degeneration that underlies clinical dementia. Certainly, genetic data point to Aβ as the central component of the Alzheimer’s pathogenic pathway. However, several other proteins are found in amyloid deposits, and some of these have been proven to alter the rate of amyloid filament formation. The best characterized of these amyloid promoters are apolipoprotein E (apoE) and α1-antichymotrypsin (ACT), which have been shown to facilitate Aβ deposition in vitro and in APP transgenic mouse models (Ma et al., 1994; Bales et al., 1997; Holtzman et al., 1999; Holtzman et al., 2000; Nilsson et al., 2001). Indeed, without apoE or ACT, even at 2 years of age, roughly 50% of homozygous PDAPP mice still do not develop filamentous amyloid (Nilsson et al., 2004). These findings agree nicely with the demonstration that postmortem AD brain of ApoE4 carriers have increased plaque density (Corder et al., 1993; Rebeck et al., 1993).

A long-standing question in AD research is whether Aβ and other proteins that are present in the peripheral circulation are involved in amyloid formation and the associated brain pathology. Plasma Aβ levels, particularly Aβ1-42, are elevated in some familial and sporadic AD patients (Scheuner et al., 1996) and have been suggested as a potential AD biomarker. In addition, serum ACT and apoE levels have been shown to be generally increased in AD patients (Licastro
et al., 1995; Lieberman et al., 1995; Blain et al., 1997), and apoE has been shown to shuttle Aβ across the blood brain barrier (Martel et al., 1997). It is therefore important to analyze whether Aβ peptides, apoE, and ACT in the peripheral circulation may have a pathogenic role in AD with respect to amyloid deposition in the brain.

Parabiosis is a technique that enables shared peripheral circulation between surgically joined animals and thus gives us the ability to address such questions (Martinez et al., 1959). It allows the influence of circulating factors or hormones to be studied, since any circulating protein produced in one mouse will be passed to its parabiosed partner and vice versa (Finerty, 1952). In the present study, we used parabiosis to investigate the effect of apoE present in the peripheral circulation on brain amyloid deposition in a transgenic model of Alzheimer’s disease. Transgenic mice expressing both the human mutant presenillin-1 (PS1^{M146L}) and the human mutant amyloid precursor protein (APP^{V717F}) genes were parabiosed. However, one of the parabiosed mice was heterozygous for endogenous apoE, while its parabiosed partner lacked apoE expression altogether.

PS1^{+/-},APP^{+/-} transgenic mice rapidly develop robust Aβ-immunoreactive deposition by 3 to 4 months of age. The pathology mainly consists of mature cored plaques, which is totally prevented in the absence of apoE in this transgenic model (Costa et al., 2004). We considered PS1^{+/-},APP^{+/-} mice, with or without apoE, to be an excellent choice for parabiosis experiments due their rapid onset of amyloid pathology. Furthermore, the strong impact of apoE on
amyloid deposition in this model is ideal, since it is likely that even minute amounts of apoE in the central nervous system will strongly affect the amyloid phenotype of a parabiosed PS1+/−,APP+/−,apoE-KO mouse. Thus, parabiosis of such transgenic mice would allow us to determine whether the pathological chaperone, apoE, needs to be synthesized within the brain or whether its presence in the peripheral circulation is sufficient to promote amyloid formation. This knowledge will allow strategies for therapeutic interventions against Alzheimer disease to be more appropriately devised.

MATERIAL AND METHODS

All described procedures were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the “Guide for the Care and Use of Laboratory Animals”. All animals were housed in shoe box cages with static microisolator tops under climate-controlled conditions on a 12 hour light/12 hour dark cycle, fed Harlan Teklad Global Diet #2018 and provided with tap water ad libitum. The animal facility maintains a specific pathogen free status based on a sentinel system including colony representatives and quarantine of animals received from non-approved vendors or other institutions, respectively.

Parabiosis

Six week-old siblings of the same sex were selected for parabiosis. Both animals were transgenic for APP (PDGF-hAPPV717F, human mutant amyloid precursor protein) and PS1 (PDGF-hPS1M146L, human mutant presenilin-1).
Subsequent crossings of these mice with an apoE<sup>−/−</sup> mouse generated the APP<sup>−/−</sup>,PS1<sup>−/−</sup>,apoE<sup>−/−</sup> genotype. This schedule allowed the generation of siblings with genotypes PS1<sup>−/−</sup>,APP<sup>−/−</sup>,apoE<sup>−/−</sup> and PS1<sup>−/−</sup>,APP<sup>−/−</sup>,apoE<sup>−/−</sup> as partners for the parabiosis. Animals were anesthetized with 100 mg/kg, Xylazine 20 mg/kg, and Acepromazine 3 mg/kg. For the parabiosis, the mice were placed in a parallel orientation, and a left lateral incision was made on one mouse while a right one was made on the partner mouse, extending from the base of the ear toward the middle of the femur of the extended pelvic extremity. The incision included skin and muscle along thorax and abdomen. Starting at the last rib, the opening was extended into the abdominal cavities to accomplish convergence. The peritonea and muscle layers of the two animals were joined by simple interrupted suture with 4-0 PDS<sup>®</sup> (polydioxanone) whereas the skin was closed via stainless steel clips. The animals were allowed to recover in a warm, clean environment before being transferred into the husbandry area. Due to the expected immunosuppression, prophylactic antibiotic treatment (Enrofloxacin, 5mg/kg), was started one day prior to surgery, and was continued for three days. All animals received analgesic/anti-inflammatory treatment (Acetyl salicylic acid 5mg/kg), for 14 days. Six months after surgery, the parabiotic pairs were euthanized for brain analysis at seven months of age.
**Immunohistochemical Procedures**

Parabiosed mice were fasted overnight and subsequently anesthetized with Nembutal (0.1mg/g body weight). Blood was collected by cardiac puncture and immediately supplemented with 0.1% (w/v) EDTA followed by centrifugation (2000xG, 15min). Plasma was aspirated and total cholesterol was measured with a colorimetric assay (Infinity Cholesterol Reagent procedure 401, Sigma). The animals were then intracardially perfused with 0.9% NaCl (25ml) followed by 50ml 4% paraformaldehyde in 1x Sorenson’s phosphate buffer. Brains were cryoprotected through sequential immersion in 10%, 20% and 30% sucrose and sectioned (25μm) using a sledge microtome. The mounted brain sections were processed through antigen retrieval in prewarmed 25mM citrate buffer (pH 7.3) at +82°C for 5 min and further processed as previously described (Nilsson et al., 2004). The sections were incubated with primary antibodies against Aβ (6E10, dil 1:5000, Signet and rAβ40, dil 1:3000, QCB) and apoE (AB947, dil 1:5000, Chemicon) overnight at +4°C. Secondary antibodies were anti-rabbit IgG or anti-mouse IgG (1:300, Vector). The immunostaining was visualized with a NovaRED substrate kit (Vector). Thioflavine S-staining was performed as previously described (Nilsson et al., 2004). For each mouse, data were collected from three equally spaced coronal tissue sections for both dorsal hippocampus and overlying parietal cortex (Bregma -1.30 to -2.30 mm). The sections were examined with a Nikon Eclipse E1000 microscope using either 4X or 10X Plan Fluor objective lenses. A Retiga 1300 CCD (Qimaging) with a Qimaging RGB LCD-slider was used to capture images. For thioflavin S stained sections, a
Nikon BV-2B fluorescence filter cube was used. Customized software, written in Visual Basic 6.0 (Microsoft) utilizing Auto-Pro function calls (Image Pro Plus, Media Cybernetics) was used to segment and quantify images. Aβ deposition was calculated as percent area of interest (=\(\text{Area Stained}_{\text{tot}}/\text{Area Measured}_{\text{tot}}\)) from no less than seven microscope fields. Results were analyzed using a two-tailed, unpaired student’s t test with Welch’s correction.

RESULTS

Apolipoprotein E is transferred to the parabiosed apoE-KO mice via blood circulation. Endogenous apoE in the plasma of nontransgenic mice can be detected at up to 100-fold dilutions. Plasma apoE expression was examined with western blot analysis to verify that apoE from the apoE-positive parabiont was able to gain access to the apoE-knockout mouse. ApoE, in PS1\(^{+/-}\),APP\(^{+/-}\),apoE-KO mice that had been parabiosed to PS1\(^{+/-}\),APP\(^{+/-}\),apoE\(^{+/-}\) mice, was detected at a level corresponding to about 5% of that found in nontransgenic mice, as determined by densitometric analysis. In contrast no apoE was found in control non-parabiosed apoE-KO mice (Figure 1A). This finding could be interpreted as restricted exchange of plasma proteins, such as apoE, between the partners. Alternatively the low plasma apoE levels in the parabiosed apoE-knockout partner could be due to the need to quickly sequester lipoprotein particles from the blood stream in these mice. This would tend to increase turnover and lower steady state levels of apoE in the plasma. We decided to determine whether the parabiosis allowed free exchange of blood cells between parabiosed partners by
analyzing serum DNA. PCR analysis revealed that parabiosed apoE-KO mice contain almost equal amounts of apoE DNA in their blood as their apoE heterozygous parabiosis partners. This DNA analysis demonstrates a successful and efficient white blood cell transfer between the two parabionts (Figure 1B).

Apolipoprotein E transferred through parabiosis prevents hypercholesterolemia in the apoE-KO mice. Mice with targeted disruption of the mouse-apoE gene are known to develop severe hypercholesterolemia and atherosclerosis (Plump et al., 1992; Zhang et al., 1992). Accordingly, the PS1 +/-,APP+/+,apoE-KO mice used in these experiments show elevated levels of serum cholesterol as compared to nontransgenic, wild type, mice and PS1 +/-,APP+/+,apoE-KO mice with the endogenous murine apoE gene (Table 1). Nontransgenic mice had, on average, 105±6 mg/dl (n=19) of total cholesterol in their plasma, while PS1 +/-,APP+/+,apoE-KO transgenic mice with only one copy of apoE (PS1 +/-,APP+/+,apoE+/-) had levels averaging near 79±6 mg/dl (n=8). In contrast, the levels of total cholesterol were roughly four times higher in PS1 +/-,APP+/+,apoE-KO mice (392±121 mg/dl, n=3) and five times higher in apoE-KO mice lacking APP expression (501±39 mg/dl, n=13). In PS1 +/-,APP+/+,apoE-KO mice that had been parabiosed with a partner harboring one copy of the murine apoE gene, cholesterol levels in the apoE knock-out mice were rescued (125 mg/dl for a 5-month PS1 +/-,APP+/+,apoE-KO mouse and 87 mg/dl for a 7 month PS1 +/-,APP+/+,apoE-KO mouse, Table 1). The latter result was reproduced in all
parabiosed pairs whose Aβ-immunoreactive and amyloid deposition was quantified.

**Apolipoprotein E in the peripheral circulation alone does not reach the brain parenchyma and is unable to promote brain amyloid deposition.** Immunohistochemical analysis of Aβ-immunoreactive deposition using the 6E10 monoclonal anti-Aβ antibody on coronal brain sections from parabiosed partners and their respective non-parabiosed controls at seven months of age revealed only minor differences (Figure 2). In the cerebral cortex no statistically significant difference in Aβ burden between the parabiosed (1.55%±0.5; n=6) and the nonparabiosed (1.50%±0.1; n=3) PS1+/−,APP+/+,apoE-KO mice was found. This suggests that apoE present in the peripheral circulation is not sufficient to promote brain Aβ-immunoreactive deposition. There also was no statistically significant difference in Aβ burden in cerebral cortex between the parabiosed (2.2%±0.5; n=6) and the nonparabiosed (3.0%±0.7; n=3) PS1+/−,APP+/+,apoE+/- mice. However, a slightly greater amount (p=0.043) of Aβ deposition was found in the hippocampus, of the nonparabiosed PS1+/−,APP+/+,apoE+/- mice (5.5%±0.7; n=3) as compared to the parabiosed PS1+/−,APP+/+,apoE+/- mice (3.3%±0.7; n=6; Figure 2F). Again, there was no statistically significant difference in Aβ burden between parabiosed and control PS1+/−,APP+/+,apoE-KO mice in the hippocampus (Figure 2F). ApoE-immunoreactivity was detected in virtually all amyloid plaques of parabiosed PS1+/−,APP+/+,apoE+/- in the brain (Figure 3a,b) and in astrocytes of nontransgenic mice (Figure 3c,d). In contrast, parabiosed
PS1\textsuperscript{+/-},APP\textsuperscript{+/-},apoE-KO with an Aβ burden showed some apoE-immunoreactive staining in the choroid plexus, but none in the brain parenchyma (Figure 3e), suggesting that apoE in the peripheral circulation is essentially unable to cross the blood brain barrier to reach the brain parenchyma thereby limiting its impact on AD neuropathology. Control experiments verified that no apoE-immunostaining was present in choroid plexus of unoperated PS1\textsuperscript{+/-},APP\textsuperscript{+/-},apoE-KO mouse (Figure 3f).

**Amyloid deposition is reduced in parabiosed mice lacking ApoE.**

Previous studies have shown a striking difference in the structure of amyloid and Aβ immunoreactive deposition in PS1\textsuperscript{+/-},APP\textsuperscript{+/-} mice expressing endogenous apoE as compared to PS1\textsuperscript{+/-},APP\textsuperscript{+/-},apoE-KO mice (Costa et al., 2004). This structural difference is not associated with similar quantitative differences in the Aβ-immunoreactive burden. In fact, immunohistochemical detection of Aβ immunoreactive deposition showed equal amounts in PS1\textsuperscript{+/-},APP\textsuperscript{+/-} mice with and without apoE, albeit with a different anatomic distribution. In the present study, Thioflavin S staining was performed to compare the levels of mature, compact, amyloid between the parabiosed mice pairs and their respective non-parabiosed controls. No statistically significant difference in the %-area of Thioflavin S-staining was found between parabiosed PS1\textsuperscript{+/-},APP\textsuperscript{+/-},apoE\textsuperscript{+/-} mice and non-parabiosed controls of the same genotype (Figure 4a). Because the deposition of compact amyloid is a specific effect of apoE, in general, only minute amounts of compact amyloid were found in apoE\textsuperscript{-/-} mice. The few, detectable,
plaques were counted at 400x magnification. Remarkably, even though apoE knock-out mice develop only a very low amount of amyloid deposition, this number was further reduced in PS1\(^{+/−}\),APP\(^{+/−}\),apoE-KO mice that had been parabiosed. Both, the hippocampal and cortical regions showed a significant difference between parabiosed PS1\(^{+/−}\),APP\(^{+/−}\),apoE-KO mice (hippocampus, \(17.2\pm5.4; \ n=6\) and cortex, \(13.0\pm3.0; \ n=6\)) and non-parabiosed PS1\(^{+/−}\),APP\(^{+/−}\),apoE\(^{-/-}\) mice (hippocampus, \(28.3\pm3.0; \ n=3\); and cortex, \(20.7\pm5.2; \ n=3\)) in the number of plaques per brain section (Fig. 4b). Thus, the amyloid plaque loads were 53% and 112% higher for the hippocampus and the cerebral cortex respectively in non-parabiosed mice versus parabiosed apoE-KO mice. Similarly, the optical density of thioflavine S plaque deposition was also significantly higher in the apoE-KO control mice as compared to those that were not parabiosed (data not shown).

**DISCUSSION**

It has long been debated to what extent proteins derived from the bloodstream contribute to Alzheimer’s disease pathology. A hematogenous origin of amyloidogenic proteins in AD is supported by the fact that systemic amyloid disorders are driven by high concentrations of these proteins in the peripheral circulation. Furthermore, the frequent presence of cerebral amyloid angiopathy (CAA) in AD postmortem brain might affect BBB permeability and further increase influx and efflux of amyloidogenic proteins such as apoE and Aβ. It is known that Aβ in the blood stream can reach the brain and adhere to amyloid
deposits, as demonstrated through intra-arterial injection of labeled [125I]-Aß in squirrel monkeys (Ghilardi et al., 1996). Serum amyloid P component, which is derived from the peripheral circulation and believed to render amyloid plaques in AD brain less prone to clearance, is an example of a peripheral protein that is suspected to impact AD neuropathology (Pepys et al., 2002). Developing an AD therapy against a peripheral target has clear pharmacological and clinical advantages.

Here we show that parabiosis is a feasible approach to determine whether a given protein in the bloodstream is involved in AD amyloidosis. This procedure allows free flux of apoE between the parabiosed partners, as assessed by the level of blood cell DNA. Despite this fact, we observed rather low steady state levels of apoE (5% of nontransgenic mice) in parabiosed apoE knockout mice. We speculate that the low steady state levels of plasma apoE are caused by the rapid turnover of apoE and the high demand for apoE to clear lipoprotein particles in the apoE knockout mice. The very rapid clearance of plasma apoE ($t_{1/2}= 20$ min), through uptake by the liver (Vogel et al., 1985; Mahley et al., 1989), does support this hypothesis.

The low amounts of apoE transferred through parabiosis are however sufficient to normalize the hypercholesterolemia of apoE-knockout mice. Previous studies have also shown that a rather small amount of apoE is sufficient to restore essentially normal cholesterol metabolism in mice. Normalization of hypercholesterolemia of apoE knockout mice has been shown by supplementation with apoE through bone marrow transplantation (Linton et al.,
1995) or from hypomorphic apoE alleles (Raffai and Weisgraber, 2002). In fact only \( \approx 3\% \) of wild-type apoE levels in plasma appear to be required to maintain normal plasma cholesterol levels, and even less plasma apoE is needed to reduce atherosclerosis and lipid deposition in the aorta (Thorngate et al., 2000).

ApoE gene dosage also strongly determines the onset and extent of amyloid deposition in amyloid precursor protein transgenic mice. The absence of apoE anatomically redistributes Aß immunoreactivity in the brain and essentially blocks amyloid deposition (Bales et al., 1999; Holtzman et al., 2000; Nilsson et al., 2004). Experiments in bigenic mice containing both presenilin-1 and APP demonstrated that the main effect exerted by apoE is on the formation of senile (Thioflavine S positive) plaques (Costa et al., 2004). Thus, we considered the crossed PS1xAPP animal model to be an ideal animal model to evaluate the role of peripheral apoE on Aß and amyloid pathology. It is reasonable to anticipate that even a very small amount of apoE entering the CNS compartment would profoundly facilitate amyloid pathology in such a transgenic model. To our surprise we did not see any enhanced amyloid deposition in parabiosed PS1\(^{+/-}\),APP\(^{+/-}\),apoE\(^{-/-}\) mice. In fact, on the contrary, we found a reduced number of Thioflavine S-positive deposits in the parabiosed mice that were devoid of apoE. These results show that very little, if any, apoE synthesized in the periphery reaches the brain and that apoE derived from the periphery does not accelerate the amyloid pathology. We were only able to detect apoE-immunoreactivity in the choroid plexus of parabiosed PS1\(^{+/-}\),APP\(^{+/-}\),apoE\(^{-/-}\) mice, which is in
agreement with studies where $^{125}\text{I}$-labeled apoE has been infused via the carotid artery in guinea pigs (Martel et al., 1997).

Amyloid deposition, at least in APP transgenic mice, is a rather dynamic process whereby the size of an individual plaque can grow or shrink dramatically within months (Christie et al., 2001). Clearly different pools of Aβ in the body exist in a dynamic equilibrium prior to onset of amyloid deposition. There is, for example, a strong correlation between Aβ levels in the CSF, plasma, and brain of young PDAPP mice (DeMattos et al., 2002b), and this equilibrium can be quickly altered by injecting a high-affinity Aβ antibody that is able to sequester soluble Aβ in the periphery (DeMattos et al., 2001; DeMattos et al., 2002a). It is interesting to note that peripherally injected Aβ is rapidly cleared by the liver in nontransgenic mice, but not in apoE knockout mice (Hone et al., 2003). Thus, it could be that our finding of reduced amyloid deposition in parabiosed PS1$^{+/−}$,APP$^{+/+}$,apoE-KO mice is due to apoE in the periphery clearing Aβ. The peripheral clearance of Aβ would then shift the equilibrium towards efflux of Aβ out of the brain and thereby reduce/delay amyloid deposition in the brains of parabiosed PS1$^{+/−}$,APP$^{+/+}$,apoE-KO mice. ApoE could thereby act as a sink to drain Aβ out of the brain and into the peripheral circulation for subsequent clearance by the liver. The sink hypothesis of Aβ metabolism in APP transgenic mice was introduced as a model to explain the effects of passive vaccination against Aβ. Anti-Aβ, but also apoE, was found to sequester Aβ in a dialysis system (DeMattos et al., 2002a). A similar mechanism might also explain the tendency of reduced Aβ and amyloid burden in the brains of parabiosed PS1$^{+/−}$.
, APP<sup>+</sup>/-, apoE<sup>+</sup>/- mice as compared to their unoperated siblings. Thus, drainage of apoE in the blood of the parabiosed PS1<sup>+</sup>/-, APP<sup>+</sup>/-, apoE<sup>+</sup>/- mice, through the shared circulation with the apoE KO mice, could be compensated for by an efflux of apoE from the brain to the periphery in these mice. Such transfer of apoE would tend to transport Aβ out of the CNS compartment in the parabiosed PS1<sup>+</sup>/-, APP<sup>+</sup>/-, apoE<sup>+</sup>/- mice, and thereby slow amyloid deposition. Previous transgenic experiments do suggest that human apoE can shift the equilibrium of Aβ between different compartments and favor transport of Aβ out of the brain. The mechanisms of Aβ efflux from the brain might be both by bulk flow of ISF into the CSF, as well as across the blood brain barrier (Shibata et al., 2000; Silverberg et al., 2003). The low-density lipoprotein receptor-related protein, which is an apoE receptor, is a suspected receptor for Aβ efflux (Zlokovic, 2004). No apoE-Aβ complexes relevant to such transport mechanism could be shown in these experiments (Shibata et al., 2000), however other findings are strongly suggestive of apoE-Aβ complexes existing in the ISF and also regulating Aβ clearance. Microdialysis experiments have shown that the presence of apoE extends the half-life of Aβ in the ISF, which could be due to the existence of a reservoir of Aβ as apoE-Aβ complexes in the ISF (DeMattos et al., 2004). Furthermore, the human glial fibrillary acidic protein promoter GFAP-apoE<sub>3</sub> transgene, when crossed to an APP/apoE-KO genotype, lowered both soluble Aβ levels in the brain and the ratio of CSF/plasma Aβ42 in a dose dependant manner prior to onset of amyloid deposition (DeMattos, 2002). The latter findings strongly suggest that apoE exerts dual functions with respect to Aβ metabolism.
by both facilitating amyloid deposition, but also by clearing Aβ from the CNS to plasma through efflux mechanisms. In sum, our findings suggest that apoE in the peripheral circulation does not promote Aβ deposition in the brain, but clears Aβ by favoring its efflux from the brain into the periphery. These findings reinforce the idea that agents that are able to strongly sequester and clear soluble Aβ monomers in the periphery, such as Aβ antibodies (DeMattos et al., 2001) or other high-affinity Aβ binders (Matsuoka et al., 2003), could be promising novel therapeutics in AD.
Table 1 – Apolipoprotein E (ApoE) derived from the blood through parabiosis restores hypercholesterolemia in Aβ-producing ApoE Knockout Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>Total Cholesterol (mg/dl)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontransgenic</td>
<td>7 months</td>
<td>105 ± 6</td>
<td>15</td>
</tr>
<tr>
<td>PS1+/−APP+/−apoE+/−</td>
<td>7 months</td>
<td>73 ± 8</td>
<td>7</td>
</tr>
<tr>
<td>PS1+/−APP+/−apoE−/−</td>
<td>7 months</td>
<td>79 ± 6</td>
<td>8</td>
</tr>
<tr>
<td>PS1+/−APP/+apoE−/−</td>
<td>7 months</td>
<td>392 ± 121</td>
<td>3</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>7 months</td>
<td>501 ± 39</td>
<td>13</td>
</tr>
<tr>
<td><strong>Parabiosed pairs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a. PS1+/−APP−/−apoE+/−</td>
<td>5 months</td>
<td>118</td>
<td>1</td>
</tr>
<tr>
<td>1b. PS1+/−APP−/−apoE−/−</td>
<td>5 months</td>
<td>125</td>
<td>1</td>
</tr>
<tr>
<td>2a. PS1+/−APP+/−apoE−/−</td>
<td>7 months</td>
<td>87</td>
<td>1</td>
</tr>
<tr>
<td>2b. PS1+/−APP+/−apoE−/−</td>
<td>7 months</td>
<td>86</td>
<td>1</td>
</tr>
<tr>
<td>3a. PS1+/−APP+/−apoE−/−</td>
<td>7 months</td>
<td>106</td>
<td>1</td>
</tr>
<tr>
<td>3b. PS1+/−APP+/−apoE−/−</td>
<td>7 months</td>
<td>90</td>
<td>1</td>
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<td>4a. PS1+/−APP−/−apoE−/−</td>
<td>5 months</td>
<td>89</td>
<td>1</td>
</tr>
<tr>
<td>4b. PS1+/−APP−/−apoE−/−</td>
<td>5 months</td>
<td>90</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1 - Total plasma cholesterol measurements in randomly selected parabiosed pairs compared to control mice of various genotypes. Apolipoprotein E derived from peripheral circulation can restore the hypercholesterolemia typical of Apolipoprotein E knockout (PS1+/−,APP+/−,apoE−/−) mice.
Figure 1 - (A) Apolipoprotein E can be detected in a parabiosed apoE-knockout mouse (lane 7) but not in a apoE knockout mouse that has not been parabiosed (lane 5). Lane 6 shows parabiosed partner that is heterozygous for the apoE gene. Lanes 1-4 are ten-fold dilutions of plasma from a nontransgenic mouse. (B) Parabiosed PS1+/−,APP+/−,apoE−/− and PS1+/−, APP+/−,apoE+/− mice show an equal extent of murine apoE and neo DNA in blood cells indicating successful and efficient white blood cell transfer between parabiosed partners.
Figure 2. Aβ-immunoreactive (6E10) staining in parabiosed or control PS1+/−,APP+/−,apoE−/− and PS1+/−,APP+/−,apoE+/− mice. The staining in PS1+/−,APP+/−,apoE−/− mice, which is essentially only diffuse Aβ deposition, is extensive in the hippocampus (B and D). In contrast, PS1+/−,APP+/−,apoE+/− mice show an abundance of amyloid plaques (A and C). The Aβ immunostaining is essentially unaffected by parabiosis (n=6) (A and B) in both the cerebral cortex (E) and the hippocampus (F), as compared to unoperated mice (n=3) (C and D). No Aβ-immunoreactive staining was detected in nontransgenic mice (data not shown).
Fig. 3 – Apolipoprotein E immunostaining in parabiosed and control PS1+/+ ,APP+/+,apoE-/- and PS1+/-,APP+/+,apoE+/- mice. ApoE is detected in amyloid plaques in parabiosed PS1+/-,APP+/+,apoE+/- mice (A and B) and PS1+/- ,APP+/+,apoE+/- controls (not shown) as well in astrocytes of nontransgenic control animals (C and D). In PS1+/-,APP+/-,apoE-/- control mice, no apoE immunoreactivity is detected (F), but parabiosed mice of the same genotype present with a small amount of staining in choroid plexus, while none is present in the brain parenchyma (E).
**Fig. 4** – Thioflavine S tissue staining for the detection of filamentous Aβ. (A) There is no statistical significant difference in the area of thioflavine S staining, as determined by densitometric analysis, in PS1$^{+/+}$,APP$^{+/+}$,apoE$^{+/+}$ between parabiosed (n=6) and control (n=3) mice in either the hippocampus or cerebral cortex. (B) The number of plaques counted per brain section in parabiosed PS1$^{+/+}$,APP$^{+/+}$,apoE$^{-/-}$ (n=6) mice is significantly smaller than that of nonparabiosed control (n=3) mice of the same genotype in both hippocampus and cerebral cortex.
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Paper III:

Environmental Enrichment Protects Alzheimer’s Disease Mice from Cognitive Impairment through Reductions in Aβ Deposition and Beneficial Changes in Gene Expression and can be Mimicked by Inhibition of Phosphodiesterase 4 (PDE4)

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ABSTRACT

Although lifelong cognitive stimulation is associated with lower risk of Alzheimer’s disease (AD), prospective long-term human studies needed to prove that environmental enrichment (EE) protects against AD are impractical. We therefore sought to investigate the preventative potential of EE using mice expressing both human mutant presenilin-1 and amyloid precursor protein (PS1/PDAPP). At weaning, mice were placed into either an enriched or standard housing (SH) environment. Behavioral testing at 4½-6 months showed that EE-PS1/PDAPP mice outperformed mice in SH, and were behaviorally indistinguishable from NT mice. PS1/PDAPP mice given both EE and behavioral testing showed greater than 50% less brain β-amyloid (Aβ), but did not exhibit changes in dendritic morphology. Microarray analysis using hippocampal tissue revealed large EE-induced changes in the expression of genes/proteins related to memory, neuroprotection, and Aβ sequestration. Inhibition of one such protein, PDE4, a cAMP-phosphodiesterase, by Rolipram treatment for two weeks, mimicked the cognitive benefits of EE.
INTRODUCTION

Alzheimer’s disease (AD) is a common neurodegenerative disorder characterized by parenchymal β-amyloid deposition, neurofibrillary tangle formation, neuronal loss, and cognitive decline. A lifelong pattern of high mental activity (Wilson et al., 2002) and educational attainment (Stern et al., 1994) correlates with lower risk of AD and may be protective. Furthermore, high levels of linguistic ability early in life are associated with a reduced risk of the disease (Snowdon et al., 1996; Riley et al., 2005). These studies suggest that extra “cognitive reserve” developed throughout life may help buffer against the consequences of later dementia. However, despite some encouraging results (Loewenstein et al., 2004) the extent to which cognitive stimulation (i.e. environmental enrichment, EE) protects against AD remains difficult to assess in humans because: 1) retrospective studies cannot unequivocally isolate environmental enrichment from other factors affecting cognition over a lifetime, and 2) long-term intervention in humans is impractical. Furthermore, epidemiological human studies give no insights about the potential mechanisms by which EE may protect against AD.

The key question about whether EE intervention can protect against AD pathology, or its associated mental decline, was addressed in the present study using a transgenic mouse model of the disease. The mice chosen express both the human mutant amyloid precursor protein (hAPPV717F) and mutant Presenilin 1 (hPS1M146L) genes, which result in moderate brain β-amyloid plaque deposition and significant behavioral impairment by 5-6 months of age. At weaning, mice
were placed into either standard housing or an enriched environment, and were behaviorally tested between 4½-6 months of age. It is known from previous studies that non-transgenic rodents subjected to cognitive stimulation (i.e. EE) perform better in water mazes, exhibit increased dendritic branching and dendritic spine formation (Globus et al., 1973; Comery et al., 1995; Turner et al., 2003), increased synaptogenesis (Ramirez-Amaya et al., 1999), and increased neuronal plasticity-related gene expression (Pinaud et al., 2001), while exhibiting decreased levels of apoptotic cell death (Young et al., 1999).

Our previous experiments determined that an EE paradigm used therapeutically in aged AD transgenic mice with severe Aβ plaque deposition provided cognitive benefits without affecting the amyloid plaque burden (Arendash et al., 2004a). Here we sought to determine the extent to which preemptive EE protects memory, impacts Aβ deposition, and affects dendritic morphology. To characterize these changes at the molecular level, hippocampal gene expression was analyzed using a whole mouse genome microarray.

We found that PS1/PDAPP mice raised in EE outperformed those raised in SH across a variety of behavioral/memory tasks, in which they were statistically indistinguishable from non-transgenic (NT) mice. Interestingly, brain Aβ deposition in Tg+ mice was not affected by EE alone, but in combination with behavioral testing (an additional enriching experience), EE resulted in large reductions in brain Aβ deposition. Although the extent of dendritic branching and dendritic spine numbers in cortex/hippocampus were unchanged by enrichment, Tg+ mice given EE did exhibit increased expression of multiple memory-related
genes, a number of neuroprotective genes involved in anti-apoptotic BAD phosphorylation, as well as increased expression of transthyretin (a known Aß sequestering molecule).

RESULTS

Behavioral Testing. Cognitive performance for both standard-housed (SH) and environmentally-enriched (EE) mice was determined between 4½-6 months of age through five cognitive-based tasks (Radial Arm Water Maze, Platform Recognition, Morris maze, Y-maze, and Circular Platform) – each measuring discreet cognitive domains. Although we found significant effects of EE on multiple tasks and measures therein, two of the tasks were particularly important. Mice were tested in the radial arm water maze (RAWM), a task designed to assay working memory. Over days 7-9 of testing, transgenic mice raised in environmental enrichment (Tg+/EE) had significantly lower escape latencies than their SH counterparts for the last acquisition trial (T4; p<0.001) and also the memory retention trial (T5; P<0.00001) (Figure 1A). Interestingly, EE also improved the overall RAWM performance of non-transgenic (NT) mice, as evidenced by their significantly lower escape latencies on T4 compared to their SH counterparts (p<0.02). The only other task in which EE improved upon the already fine performance of NT/SH mice was the circular platform task of spatial learning, wherein NT/EE mice had significantly shorter escape latencies overall compared to NT/SH mice (data not shown). Since mice raised in an enriched environment had larger cages and access to mazes and running wheels, their
potential athletic ability could have been superior to mice raised in standard housing. This was not the case, however, since analysis of the number of seconds taken per arm choice revealed no statistical differences in swim speed between SH and EE groups for either NT or Tg+ mice. Furthermore, Tg+/EE mice made significantly fewer RAWM errors than Tg+/SH mice, and again were not statistically different from NT mice (SH or EE) (Data not shown). In the platform recognition task, which requires mice to use a search/identification strategy rather than the spatial strategy of Morris maze and RAWM, similar behavioral benefit was observed for Tg+ mice. Tg+/EE mice had significantly lower escape latencies than Tg+/SH mice over all days of testing (p<0.005), as well as on all but the initial day of testing (P<0.01) (Figure 1B). Furthermore, Tg+/EE mice were not statistically significantly different from both NT groups in this task.

To determine the effects of EE on overall cognitive performance, we performed discriminant function analysis (DFA) across 8 cognitive measures taken from our test battery that represent multiple cognitive domains (working memory, reference learning/memory, identification/recognition). DFA determines whether groups can be distinguished from one another based on their overall behavioral performance across multiple cognitive measures. As shown in Figure 1C, DFA was easily able to distinguish the impaired overall cognitive performance of Tg+/SH mice from the much better performance of both Tg+/EE mice and NT/SH controls (p<0.001 for both comparisons). The latter two groups
could not be distinguished from one another by DFA. Thus, the effect of EE in Tg+ mice was global in spanning multiple cognitive domains.

*Alzheimer’s Disease Pathology.* To study the effect of environmental enrichment on AD pathology, overall Aβ immunoreactivity and mature β-amyloid (Aβ) deposition were measured. In order to eliminate behavioral testing as an interfering form of cognitive stimulation that could alter brain pathology, we raised two cohorts of mice in both SH and EE conditions through 6 months of age. Aβ immunohistochemistry was measured in both cohorts, but only one cohort underwent behavioral testing between 4½-6 months of age. Total Aβ immunoreactivity, using the 6E10 monoclonal antibody, revealed that there was no difference in total Aβ load between Tg+/EE and Tg+/SH mice in either cerebral cortex or hippocampus for the “non-behaviorally tested” group (Figure 2A,B). Aβ 1-42 enzyme linked immunosorbent assay (ELISA) further verified that the Aβ levels (either soluble or insoluble) were not statistically significantly different between non-behaviorally tested SH vs. EE transgenic groups (Data not shown). Since some pathological chaperones, such as apolipoprotein E, exert their contributory effect on AD pathology by promoting compact plaque formation rather than overall Aβ levels (Costa et al., 2004), it is not unreasonable to expect EE to affect a change in only mature (compact) Aβ deposition. We find however, that mature Aβ plaque loads (as measured by thioflavine S staining) were also not significantly different between non-behaviorally tested EE and SH transgenic groups in either cerebral cortex or hippocampus (Figure 2C,D).
Importantly, we did observe that behaviorally-tested EE mice had significantly lower Aβ deposition than those mice that underwent only EE. This was true for both total and compact Aβ deposition and for both cortex and hippocampus (Figure 2A-D). For behaviorally-tested Tg+/EE mice, their 57.6% and 69.0% decreases in compact Aβ deposition in cortex and hippocampus, respectively, compared to non-behaviorally tested Tg+EE mice are particularly noteworthy. Our Aβ deposition results from both cohorts thus indicate that EE or behavioral testing alone is not sufficient to alter Aβ pathology, but when they are combined there is a large statistically significant effect provided by both of these forms of cognitive stimulation in concert with one another. Although Aβ deposition was reduced by a combination of EE and cognitive testing, there were no statistically significant correlations in the Tg+/EE mice between such AD pathology and any behavioral measure (data not shown), indicating that the mice had experienced EE-induced cognitive improvement that was at least partially independent of any reduction in Aβ pathology.

The extent and distribution of dendritic arborization in Layer V cortical neurons was measured in behaviorally tested mice through Golgi staining. Using the Sholl method of quantification, there was no observed difference in dendritic branching between transgenic EE and SH groups (Figure 2E). However, both Tg+/SH and Tg+/EE groups had significantly reduced dendritic arborization compared to non-transgenic single-housed (NT/SH) mice (p<0.001), indicating that EE could not increase dendritic arborization in Tg+ mice to the level of NT controls. Additional evidence that EE did not affect dendritic morphology is
provided by analysis of dendritic spine counts in both cortex and hippocampus of Tg+ mice (Figure 2F). No EE-induced increase in dendritic spines was evident in Tg+ mice for either cortical Layer V pyramidal neurons or for hippocampal CA1 neurons. Thus, Tg+/EE mice performed identically to NT/SH controls despite having reduced dendritic branching and synaptic spine counts that were identical to Tg+/SH mice. Evidentially, EE must induce changes in the brains of PS1/PDAPP Tg+ mice that allow them to compensate for any cognitive damage that may be imparted by the dendritic branching deficits and the Aβ pathology typical of these transgenic mouse lines.

Microarray. To begin to investigate the mechanism(s) by which EE protects against AD-induced cognitive decline, we used microarray analysis to examine gene expression changes in the hippocampus that might contribute to the aforementioned memory enhancement. Non-behaviorally tested EE (n=4) and SH (n=4) transgenic mice were analyzed using an Affymetrix GeneChip designed for the mouse genome. In all, over 120 genes showed statistically significant expression changes in response to EE. Of these genes, roughly 70 were changed by a factor of 2.0 or more. Many of the more robust changes occurred in genes already implicated in one or more aspects of memory or AD (TABLE 1). For example, insulin-like growth factor (IGF-2), which has also been shown to play a neuroprotective role against Aβ (Dore et al., 1997) was up-regulated in EE mice by 2.5 fold. This gene, along with Insulin-like growth factor binding protein-2 (IGFBP-2) (up-regulated 3.0 fold in EE), and the prolactin receptor (PRLR) (up-regulated 21.1 fold in EE) have all been implicated as
neuroprotective molecules acting in concert upstream of the Akt and Erk1/2 pathways that ultimately lead to BAD phosphorylation, an event shown to be both neuroprotective and anti-apoptotic (Bonni et al., 1999; Thompson and Thompson, 2004). A 10-fold increase in transthyretin (TTR), an Aβ binding protein shown to sequester Aβ and inhibit amyloid formation, was also observed in the EE mice. These microarray results were verified by quantitative real-time PCR (Table 2). Two genes involved in memory improvement, phospholipase A2 and cytochrome C oxidase were up-regulated in EE mice by 6.7 and 3.1 fold respectively. Furthermore, phosphodiesterase 4B was down-regulated 2.2 fold and the cholecystokinin B receptor was down-regulated 3.2 fold. Both of these genes, when experimentally inhibited, are known to improve memory (Lemaire et al., 1994; Zhang et al., 2000; Bourtchouladze et al., 2003; Zhang et al., 2004).

Calcineurin knockout mice exhibit increased tau phosphorylation, abnormal neuronal cytoskeleton, and cognitive deficits, making the increased expression of this gene by EE also interesting (Kayyali et al., 1997; Zeng et al., 2001).

An examination of the functions of the proteins whose expression is changed by EE reveals that many participate in a signaling pathway that can confer neuroprotection in several experimental systems (See Figure 3). The benefit of deriving such a neuroprotective pathway from the microarray results is that potential targets of therapeutic intervention can be identified. For example, if the down-regulated genes identified by microarray analysis are responsible for the improvement in cognitive function, we would predict that pharmacological
inhibition of the products of one or more of these genes should have similar beneficial effects.

To test this hypothesis, we attempted to pharmacologically intervene in the pathway shown in Figure 3 in hopes of emulating the beneficial memory effects of EE. In our initial experiments, we chose to inhibit PDE4 since it is down-regulated by EE, and there exists a highly specific PDE4 inhibitor, Rolipram, which has already been found to alleviate memory deficits in rodent models.

To test this possibility, we initiated experiments in which Rolipram, a specific inhibitor of cAMP phosphodiesterase 4 (PDE4) (Figure 3, Purple), has been injected over a two-week period into. Cognitively deficient PS1/PDAPP mice were injected with Rolipram over a two-week period, and then subjected to 6 days of RAWM testing. The injected mice had significantly lower escape latencies than those mice receiving vehicle alone for the last acquisition trial (T4; p<0.05) and also the memory retention trial (T5; P<0.05), indicating that Rolipram treatment effectively mimics the cognitive benefits of environmental enrichment (Figure 4). Indeed, a comparison of the results shown in Figures 1 and 4 reveals that only two weeks of Rolipram treatment is able to restore normal function to severely impaired AD mice.
DISCUSSION

Our previous studies, and studies from others, have shown that mice that overexpress a mutant human APP gene linked to AD are significantly impaired in a number behavioral tasks (Arendash et al., 2001b; Arendash et al., 2004b; Nilsson et al., 2004; Jensen et al., 2005; Ognibene et al., 2005). Here we show that doubly transgenic PS1/PDAPP Alzheimer’s mice raised in a mentally-stimulating environment were “protected” from otherwise certain cognitive impairment, as evidenced by their superior performance in a variety of behavioral tasks compared to transgenic mice raised in standard housing conditions. In fact, long-term EE produced transgenic mice whose “overall” performance across multiple cognitive measures was indistinguishable from non-transgenic mice. Thus, the cognitive benefits of EE were global in nature, affecting multiple cognitive domains. Only by adding subsequent behavioral-testing to EE were profound reductions in brain Aß deposition achieved, suggesting an Aß limiting/sequestering action of EE. Moreover, EE produced beneficial changes in expression of multiple genes involved with Aß sequestration, memory, and neuroprotection. Our results not only provide the first unequivocal evidence that EE can protect against Alzheimer’s-like cognitive impairment, but also provide insight into the multi-faceted mechanisms involved in that protection.

Our previous work has shown that the levels of amyloid deposition in AD (i.e. APP) transgenic mice correlate with extent of cognitive impairment (Arendash et al., 2004b; Leighty et al., 2004; Nilsson et al., 2004). For example, when different Aß promoting genes, namely Apolipoprotein E (apoE) and α-1
antichymotrypsin (ACT) are introduced into a mouse with an APP, apoE-KO, ACT<sup>-/-</sup> background, the severity of amyloid deposition depends on the presence of these promoters, and, more importantly, directly correlates with the levels of cognitive decline (Nilsson et al., 2004). In our current study, we determined that neither EE nor the 6-week behavioral test period alone was sufficient to affect brain Aβ deposition. However, combining both these forms of cognitive stimulation (i.e. long-term EE and intense behavioral testing during the final 6 weeks) resulted in dramatic (up to 69%) reductions in both total and mature (compact) Aβ deposition. Since it is unlikely that Aβ deposition during the final 6 weeks of EE would have increased by 2-3 fold in non-tested mice, it is reasonable to conclude that the combination EE experience actually resulted in removal/sequestration of brain Aβ from both diffuse and compact deposits. We hypothesize that long-term EE primed molecular/genetic pathways in the brain (see below) for complementary and/or synergistic actions provided by behavioral testing, resulting in profound reductions in brain Aβ deposition. These results are in direct conflict with another recent study (Jankowsky et al., 2003) that focused on Aβ deposition changes in another transgenic mouse model of AD (APPsw/PS1dE9), and reported that EE promotes Aβ burden and deposition. Since Jankowsky et al. (2003) did not behaviorally test their animals and used an atypical EE methodology, a direct comparison between the two studies is not practical. In contrast to our EE methodology of maintaining the same stable group of animals in any given EE cage, Jankowsky et al. (2003) continually introduced young animals and removed older animals from their EE cages. This
likely added a continual source of stress, which could have contributed to their observed increase in Aβ burden.

EE was not able to protect against the dendritic branching defects observed in PS1/PDAPP mice despite cognitive benefits observed in the same animals. Analysis of Golgi-stained Layer V pyramidal cells in cortex revealed Tg+/SH and Tg+/EE were nearly identical in their extent of dendritic branching, with both groups having significantly less dendritic branching than NT/SH controls. Dendritic spine counts in both cortex and hippocampus further underscored the lack of EE effects on dendritic morphology in Tg+ mice. By contrast, prior studies involving normal rodents have provided evidence for EE-induced changes in dendritic/synaptic morphology. In those studies, EE induced: 1) greater synaptophysin levels (an index of synaptic surface area, but not number of synapses) in hippocampus and neocortex (Frick and Fernandez, 2003), and 2) an increase in Golgi-stained dendritic branches and dendritic spines in neocortex (Comery et al., 1995; Turner et al., 2003). The inability of EE in the present study to increase dendritic branching or dendritic spines in Alzheimer’s Tg+ mice suggests that EE’s protective effect in those same mice does not primarily involve changes in dendritic/synaptic morphology. Nonetheless, subtle morphologic changes may have occurred or neuronal populations, other than those presently evaluated, may have been effected by EE.

To study possible biochemical/genetic changes that might be independent of, or additive to, Aβ reducing/sequestering effects of EE in transgenic mice, we
performed microarray analysis using an Affymetrix 430 2.0 mouse GeneChip® which probes over 39,000 known unique transcripts of the mouse genome. Hippocampal RNA from eight non-behaviorally tested transgenic mice (EE n=4, SH n=4) was pooled in pairs, and used to probe four microarrays. The resultant expression should reflect the effects of environmental enrichment and identify genes that are either up- or down-regulated by EE and that may underlie the cognitive protection evident in behaviorally-tested transgenic mice. Of the approximately 70 known genes that were either up or down regulated by at least 2-fold due to EE, we focused our attention on those that were most likely to impact AD and/or cognitive function.

A number of genes known to enhance or inhibit memory were up-regulated or down-regulated, respectively. In particular, phospholipase A2 (PLA2), a protein involved in the inflammatory response, membrane remodeling, and phospholipid metabolism (Sun et al., 2004) and a proposed target for the treatment of inflammatory brain related disorders (Strokin et al., 2004) was up-regulated approximately 6.7-fold in AD mice raised in EE conditions. Expression of a mutated PLA2 homolog in drosophila introduces memory defects (Chiang et al., 2004), as does treatment of mice with bromoenol lactone, a potent PLA2 inhibitor (Fujita et al., 2000). Furthermore, reduced PLA2 expression has been reported in human AD brains (Gattaz et al., 2004). We also observed a 3.1 fold increase in cytochrome C oxidase (CO) message. Often used as a metabolic marker for neuronal activity, CO, an important enzyme involved in mitochondrial electron transport, is reportedly lowered in AD patients (Mutisya et al., 1994). In
addition, mice trained in spatial working memory tasks show increased CO activity in the mammillary bodies, a region of the brain closely related to the hippocampus and memory formation. It is also interesting that transthyretin was up-regulated between 6- and 10-fold in transgenic mice raised in EE. TTR is a plasma and CSF protein that is known to bind to (Tsuzuki et al., 1997; Tsuzuki et al., 2000) and sequester (Schwarzman et al., 1994) Aß, thus inhibiting plaque formation. It is hypothesized that high levels of kidney TTR prevent Aß plaque formation despite the fact that kidneys have the highest levels of Aß 1-40 and 1-42 in the body, after the brain (Tanzi et al., 1987; Tsuzuki et al., 2000). In vitro work has shown that TTR co-localizes with amyloid plaques, and in vivo inhibition of TTR increases Aß deposition (Stein et al., 2004). Previous microarray experiments have shown an increase in TTR in an APPsw mouse model of AD as compared to NT mice, and this is hypothesized to be partly responsible for the lack of neurodegeneration typically characteristic of AD mouse models (Stein and Johnson, 2002). Similarly, we show an increase in TTR expression due to EE. Perhaps the reduced levels of TTR in human AD patients (Serot et al., 1997) contribute to their neurodegeneration and may therefore be alleviated by cognitive stimulation. Despite TTR’s known Aß–sequestering activity, the 6-10x expression increase in non-behaviorally tested transgenics was unable to limit/decrease their brain amyloid deposition.

Converse to EE-induced gene up-regulation, two genes associated with memory repression - phosphodiesterase 4B and cholecystokinin B - were down-regulated by 2.2- and 3.2-fold, respectively, in transgenic mice raised in EE.
Inhibiting PDE4 in a mouse model of Rubinstein-Taybi syndrome, a disease characterized by mental retardation, ameliorates the long-term memory deficits typical of this disorder. Similarly, a recent study has shown that specifically inhibiting PDE4 in an APPsw/PS mouse model of AD ameliorates both deficits in long-term potentiation and memory (Gong et al., 2004). Likewise, CCK-B receptor antagonists are shown to enhance memory in rats (Lemaire et al., 1994). In sum, the data show that environmental enrichment increases relative levels of memory enhancing transcripts while lowering the levels of multiple known inhibitors of memory. Together, these genetic changes may help explain the cognitive enhancement induced by environmental enrichment.

The change in PDE4 suggests that the ERK/MAPK signaling cascade, known to be essential for memory consolidation (Impey et al., 1999; Schafe et al., 2000) may contribute to the observed EE effect. MEK inhibition-induced memory impairment in a mouse model can be reversed by treatment with a PDE4 inhibitor (Zhang et al., 2004), indicating the potential involvement of PDE4 in MEK/ERK signaling. In addition, inhibition of PDE4 has been shown to increase cAMP concentrations (Barad et al., 1998), and the cAMP/PKA pathway is also known to modulate memory formation (Frey et al., 1993). Environmental enrichment significantly lowers PDE4 levels, and therefore likely promotes both the ERK/MAPK and cAMP/PKA pathways. We noticed a pattern emerge, in that many of the genes changed on the microarray (i.e. PDE4, PRLR, IGF-2, and IGFBP2) fit into a few related metabolic pathways with a common end-point (Figure 3). Interestingly, these kinase cascade pathways culminate in the
phosphorylation of BAD, an event known to suppress apoptosis and promote cell survival. The neuroprotective nature of BAD is evidenced by mouse experiments in which high dietary levels of docosahexanoic acid (DHA; an essential omega-3-fatty acid whose consumption is associated with a reduced risk of AD) increased PI3-K, BAD phosphorylation, and provided some protection against behavioral deficits found in mice fed a DHA-deficient diet (Calon et al., 2004). Activation of PI3-K is an IRS-1 mediated event. We have found that the PRLR, IGF-2, and IGFBP-2 are all up-regulated in the EE mice. IGF-2 and IGFBP-2 bind to the IGF-1 receptor and, together with the action of the PRLR, are able to activate IRS-1, leading to AKT-mediated BAD phosphorylation. Our findings that PS1/PDAPP mice raised in EE have altered expression of PDE4, PRLR, IGF-2, and IGFBP-2 suggests that these proteins and the pathway linking them together (See Figure 3) may constitute a neuroprotective mechanism that is inducible by environmental enrichment.

Finally, we determined that the combination of two forms of mental stimulation (long-term EE and intense behavioral testing) resulted in dramatic reductions in both total and mature (compact) Aβ deposition occurring over the 6 week period of behavioral testing. Since neither EE nor behavioral testing alone was sufficient to decrease Aβ deposition, the EE-induced changes in gene expression depicted in Figure 3 may have primed the brain for “behavioral testing-induced” synergistic and/or complementary effects that resulted in reduced brain Aβ and increases cognitive function. We propose that both Aβ-lowering and favorable gene expression pathways are involved in the cognitive
protection afforded by EE. Thus, therapeutics designed to impact these pathways may provide cognitive protection against, or viable treatment for, Alzheimer’s disease. This prediction has been strikingly supported by the finding that pharmacological inhibition of PDE4 by Rolipram can mimic the cognitive benefits of EE.

**EXPERIMENTAL PROCEDURES**

*Construction of transgenic mice.* All procedures involving experimentation on animal subjects were done in accord with the guidelines set forth by the University of South Florida’s Institutional Animal Care and Use Committee. Heterozygous PDGF-hAPP(V717F) mice [Swiss-Webster x C57BL/6] were crossed with PDGF-hPS1(M146L) heterozygotes [Swiss-Webster x C57BL/6] to generate mice with an APP⁺/⁻, PS1⁺/⁻ genotype. All offspring were screened by PCR to identify the PDGF-hAPP (Games et al., 1995) and the PDGF-hPS1 gene (Duff et al., 1996).

*Environmental Enrichment.* At weaning, mice were place into two groups that were exposed to standard single housing (PS1/PDAPP n=32; NT=23) or environmentally enriched housing (PS1/PDAPP n=27; NT n=19) for 4½-5½ months. All SH animals were housed in shoe box cages with static microisolator tops under climate-controlled conditions on a 12 hour light/12 hour dark cycle, fed Harlan Teklad Global Diet #2018 and provided with tap water ad libitum. Although some enrichment studies in normal rodents have involved socially-housed animals as the standard housing control, we have found that both single- and socially-
housed double transgenic mice are equally impaired across all of our cognitive-based tasks vs. NT mice, and have elected to utilize standard single housing as our housing control for this study. Enriched mice had the same diet and light cycle as SH mice, but were socially housed by sex (6-8 to a cage) in cages containing toys, tunnels, running wheels, etc. (Arendash et al., 2004a). In addition to their enriched housing, EE mice were placed in novel complex environments 3 times weekly for several hours over the enrichment period.

**Behavioral Testing.** Beginning at 4.5 to 6 months of age, while continuing in either EE or SH housing, approximately half of the mice (n=57) were tested in five cognitive-based tasks as previously described in detail (Arendash et al., 2001a): Y-maze, Morris water maze, circular platform, platform recognition, and radial arm water maze (RAWM) in that order. All testing was conducted during the light phase. Because this report only presents results from platform recognition and RAWM in detail, the methodology of those tasks is described. The platform recognition task measures the ability to search for and identify/recognize a variably-placed elevated platform. It requires animals to ignore the spatial cues present around a 100 cm circular pool, which was the same pool used in earlier Morris water maze testing. Mice were given four successive trials/day over a 4-day period. Latencies to find an elevated platform (9 cm dia.), bearing a prominent cone-shaped Styrofoam ensign on a wire pole, were determined. For each trial (60 sec. max.), animals were placed into the pool at the same location and the platform was moved to a different one of four possible locations.
For the RAWM task of working memory, an aluminum insert was placed into the above pool to create 6 radially-distributed swim arms emanating from a central circular swim area. The latency to locate which one of the 6 swim arms contained a submerged escape platform (9 cm dia.) was determined for 5 trials/day over 9 days of testing. There was a 30 min. time delay between the 4th trial (T4; final acquisition trial) and 5th trial (T5; memory retention trial). The platform location was changed daily to a different arm, with different start arms for each of the 5 trials semi-randomly selected from the remaining 5 swim arms. During each trial (60 sec. max.), the mouse was returned to that trial’s start arm upon swimming into an incorrect arm. If the mouse did not find the platform within a 60s trial, it was guided to the platform for the 30s stay. Escape latencies during Trials 4 and 5 are both considered indices of working memory.

For both of the above tasks, statistical analysis involved either one-way or two-way repeated measure ANOVAs, followed by post-hoc planned comparisons between groups using the Fisher LSD test. Discriminant Function Analysis (DFA) was performed across all 8 cognitive measures, from multiple tasks, that loaded together as the primary cognitive factor in Factor Analysis. DFA was performed using the DISCRIM subroutine of the Systat software package. Following completion of the behavioral testing at 6 to 7.5 months of age, mice were then anesthetized with Nembutal (0.1mg/g). The animals were intracardially perfused with 0.9% NaCl (25ml), and their brains were removed.

**Immunohistochemical Procedures.** Brains were immersion fixed for 24 hours in 4% paraformaldehyde in 1x Sorenson’s phosphate buffer, followed by
cryoprotection via three sequential overnight sucrose immersions, ending in 30% sucrose. 25μm sections were cut using a sliding microtome (Spencer Lens Co.) and a freezing stage (Physitemp). The sections were mounted and processed through antigen retrieval in prewarmed 25mM citrate buffer (pH 7.3) at +82°C for 5 min. and further processed as previously described (Nilsson et al., 2004). The sections were incubated with primary antibodies against Aβ (6E10, dil. 1:5000) overnight at +4°C. Secondary antibody was anti-mouse IgG developed with NovaRED substrate kit (Vector). Thioflavine S staining was accomplished using a 5 min. incubation in 1% Thioflavine S followed by 5 min. differentiation in 70% ethanol.

Image Analysis. Data were collected from three equally spaced coronal tissue sections for both dorsal hippocampus and overlying parietal cortex (Bregma -1.30 to -2.30 mm) for each mouse. The sections were examined with a Nikon Eclipse E1000 microscope using either 4X or 10X Plan Fluor objective lenses. A Retiga 1300 CCD (QImaging) with a QImaging RGB LCD-slider was used to capture images. For thioflavine S, a Nikon BV-2B fluorescence filter cube was utilized. Customized software written in Visual Basic 6.0 (Microsoft) utilizing Auto-Pro function calls (Image Pro Plus, Media Cybernetics) was used to segment and quantify images. Aβ deposition was calculated as percent area of interest (=Area Stained\text{tot}/Area Measured\text{tot}). Results were analyzed using a two-tailed, unpaired student’s t test with Welch’s correction.

Golgi Staining. Coronal brain slices 2-3 mm thick were stained en bloc using the Rapid Golgi modification of (Valverde, 1993). Tissue blocks were
initially immersed in a mixture of osmium tetroxide and potassium dichromate for 5-6 days, then rinsed and blotted, and then subsequently immersed in a silver nitrate solution for 36-48 hours. The blocks were then dehydrated and embedded in nitrocellulose. The stained blocks were then cut at 120 µm on a sliding microtome, cleared in alpha-terpineol, rinsed in xylene, and coverslipped under Permount. All slides were coded for subsequent quantitative analyses. For dendritic branching analysis, randomly selected layer V pyramids of the parietal cortex (6 neurons per brain) had camera lucida drawings prepared of their basilar dendritic arbors. These were subsequently quantified for the amount and distribution of their dendritic domains using the Sholl analysis (Method of Concentric Circles; (Sholl, 1953)). Pair-wise statistical comparisons of the Sholl profiles utilized the repeated measures ANOVA with the post-hoc tuckey test. For dendritic spine analysis, from the coded slides, spines were counted directly on the Zeiss research microscope using 100x long-working distance oil-immersion objectives. Spines were counted from two neuronal populations: along 30 micron terminal tip segments of the basilar tree of the layer V pyramids, and along 30 micron long terminal tip segments of the basilar tree of CA1 pyramids of the hippocampus. Spines were counted on 3-5 terminal tip segments from each of 6 neurons per brain in each brain region. Only flanking dendritic spines were counted, e.g., spines which were not obscured by the dendritic branch itself. Statistical analysis of the spine counts was carried out using ANOVA with a post-hoc tuckey test.
**ELISA Analysis of Aβ levels.** Protein extracts from the above eight hippocampi were used for ELISA and western blot analysis. ELISA was performed as previously described (Dodart et al., 2002), with the modification that PBS rather than TBS was used to extract soluble Aβ.

**Microarray.** Both experimental groups, EE and SH, were represented by two microarrays each, and each microarray had RNA pooled from two mice (n=8 total). Immediately following saline perfusion, brains were micro-dissected and the hippocampus isolated and frozen in liquid nitrogen. Total RNA was isolated from each hippocampus using an RNeasy kit (Qiagen). The microarray used, GeneChip 430 2.0 Mouse Expression Set, contains over 45,000 probe sets designed from GenBank, dbEST, and RefSeq sequences clustered based on build 107 of the UniGene database. The clusters were further refined by comparison to the publicly available draft assembly of the mouse genome. An estimated 39,000 distinct transcripts are detected including over 34,000 well substantiated mouse genes. Five micrograms of total RNA pooled from each brain sample served as the mRNA source for microarray analysis. The poly(A) RNA was specifically converted to cDNA and then amplified and labeled with biotin following a previously described procedure (Van Gelder et al., 1990). Hybridization with the biotin labeled RNA, staining, and scanning of the chips followed the proscribed procedure outlined in the Affymetrix technical manual and has been previously described (Warrington et al., 2000). Scanned output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix Microarray 5.1 software. Signal intensity was scaled to an average
intensity of 500 prior to comparison analysis. Using the default settings, MAS 5.1 software identifies the increased and decreased genes between any two samples with a statistical algorithm that assesses the behavior of 16 different oligonucleotide probes designed to detect the same gene (Liu et al., 2002). Probe sets that yielded a change $p$-value less than 0.0045 were identified as changed (increased or decreased) and those that yielded a $p$-value between 0.0045 and 0.006 were identified as marginally changed. A gene was identified as consistently changed if it was identified as changed in all replicate experiments.

**Rolipram Administration.** Twelve-month-old PS1/PDAPP/APOE+/- were subcutaneously injected daily with either 0.03 mg/kg Rolipram (n=5) or vehicle only (n=4) for two weeks. After one day without treatment, all mice were behaviorally tested for days in the radial arm water maze.

**Real-Time quantitative PCR.** RNA samples were treated with DNase (DNA-free, Ambion), to reduce the chance of genomic DNA contamination. cDNA synthesis was achieved using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen) according to the manufacturer’s instructions. Primer pairs for real-time PCR were designed using the web-based applications Primer3 (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the Oligo Toolkit (Operon Technologies). The following primer pairs were used: Prolactin Receptor primer 1-ATCATTGTGGCCGTTCTCTC, Primer 2-TGGAAAGATGCAGGTCATCA; Transthyretin, primer 1-ATGGCTTCCCTTCGACTCTT, primer 2-GCATCCAGGACTTGGACC; Insulin
like growth factor 2, primer 1-CCCTCAGCAAGTGCTAAAG, primer 2-
TTAGGGTGCGCTCGAGATGTT; Insulin-like growth factor binding protein 2,
primer 1-GCCGGTACACCTTAAGCAG, primer 2-
GTGTTGGGTTTCACACACC; 18s RNA, primer 1-
GTAACCCGTTGAAACCCATT, primer 2- CCATCCAATCGGTAGTAGCG. 25μl
PCR reactions were prepared using iQ SYBR Green Supermix (Bio-Rad), and
repeated in triplicate. A two-step PCR reaction was run using an iCycler iQ Real-
Time PCR detection system (Bio-Rad) as follows: 1 cycle of 95°C for 3 min.
followed by 40 cycles of 95°C for 30 s. and 60°C for 30 s. All primer sets yielded
a single fluorescence peak via melt curve analysis, indicating a lack of mis-
priming or primer-dimer artifacts. Fold-change values were calculated for all
experimental wells using the comparative C_{T} method (2^{-ΔΔC_{T}}) (Livak, 1997).
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Figure 1. Environmental enrichment (EE) protects PS1/PDAPP mice against cognitive impairment across multiple behavioral tasks administered between 4½-6 months of age.

(A) In the radial arm water maze task of working memory, SH Tg+ mice given showed impaired performance across days 7-9 of testing on working memory Trials 4 and 5 (T4, T5), while EE Tg+ mice exhibited working memory on trials 4 and 5 that was indistinguishable from non-transgenic standard-housed (Tg-/SH) mice. On working memory T4, EE also significantly improved performance of non-transgenic mice raised in EE compared to NT/SH mice. All groups were similar in escape latencies during the semi-randomized initial trial (T1)

(B) In the platform recognition task of search/identification, the performance of Tg+/EE mice over all 4 days of testing was significantly better than impaired Tg+/SH controls and identical to Tg-/SH mice. This effect was evident on individual Days 2-4 of testing.

(C) Environmental enrichment completely protected mice from overall cognitive impairment across 8 behavioral measures evaluated by discriminant function analysis (DFA). This Canonical Scores Plot depicts a linear representation of the two functions resulting from DFA (Wilks’ lambda for overall discrimination was p<0.0001), representing the overall poorer cognitive performance of control Tg+/SH mice relative to both enriched Tg+/EE mice and control NT/SH mice, which could not be discriminated from one another. In (A) and (B), means±SEM’s are plotted. * = p<0.002 or higher level of significance for Tg+/SH vs. both Tg+/EE and NT/SH; # = p<0.02 for NT/SH vs. NT/EE; ** = p<0.05 or higher level of significance for Tg+/SH vs. all other groups for that day.
Figure 2. Aβ and compact amyloid plaque levels are severely reduced in Tg+ mice both environmentally enriched and behaviorally tested despite dendritic deficits.

(A) Immunohistochemical analysis, using the monoclonal antibody 6E10, reveals a 51.5% reduction in area immunostained cerebral cortex (P=0.025) in those mice behaviorally tested and raised in EE (n=8) vs. mice only raised in EE (n=11). (B) Similarly, in hippocampus, mice behaviorally tested and raised in EE had a 44.1% (P=0.022) reduction in immunostaining vs. those only raised in EE. There was no statistically significant difference in immunostaining in either cortex or hippocampus between EE and SH mice in either tested or non-tested cohorts.

(C) Compact plaque deposition, as measured by thioflavine S staining, shows, in cerebral cortex, a 57.6% reduction in mature plaque deposition (p=0.008) and (D) a 69.0% reduction (P<0.0001) in hippocampal deposition in mice behaviorally tested and raised in EE vs. those only raised in EE. As with 6E10 immunostaining, there was no statistically significant difference in thioflavine S staining in either cortex or hippocampus between EE and SH mice in either tested or non-tested cohorts. (E) EE does not rescue the transgene-induced dendritic arborization deficits, as shown by Golgi staining (p<0.0001) (Sholl method). (F) Dendritic spine counts further verify that EE has no affect on dendritic morphology.
Figure 3. Environmental Enrichment results in a set of genetic changes that culminate in anti-apoptotic/neuroprotective BAD phosphorylation.

A number of proteins (represented as red in this figure) that affect BAD phosphorylation were changed in their expression due to EE. Insulin-like growth factor (IGF-2) was up-regulated 2.5 fold, and Insulin-like growth factor binding protein-2 (IGFBP-2) was up-regulated 3.0 fold in Tg+/EE mice, which acts through the Akt and Erk1/2 pathways to phosphorylate BAD. Working only through the Akt pathway, the prolactin receptor (PRLR) was up-regulated 21.1 fold in Tg+/EE mice. Phosphodiesterase 4B was down-regulated 2.2 fold, resulting in a diminishing of cAMP inhibition and a resultant increase in Mek/Erk mediated BAD phosphorylation. Also shown is Rolipram, a specific PDE4 inhibitor, whose ability to mimic EE was tested (Figure 4). A 10-fold increase in transthyretin (TTR) was also observed in the Tg+/EE mice, an Aβ binding protein known to sequester Aβ and inhibit amyloid formation. (Bole-Feyssot et al., 1998; Bergmann, 2002; Stein and Johnson, 2002). The figure shows how the protective affects of BAD phosphorylation and TTR may inhibit neuronal degeneration and dysfunction caused by Alzheimer's-related changes in Aβ peptide and τ-phosphorylation.
Figure 4. Administration of Rolipram to Tg+ mice results in memory improvement which mimics environmental enrichment.

(A) In the radial arm water maze task of working memory, Tg+ mice injected with Rolipram showed significantly improved performance across days 4-6 of testing on working memory Trials 4 and 5 (T4, T5), while Tg+ injected with vehicle only exhibited working memory deficits typical of PS/PDAPP mice. Both groups were similar in escape latencies during the semi-randomized initial trial (T1).
Vehicle Rolipram Latency (sec) Ret. Trial

Latency (sec)

Vehicle
Rolipram

T1 T4 T5 Ret. Trial

* *
Table 1. Comparative microarray analysis of hippocampal gene expression between environmentally enriched and standard housed PS1/PDAPP mice

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene</th>
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<th>Affymetrix ID</th>
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SLR, Signal log ratio; FC, Fold change (approximate) = 2^{SLR}
Table 2. qRT-PCR verification of select transcripts from hippocampal microarray analysis.

<table>
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<th>Gene</th>
<th>ΔΔCT ± SD</th>
<th>Fold Change ($2^{ΔΔCT}$)</th>
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<tr>
<td>Prolactin Receptor</td>
<td>-3.59 ± 1.02</td>
<td>12.06</td>
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<tr>
<td>Transthyretin</td>
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<tr>
<td>Insulin-like growth factor 2</td>
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<tr>
<td>Insulin like growth factor binding protein 2</td>
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Comparative threshold cycle: $\Delta\Delta C_T = \Delta C_T(EE) - \Delta C_T(SH)$

$\Delta C_T = C_T$ (target) - $C_T$(reference)
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Pinaud R, Penner MR, Robertson HA, Currie RW (2001) Upregulation of the immediate early gene arc in the brains of rats exposed to environmental


associated with NMDA receptor antagonism. Neuropsychopharmacology 23:198-204.

DISCUSSION

*In vitro* (Ma et al., 1994) and *in vivo* (Nilsson et al., 2001) studies have found that the chief pathogenic chaperone found in amyloid β plaques, apoE, is able to catalyze the polymerization of Aβ into neurotoxic amyloid filaments and change the anatomical distribution of immunoreactivity in the brain. Furthermore, the deposition of brain AD pathology is strongly influenced, both in intensity and age of onset, by the gene dosage of apoE. Specific binding experiments, site directed mutagenesis, and structural analysis have identified the primary molecular interactions between the pathological chaperone, apoE, and Aβ (Potter, 1991; Strittmatter et al., 1993; Ma et al., 1996; Janciauskiene et al., 1998). A previous study from our laboratory provides the first *in vivo* demonstration that the increased Aβ immunoreactive and mature plaque deposition caused by expression of the amyloid promoter, apoE, is associated with impaired spatial learning (Nilsson et al., 2004, APPENDIX B).

We found that apoE promotes the development of both diffuse, immunoreactive, Aβ deposits and mature amyloid plaques in Aβ-overexpressing mice without affecting the levels of monomeric Aβ itself and that impaired cognitive performance in two different spatial tasks requires both Aβ peptide and *either* ApoE or ACT as an amyloid promoter, with apoE showing a higher catalytic activity. Furthermore, both diffuse Aβ deposits and mature amyloid
plaques were correlated with impaired spatial learning ability. Indeed, 18 month old APP mice that didn’t express ApoE deposited very little Aβ and displayed no cognitive deficits in the RAWM or Morris Water Maze, similar to nontransgenic mice. These findings imply that the process and/or product of ApoE catalyzed amyloid formation is more critical for the cognitive decline in AD than is the amount of monomeric Aβ-peptide. In sum, our previous findings of a strong association between cognitive decline and both diffuse Aβ deposits and mature amyloid plaques in transgenic mice indicate that a major pathological role of ApoE in Alzheimer’s disease is to promote Aβ polymerization and deposition, possibly with slightly different effects in terms of time course and anatomical distribution of the pathology.

Given that the doubly transgenic AβPP/PS1 mouse model used in the currently reported study expresses severely elevated levels of brain Aβ1-42, most likely superseding any possible effect of apolipoprotein E on Aβ peptide production or clearance (if there are indeed any), we offer clear proof that apoE directly catalyzes the polymerization of Aβ into β-sheet fibrils and its consequent aggregation to form mature, Thioflavine S-positive, amyloid plaques. This directly contrasts with previous publications only utilizing singly transgenic, PDAPP, animals, whereby apoE had been shown to promote both total Aβ deposition and amyloid formation (Nilsson et al., 2004, APPENDIX B).

Transgenic mice expressing both human PS1M146L and AβPPV717F mutations do not develop significant fibrillar amyloid deposits by 7 months of age, in the absence of apoE, despite copious amounts of immunoreactive Aβ
deposition. We find that mature, Thioflavine-S positive, amyloid deposits are allowed to form only the presence of apoE, and are the first authors, to our knowledge, to describe the gross morphological changes of amyloid plaques, using electron microscopy, due to apoE.

Although we find that ApoE clearly promotes Aβ polymerization directly, this protein likely has additional roles to play in AD and other brain diseases. For example, other authors have shown that ApoE affects the level or location of APP processing so as to generate fewer Aβ peptides and more potentially toxic C-terminal fragments (in animals slightly older than ours’ and at an age known to show the initial stages of amyloid deposition) (Dodart et al., 2002). Particularly interesting is the finding that ApoE4 is less neuroprotective than ApoE3 both in vitro and in vivo (Buttini et al., 2002).

ApoE is also known to affect cholesterol metabolism (Poirier, 2000), for ApoE knockout mice develop hypercholesterolemia and atherosclerosis that could have an effect on amyloid formation. It is therefore wise to consider the possible direct and compensatory consequences of expressing or not expressing ApoE during development. However, heterozygous PDAPP/ApoE+/- mice have normal serum cholesterol levels and develop amyloid deposits at a rate intermediate between full PDAPP/ApoE-KO and PDAPP/ApoE+/+ animals (Bales et al., 1999), suggesting that the lower amyloid deposition induced by knocking out ApoE is not caused by high serum cholesterol due to ApoE deficiency. Furthermore, except for hypercholesterolemia in ApoE-KO mice, we have not
observed gross differences in physiology or brain morphology due to knocking out ApoE.

Preventing apoE from binding to Aβ and stimulating its polymerization into β-sheet filaments may prove to be a promising therapeutic approach for AD drug development. It is possible that the process of amyloid formation, or an intermediate formed therein, rather than the end product (mature plaques), causes the typical neuronal dysfunction and cognitive decline of AD. These results showing the essential role of ApoE in amyloid fibril formation, together with the previous findings that apoE promotes cognitive decline and does not alter the steady state level of Aβ, suggest that pharmacologic inhibition of Aβ/ApoE interactions should be a prime target for therapeutic intervention in AD. Previous experiments indicate that such an approach may be effective, for small fragments of the Aβ peptide were shown to effectively prevent ApoE-catalyzed polymerization in vitro (Ma et al., 1994; Ma et al., 1996).

A pharmacological target in the periphery as a therapy against AD has clear pharmacological and clinical advantages. There is much debate in the field of Alzheimer’s disease regarding the contribution of peripheral circulating proteins to disease pathology. The fact that disease-related proteins such as Aβ and apoE are elevated in the serum of AD patients, and that there exists a notable prevalence of vascular cerebral amyloid angiopathy indicates that circulating proteins are at least linked causally to the disease process. Since we observed such a drastic catalytic affect of apoE on the polymerization of Aβ, and since apoE is known to shuttle Aβ across the blood brain barrier, we surmised
that if circulating apoE has an affect on brain pathology it would be easily detectable in our AβPP/PS1 transgenic mouse model.

We demonstrate here that the unorthodox surgical technique of parabiosis is a feasible approach for studying the contribution of circulating proteins and hormones to Alzheimer's disease pathology. We found that apoE is efficiently transferred from one parabiont to another using PCR analysis of white blood cells. Though transferred efficiently, the levels of circulating apoE protein determined via western blot were only 5% of that found in a nontransgenic mouse. These low steady state levels of apoE in the apoE-KO parabiont are most likely due to massive sequestration of apoE for use in lipoprotein particles and cholesterol transport. This is suggested by the fact that the hypercholesterolemic phenotype of apoE-knockout mice is rescued in these same mice when parabiosed to a mouse expressing apoE. It should be mentioned that work by other authors has shown that it takes less than 3% of normal levels of apoE to prevent atherosclerotic plaque deposition and to maintain typical cholesterol levels (Thorngate et al., 2002).

Based upon our findings regarding apoE-mediated thioflavine-S catalysis, we anticipated that even minute amounts of apoE entering the central nervous system of PS1/APP/apoE-KO mice could promote large amounts of fibrillar amyloid deposition. Surprisingly, there was no increase in Aβ deposition, diffuse or fibrillar, whatsoever. Conversely, we found fewer fibrillar, Thioflavine S-positive, deposits in the parabiosed mice that expressed apoE as compared to control mice. Immunohistochemical analysis reveals that peripheral apoE is only
detectable in the choroids plexus which is in agreement with another previous study (Martel et al., 1997).

Since Aβ injected into the circulation of mice is cleared rapidly, the observed lowering of Aβ deposition in parabiosed PS1+/+,APP+/+,apoE+/- mice may be due to apoE-mediated peripheral Aβ clearance. It has been shown that there exists a dynamic equilibrium between brain Aβ levels and the levels in the CSF, which was demonstrated by using a peripheral antibody injection to sequester Aβ (DeMattos et al., 2002). Besides apoE’s known role as a promoter of fibrillization, there is increasing evidence for a dual role of apoE, in that it may also promote the clearance of Aβ. For example, in vitro astrocytic internalization and degradation of Aβ is found to be dependent on the presence of apoE (Koistinaho et al., 2004). Furthermore, intra-venous injections of Aβ, which would normally be quickly cleared by the liver, are not detectably cleared in mice devoid of apoE (Hone et al., 2003). Therefore our study, and those of others, suggests that apoE may be involved in the peripheral clearance/sequestration of Aβ, acting as a sink which may change the equilibrium between brain and peripheral Aβ. This could therefore alter the circulating:CSF/brain Aβ equilibrium, shifting it towards the blood, resulting in an efflux of Aβ from the brain, consequently resulting in the observed reduction in amyloid deposition. This shift in Aβ equilibrium between discreet compartmentalized pools has also been suggested by the work of others (Silverberg et al., 2003).

We conclude that peripheral apolipoprotein E does not contribute to the brain pathology found in Alzheimer’s disease, and may actually help promote the
clearing/efflux of brain Aβ by a mechanism that remains open to speculation. Based on the amelioration of hypercholesterolemia due to parabiosis, we are confident that this uncommon technique has merit for studying the contribution of circulating factors to Alzheimer’s and other neurodegenerative diseases.

Another, seemingly unrelated, primary topic of study in our laboratory is that of environmental enrichment as an intervention strategy for the treatment of AD. Initial environmental enrichment studies from our laboratory indicate that a long-term period of EE in aged AD transgenic mice results in superior overall cognitive performance as determined by a number of behavioral measures (Arendash et al., 2004, APPENDIX B). Although a reduction in brain Aβ deposition has been associated with behavioral benefit of Aβ immunotherapy in similar AD transgenic mice (Corder et al., 1993), our initial results indicated that mechanisms independent of Aβ deposition are sufficient for behavioral benefit, for we observed no changes in brain pathology in EE mice. Along this line, EE-induced enhancements in neurogenesis (Anderson et al., 1998), synaptogenesis (Anderson and Higgins, 1997), growth factor levels (Arendash et al., 2001b), and gene expression (Arendash et al., 2001a) have all been seen in normal mice and are thus potentially involved in the behavioral benefits of EE we reported in aged APPsw mice (Arendash et al., 2004, APPENDIX B).

In this prior study, designed to evaluate the potential of EE as a therapeutic means in aged AD transgenic mice, our results suggested that long-term intensive enrichment/cognitive stimulation could be useful in stabilizing or slowing the cognitive decline of AD and its predecessor, mild cognitive
impairment (MCI) without a need to reduce Aβ burden. Largely because of practical considerations, prospective longitudinal studies have not been done in humans to confirm controversial retrospective studies reporting AD risk reduction with "lifelong" education/occupation-related intellectual activity (Bales et al., 1997; Bales et al., 1999). A recent 5-year longitudinal study involving non-demented 75+ year olds did find participation in cognitively-stimulating leisure activities to be associated with a lower risk of dementia (Dodart et al., 2002). However, a cause and effect relationship cannot be established because the leisure activities were self-chosen.

The current study sought to both eliminate any cause/effect ambiguity by randomly assigning transgenic mice destined to develop AD and their NT counterparts to either EE or SH and to ascertain the preventative benefits of long term enrichment. We find that mice raised in a cognitively stimulating environment were protected from certain cognitive decline as measured by multiple behavioral assays. Our results also indicate that, alone, neither environmental enrichment nor behavioral testing could alter brain Aβ levels. The cognitive benefits of preventative EE are therefore at least partly independent of AD brain pathology, for we observed what may be a cognitive stimulation threshold for the clearance of Aβ that does not affect cognitive measures performance, for only the combination of both long-term environmental enrichment and intense behavioral testing resulted in a lowered level of brain Aβ deposition. In fact, we observed up to 69% decreases in both diffuse and compact Aβ levels, and surmise that a decrease of this magnitude could not
possibly be from only a halting in Aβ deposition, but from an as yet unknown removal or sequestration mechanism. EE has been shown to prime the brain for increased maze training-induced choline acetyltransferase activity (Levi et al., 2003), and we observe what is most likely a similar priming by EE for behavioral testing-induced pathology changes. Additionally, microarray analysis revealed a number of beneficial gene expression changes related to memory, neuroprotection, and Aβ sequestration.

Previous studies have revealed that EE is capable of inducing beneficial structural changes such as increases in the number of dendritic spines and branching complexity (Comery et al., 1995; Turner et al., 2003). In our experiment, we observed no such changes due to EE. In fact, all the transgenic mice in our study displayed dendritic defects, as determined by Golgi staining, and these were unaffected by EE, indicating that 1) these physical defects could not be rescued by EE, and 2) that the observed memory improvement due to EE in these mice was independent of dendritic structural changes.

Microarray analysis was performed to study the possible genetic changes that could be responsible for the improved cognitive performance and the observed lowering of AD brain pathology due to cognitive stimulation. We focused on the roughly 70 known genes that were significantly changed, either up or down, by at least 2-fold due to EE, and provided an in-detail inquiry for those transcripts most likely to affect Alzheimer’s disease or cognitive function.

We found that a number of genes known to be involved in pathways which culminate in the phosphorylation of BAD, an event known to be anti-apoptotic
and neuroprotective, had altered expression levels. Previous experiments wherein mice where fed a diet rich in a compound (docosahexanoic acid) known to promote BAD phosphorylation helped protect mice against cognitive deficits (Calon et al., 2004). In particular, we found four transcripts upstream of bad phosphorylation that were changed due to EE: phosphodiesterase 4B, insulin-like growth factor-2, insulin-like growth factor binding protein-2, and the prolactin receptor. Docosahexanoic acid-induced BAD phosphorylation is an IRS-1/PI3-K mediated event, and three transcripts for the proteins, IGF-2, IGFBP2, and PRLR which are also known to promote BAD phosphorylation via the IRS-1/PI3-K pathway were significantly upregulated. IGF-2 and IGFBP2 are also able induce BAD phosphorylation through an alternative MEK/ERK mediated pathway which has been show to be imperative in the formation of long term memory (Impey et al., 1999; Schafe et al., 2000). Interestingly, PDE4, which was downregulated by EE, acts as an inhibitor of this same pathway (Zhang et al., 2004), and its inhibition has recently been shown to promote both memory and LTP in a mouse model of AD (Gong et al., 2004). By injecting Rolipram, an inhibitor of PDE4, into 8-month-old transgenic mice we have rescued the transgene-induced cognitive deficits, effectively mimicking the effect of environmental enrichment. Taken as a whole, the changes in these related transcripts suggest the memory improvement in mice that were exposed to EE may be due an inducible mechanism that confers neuroprotection.

A molecule know to bind to (Tsuzuki et al., 1997; Tsuzuki et al., 2000) and sequester (Schwarzman et al., 1994) Aβ, transthyretin, was up-regulated roughly
10-fold in transgenic mice raised in EE. TTR binds to Aβ and is thought to inhibit Aβ aggregation, for inhibition of TTR has been shown to increase Aβ deposition (Stein et al., 2004). In fact, TTR is thought responsible for the prevention of peripheral Aβ deposition, for the kidney's level of this protein is quite high. Furthermore, TTR levels are reduced in human AD patients, and are hypothesized to be at least partly responsible for the accumulation of Aβ and the resultant neurodegeneration, so there exists a discrepancy, for in those mice only undergoing EE there was no decrease in pathology despite cognitive improvements.

Besides these changes, some transcripts were altered due to EE that are known to improve memory. For example, CCK-B was down-regulated, and past experiments have shown that inhibiting the CCK-B receptor enhances memory in rats (Lemaire et al., 1994). Another protein, which is known to be reduced in human AD patients, phospholipase A2, was upregulated due to EE, and memory defects are known to be induced by its pharmacological inhibition.

A number of other genes or gene groups experienced significant changes, but their direct relevance to our model and AD is unclear. For example, STEAP (six transmembrane epithelial antigen of the prostate), a seemingly AD- and memory-unrelated transcript was up-regulated by approximately 43-fold, the largest observed change on the microarray. Also a number of procollagen and procollagen-related transcripts were also up-regulated. These proteins are potentially important for Schwann-cell extracellular matrix (Greenberg et al., 1980; Roytta et al., 1988), but their role in memory formation or improvement is
unknown. Klotho, a gene encoding a membrane protein known to cause premature aging in mice when mutated (Kuro-o et al., 1997), was also up-regulated by 4.5-fold. This could be an EE-induced compensatory mechanism against an aspect of aging that has yet to be determined.

A recent report of a similar experiment run in parallel by another group (Lazarov et al., 2005) using a different mouse model (APPsw/PS1dE9), further confirms our findings that EE reduces amyloid deposition. It should be noted that we do not see any changes in the levels of neprilysin, an Aβ degrading enzyme that these authors attribute to the EE-induced lowering of Aβ, therefore indicating that other sequestration/degradation mechanisms are most likely responsible for the changes in pathology that we observed. Furthermore, a differential sensitivity to Aβ reduction due to EE likely depends on genotype, possibly explaining the lowered Aβ deposition only in animals that underwent EE and intense behavioral testing. Jankowsky et al., in direct contrast to both of the above findings, reported that EE promotes Aβ deposition in an APPsw/PS1dE9 mouse model of AD. Unfortunately, these authors chose an unusual enrichment methodology whereby new mice were continually introduced to the cages, adding a confounding source of stress which may explain the observed increase in pathology. This therefore makes comparisons between this study and ours’ of little value.

In conclusion, we found that the combination of both long-term EE and intense behavioral testing, both forms of cognitive stimulation, can reduce the levels of Aβ deposition. Furthermore, the gene expression changes due to EE
may prime the brain for further changes in AD pathology induced by additional intense cognitive stimulation. Therefore, pharmacological alteration of these genetic pathways, as exemplified by our inhibition of PDE4 by Rolipram, may prove to be valuable targets in the fight against the cognitive decline of Alzheimer’s disease.
FUTURE DIRECTIONS

The paths for a number of future avenues of study have been cleared by the recent research of our laboratory. For example, we show that parabiosis is a useful method for determining the contribution of a circulating protein (in the case of our current research, apoE) to AD pathology. Further experiments should be repeated with different parabiont combinations. For example, a mouse expressing circulating Aβ, such as our PS1/APP mouse, should be parabiosed to a non-transgenic mouse, for any brain pathology present in the transgenic partner would be a result of circulating Aβ, therefore answering the question of whether peripheral Aβ contributes to brain pathology. If so, antibody injections or the administering of Aβ-sequestering agents may prove useful as a stand-alone, or more likely as an adjunct AD therapy. If on the other hand there is no affect on brain pathology, efforts to curb brain pathology via the peripery should possibly be reevaluated. Similarly, experiments using mice which overexpress peripheral α1-antichymotrypsin, another pathogenic chaperone, should also be initiated for the same reasons.

Since our environmental enrichment paradigm has a number of differing aspects which may be responsible for the observed memory improvement and genetic changes, it will be useful to parse out the aspect or aspects of EE which
are most responsible for these beneficial changes. Our “whole” EE setting entails social, physical, and cognitive elements, and by systematically isolating and differentiating which element exerts the greatest cognitive and genetic benefits, using behavioral testing and further microarray analysis, both recommendations for using enrichment therapy in humans and more appropriate pharmacological targeting can be devised with greater accuracy.

Since pharmacological inhibition of PDE4 using Rolipram rescued the cognitive deficits in our transgenic mouse model of AD, further animal trials using other available phosphodiesterase inhibitors (e.g. Aminophylline, cilomilast, etc…) should be initiated, and if successful should lead to eventual clinical trials for the treatment or prevention of Alzheimer’s disease. This strategy should furthermore be applied to the pathways and proteins whose expression changed due to EE that converge in BAD phosphorylation such as IGF-2, IGFBP2, and PRLR.

Lastly, it has been shown that environmental enrichment-induced memory and learning improvements in transgenic mice are apoE allele-dependant (Levi et al., 2003), so future studies should therefore concentrate on combining the two main foci of my work, apolipoprotein E and environmental enrichment, in order to elucidate the differing genetic changes and behavioral changes of the various apoE alleles among varying environmental enrichment conditions so as to develop a clearer understanding of the relevant pathways and define the most efficacious and personalized treatment regimens possible in relation to ones’ given apoE allele.
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induced toxicity in rat hippocampal pyramidal neuronal cultures. J Neurosci 18:195-204.


Letenneur L, Gilleron V, Commenges D, Helmer C, Orgogozo JM, Dartigues JF (1999) Are sex and educational level independent predictors of dementia and


APPENDIX A

Source Code for High Output Morphometry Examination Routine (HOMER)

Form:

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'IPPMACRO.frm

' HOMER Version 1.0
' DAVID COSTA 2003
'
Option Explicit

'File Management Variables
Dim vstrBackgroundImage As String
Dim vstrSegmentationImage As String
Dim vstrSegmentationFile As String
Dim vstrExcelFile As String
Dim vstrTargetImageDirectory As String
Dim vstrTargetImage As String * 255
Dim vstrtxtIcComment As String
Dim vintRangeMin As Integer
Dim vlngRangeMax As Long
Dim vsngExcelArray(10) As Single
Dim vintTweakChoice As Integer
Dim vintExcelRow As Integer  'index for ron number for Excel data export
Dim vintDirectoryIndex As Integer  'in cmdCalculate - loop index for IpStSearchDir()
Dim vintDirectoryStatus As Integer  'in cmdCalculate - return value for IpStSearchDir()

'Excel Variables
Dim xlapp As Object
Dim xlbook As Object
Dim xlSheet As Object
Dim xlRange As Object
Dim n As Integer  'Index for excel export of data

Dim i As Integer
Dim j As Integer
Dim xlData() As Single
Dim xlLabels() As String
Dim dInfo As IPDOCINFO

'InitExcel() initializes the OLE link to Excel and returns the Object 'in the xlApplication parameter. Return value: 0 = success; -1 = failure Function InitExcel(ByRef xlApplication As Object) As Integer

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APPENDIX A (Continued)

InitExcel = 0    ' Make sure the function is initialized

On Error Resume Next ' continue if an error is generated
Err.Clear                   ' clear the error flag

Set xlApplication = GetObject(, "Excel.Application")

' GetObject will return an error if Excel isn't open
If Err.Number <> 0 Then
    ' Start Excel with CreateObject. If this fails, we exit the macro.
    Err.Clear
    Set xlApplication = CreateObject("Excel.Application")
    If Err.Number <> 0 Then
        MsgBox "Can't find Excel.", vbOKOnly + vbCritical, "OLE Error"
        InitExcel = -1
    Exit Function
    End If
End If

' show Excel (don't run in background)
xlApplication.Visible = True

If Err.Number <> 0 Then
    MsgBox "Error showing Excel!", vbOKOnly + vbCritical, "OLE Error"
    InitExcel = -2
End If

End Function

' Opens Excel spreadsheet and defines rows and columns for data input
Function fncOpenExcel()

'startup Excel for OLE data dump
If InitExcel(xlapp) < 0 Then
    Exit Function
End If

'create a new workbook for your data
Set xlbook = xlapp.Workbooks.Add
    xlbook.Activate
APPENDIX A (Continued)

Set xlSheet = xlbook.ActiveSheet
   xlSheet.Activate
   xlapp.Visible = True
   xlapp.WindowState = vbMaximized

'Note: due to the design of OLE, it is much faster to transfer data
' to Excel in a 2 dimensional array, even if you are only filling
' one cell
ReDim xlLabels(1 To 1, 1 To 3) As String        '(row,column)

'Adds Summary sheet to excel file
xlapp.worksheets.Add
Set xlSheet = xlbook.ActiveSheet
   xlSheet.Activate
   xlSheet.Name = "Result Summary"

'Adds AOI sheet to excel file if area is checked
If chkArea.value = vbChecked Then
   xlapp.worksheets.Add
   Set xlSheet = xlbook.ActiveSheet
   xlSheet.Activate
   xlSheet.Name = "AOI"

'Labels and Formats "AOI" Sheet
With xlapp
   .ActiveWindow.Zoom = 75
   .Range("A1").Select
   .ActiveCell.FormulaR1C1 = "Image"
   .Range("B1").Select
   .ActiveCell.FormulaR1C1 = "Sum"
   .Range("C1").Select
   .ActiveCell.FormulaR1C1 = "Std. Deviation"
   .Range("D1").Select
   .ActiveCell.FormulaR1C1 = "Num. of Objects"
   .Range("E1").Select
   .ActiveCell.FormulaR1C1 = "Comments"
   '.Columns("E:E").Select
   '.selection.ColumnWidth = 50

   .Rows("1:1").Select

   With .Selection
APPENDIX A (Continued)

'Adds % Area sheet to excel file if area is checked
If chkArea.value = vbChecked Then
    xlapp.worksheets.Add
    Set xlSheet = xlbook.ActiveSheet
    xlSheet.Activate
    xlSheet.Name = "% Area"

'Labels and Formats "% Area" Sheet
With xlapp
    .ActiveWindow.Zoom = 75
    .Range("A1").Select
    .ActiveCell.FormulaR1C1 = "Image"
    .Range("B1").Select
    .ActiveCell.FormulaR1C1 = "Mean Value"
    .Range("C1").Select
    .ActiveCell.FormulaR1C1 = "Std. Deviation"
    .Range("D1").Select
    .ActiveCell.FormulaR1C1 = "Min. Measurement"
    .Range("E1").Select
    .ActiveCell.FormulaR1C1 = "Maximum Measurement"
    .Range("F1").Select
    .ActiveCell.FormulaR1C1 = "Range"
    .Range("G1").Select
APPENDIX A (Continued)

ActiveCell.FormulaR1C1 = "Sum"
.Range("H1").Select
ActiveCell.FormulaR1C1 = "Num. of Objects"
.Rows("1:1").Select

With .Selection
  .Font.Underline = 2 'xlUnderlineStyleSingle
  .Font.Bold = True
  .ColumnWidth = 22
  .HorizontalAlignment = 3 'xlCenter
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .IndentLevel = 0
  .ShrinkToFit = False
  .ReadingOrder = xlContext
  .MergeCells = False
End With

.Range("C6").Select
.Columns("E:E").ColumnWidth = 23.57
End With
End If

'Adds Density sheet to excel file if Density is checked
If chkDensity.value = vbChecked Then
  xlapp.worksheets.Add
  Set xlSheet = xlbook.ActiveSheet
  xlSheet.Activate
  xlSheet.Name = "Density"
End With

With xlapp
  .ActiveWindow.Zoom = 75
  .Range("A1").Select
  ActiveCell.FormulaR1C1 = "Image"
  .Range("B1").Select
  ActiveCell.FormulaR1C1 = "Density Mean: Mean"
  .Range("C1").Select
  ActiveCell.FormulaR1C1 = "Density Mean: Std.Dev"
  .Range("D1").Select
APPENDIX A (Continued)

.Author.Cell.FormulaR1C1 = "Density Mean: Sum"
.Range("F1").Select
.Author.Cell.FormulaR1C1 = "Density Sum: Mean"
.Range("G1").Select
.Author.Cell.FormulaR1C1 = "Density Sum: Std.Dev"
.Range("H1").Select
.Author.Cell.FormulaR1C1 = "Density Sum: Sum"
.Rows("1:1").Select
With .Selection
  .Font.Underline = 2 'xlUnderlineStyleSingle
  .Font.Bold = True
  .ColumnWidth = 22
  .HorizontalAlignment = 3 'xlCenter
  .VerticalAlignment = xlBottom
  .WrapText = False
  .Orientation = 0
  .AddIndent = False
  .IndentLevel = 0
  .ShrinkToFit = False
  .ReadingOrder = xlContext
  .MergeCells = False
End With

.Columns("E:E").Select
  .Selection.ColumnWidth = 5
End With

End If

'Delete Unwanted sheets
With xlapp
  .Sheets("Sheet1").Select
  .SendKeys "Y"
  .ActiveWindow.SelectedSheets.Delete
  .Sheets("Sheet2").Select
  .ActiveWindow.SelectedSheets.Delete
  .Sheets("Sheet3").Select
  .ActiveWindow.SelectedSheets.Delete
'End With

'Label the columns
'    xlLabels(1, 1) = "Data 1"
'    xlLabels(1, 2) = "Data 2"
'    xlLabels(1, 3) = "Data 3"

' Set xlSheet = Nothing
' Set xlbook = Nothing
' Set xlapp = Nothing

End Function

Function fncExcelDataExport(vstrSheetName As String, vintYindex As Integer)

Select Case vstrSheetName

Case "% Area"

    'declare n as index integer
    n = 0

    'Get statistics from current AOI and send to Array
    ret = IpBlbGet(GETSTATS, 0, BLBM_AREA, vsngExcelArray(n))

    'make % area active sheet and sends area data to excel
    xlapp.Sheets(vstrSheetName).Select
    Set xlSheet = xlbook.ActiveSheet

    'Mean, Std.Dev, Min, Max, Range, Sum
    For n = 0 To 5
        With xlSheet
            .Range(.cells(vintYindex, 2 + n), .cells(vintYindex, 2 + n)).value = vsngExcelArray(n)
        End With
    Next n

    ' # of Objects
    n = n + 2
    With xlSheet
        .Range(.cells(vintYindex, n), .cells(vintYindex, n)).value = vsngExcelArray(n)
    End With

End Case

Case Else

End Select

End Function
End With
n = 0
' name of current file to excel
With xlSheet
  .Range(.cells(vintYindex, 1), .cells(vintYindex, 1)).value =
vstrTargetImage
End With

Case "AOI"

  n = 0
  ' Get statistics from current AOI and send to Array
  ret = IpBlbGet(GETSTATS, 0, BLBM_AREA, vsngExcelArray(n))

  ' Make AOI active sheet and sends area data to excel
  xlapp.Sheets(vstrSheetName).Select
  Set xlSheet = xlbook.ActiveSheet

  ' Sum
  With xlSheet
    .Range(.cells(vintYindex, 2), .cells(vintYindex, 2)).value = _
      vsngExcelArray(5)
  End With

  ' Std.Dev
  With xlSheet
    .Range(.cells(vintYindex, 3), .cells(vintYindex, 3)).value = _
      vsngExcelArray(1)
  End With

  ' # of Objects
  With xlSheet
    .Range(.cells(vintYindex, 4), .cells(vintYindex, 4)).value = _
      vsngExcelArray(8)
  End With

If txtIcComment.text <> "" Then
  vstrtxtIcComment = txtIcComment.text
  With xlSheet
    .Range(.cells(vintYindex, 5), .cells(vintYindex, 5)).value = _
      vstrtxtIcComment
  End With
APPENDIX A (Continued)

End If

n = 0

' name of current file to excel
With xlSheet
   .Range(.cells(vintYindex, 1), .cells(vintYindex, 1)).value = _
       vstrTargetImage
End With

Case "Density"
   n = 0

   ' Get statistics from current AOI and send to Array
   ret = IpBlbGet(GETSTATS, 0, BLBM_DENSITY, vsngExcelArray(n))

   ' make % area active sheet and sends area data to excel
   xlapp.Sheets(vstrSheetName).Select

   Set xlSheet = xlbook.ActiveSheet

   ' Mean, Std. Dev
   For n = 0 To 1
      With xlSheet
         .Range(.cells(vintYindex, 2 + n), .cells(vintYindex, 2 + n)).value = _
            vsngExcelArray(n)
      End With
   Next n

   With xlSheet
      .Range(.cells(vintYindex, 4), .cells(vintYindex, 4)).value = _
         vsngExcelArray(5)
   End With

   n = 0

   ' Get statistics from current AOI and send to Array
   ret = IpBlbGet(GETSTATS, 0, BLBM_DENSSUM, vsngExcelArray(n))

   ' make % area active sheet and sends area data to excel
   xlapp.Sheets(vstrSheetName).Select

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APPENDIX A (Continued)

Set xlSheet = xlbook.ActiveSheet

'Mean, Std. Dev
For n = 0 To 1
  With xlSheet
    .Range(.cells(vintYindex, 6 + n), .cells(vintYindex, 6 + n)).value = _
    vsngExcelArray(n)
  End With
Next n

With xlSheet
  .Range(.cells(vintYindex, 8), .cells(vintYindex, 8)).value = _
  vsngExcelArray(5)
End With

End Select

End Function

'Determines percent area of segmented portions of image
Function fncPercentArea()

'loads segmentation file
ret = IpSegLoad(vstrSegmentationFile)
ret = IpSegShow(0)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegSetAttr(Channel, 0)
ret = IpSegPreview(ALL_C_T)
ret = IpSegShow(0)
ret = IpBlbShow(1)
'selects manual autorange
ret = IpBlbSetAttr(BLOB_AUTORANGE, 0)
'Green Fill Color
ret = IpBlbSetAttr(BLOB_OUTLINECOLOR, 3)
'Style = Filled
ret = IpBlbSetAttr(BLOB_OUTLINEMODE, 3)
'Set Label Mode to NONE
ret = IpBlbSetAttr(BLOB_LABELMODE, 0)
'Sets Cleanborder Mode
ret = IpBlbSetAttr(BLOB_CLEANBORDER, 0)

If chkApplyRanges.value = vbChecked Then
    vlngRangeMax = txtSizeMax.text
    vintRangeMin = txtSizeMin.text

    'set filter ranges
    ret = IpBlbSetFilterRange(BLBM_AREA, vintRangeMin, vlngRangeMax)
    'apply filter ranges
    ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)
Else
    vlngRangeMax = 10000
    vintRangeMin = 10

    'set filter ranges
    ret = IpBlbSetFilterRange(BLBM_AREA, vintRangeMin, vlngRangeMax)
    'apply filter ranges
    ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)
End If

ret = IpBlbCount()
ret = IpBlbUpdate(0)

End Function

'Determines Integrated Optical Density
Function fncDensity()

End Function

'Determines total area of chosen AOI
Function fncAOI()

    'set large filter range
    vlngRangeMax = 10000000
    vintRangeMin = 0

    'selects manual autorange
    ret = IpBlbSetAttr(BLOB_AUTORANGE, 0)
    'Green Fill Color
APPENDIX A (Continued)

ret = IpBlbSetAttr(BLOB_OUTLINECOLOR, 3)  
'Style = Filled
ret = IpBlbSetAttr(BLOB_OUTLINEMODE, 3)  
'Set Label Mode to NONE
ret = IpBlbSetAttr(BLOB_LABELMODE, 0)  
'Sets Cleanborder Mode
ret = IpBlbSetAttr(BLOB_CLEANBORDER, 0)

'set filter ranges
ret = IpBlbSetFilterRange(BLM_AREA, vintRangeMin, vintRangeMax)
'apply filter ranges
ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)

'Set segmentation to Max
ret = IpBlbShow(1)
ret = IpSegShow(1)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegSetAttr(Channel, 0)
ret = IpSegPreview(ALL_C_T)
ret = IpSegShow(2)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegPreview(ALL_C_T)
ret = IpSegShow(1)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegSetAttr(Channel, 0)
ret = IpSegPreview(ALL_C_T)
ret = IpSegShow(2)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegPreview(ALL_C_T)
ret = IpSegSetRange(0, 0, 255)
ret = IpSegView(ALL_C_T)
ret = IpSegSetAttr(Channel, 1)
ret = IpSegSetRange(1, 0, 255)
ret = IpSegView(ALL_C_T)
ret = IpSegSetAttr(Channel, 2)
ret = IpSegSetRange(2, 0, 255)
ret = IpSegView(ALL_C_T)
APPENDIX A (Continued)

ret = IpSegShow(0)
ret = IpBlbCount()
ret = IpBlbUpdate(0)
ret = fncExcelDataExport("AOI", vintExcelRow)
End Function

'measurement function that calls Area, Density, and AOI functions
Function fncMeasure()

'capture AOI
ret = IpMacroStop("Please select Area of Interest", 0)

'open count/size dialog
ret = IpBlbShow(1)

'Percent Area
If chkArea.value = vbChecked Then
  fncPercentArea
End If

End Function

Private Sub Capture_Click()
Dim y As Integer
y = 1
ret = IpAcqShow(ACQ_SNAP, 1)
ret = IpAcqShow(ACQ_SNAP, 3)
ret = IpAcqShow(ACQ_LIVE, 1)
Load frmAutoName
frmAutoName.Show
End Sub

'MAIN SUB
Private Sub cmdCalculate_Click()

vintExcelRow = 3 'These 3 vars. are initialized here.
vintDirectoryIndex = 0 'For IpStSearch function - Load image index for Dir
vintDirectoryStatus = 1 'For IpStSearch function - if 1, images still in Dir

vstrSegmentationFile = txtSegmentationFile.text 'segmentation file path to variable

'--------------------------------Input Checks
'Checks to see if user entered file path in background text box

If txtBackgroundImage = "" Then
    MsgBox ("Please Enter Background Image")
    Exit Sub
End If

If txtSegmentationImage = "" Then
    MsgBox ("Please Enter Segmentation Image")
    Exit Sub
End If

If txtSegmentationFile = "" Then
    MsgBox ("Please Enter or Create New Segmentation File")
    Exit Sub
End If

If txtExcelFile = "" Then
    MsgBox ("Please Enter Name of Excel Spreadsheet")
    Exit Sub
End If

If txtTargetImageDirectory = "" Then
    MsgBox ("Please Enter Target Image Directory")
    Exit Sub
End If

'--------------------------------End Input Checks
APPENDIX A (Continued)

    cmdCalculate.Visible = False
    lblCurrentImageLabel.Visible = True
    lblCurrentImage.Visible = True

    IPPMACRO.Height = 6855

    'sends targetimagedir text box to string variable
    vstrTargetImageDirectory = txtTargetImageDirectory.text

    'sends background text box to string variable and loads it using IPbasic function
    vstrBackgroundImage = txtBackgroundImage.text
    ret = IpWsLoad(vstrBackgroundImage, "tif")
    ret = IpBlbEnableMeas(BLBM_DENSITY, 1)
    ret = IpBlbEnableMeas(BLBM_DENSSUM, 1)
    ret = IpBlbEnableMeas(BLBM_AREA, 1)

    fncLoadDir

    If vintDirectoryStatus = 1 Then
        fncMeasure
    End If

End Sub

Function fncLoadDir()

    vintDirectoryStatus = IpStSearchDir(vstrTargetImageDirectory, ".*\.(tif), _
    vintDirectoryIndex, vstrTargetImage)

    If vintDirectoryStatus <> 1 Then
        ret = IpMacroStop("No More Images in Directory", MS_STOP)
        cmdIcContinue.Enabled = False
        cmdIcContinue.Font.Strikethrough = True
        Exit Function
    End If

    lblCurrentImage.caption = vstrTargetImage

    'sends background text box to string variable and loads it using IPbasic function
APPENDIX A (Continued)

' vstrBackgroundImage = txtBackgroundImage.text
' ret = IpWsLoad(vstrBackgroundImage, "tif")

'loads target image to IPP
ret = IpWsLoad(vstrTargetImage, "tif")

'Background Correction
ret = IpOpShow(3)
ret = IpOpBkgndSubtract(0, 0)
ret = IpOpShow(2)

'Apply Filters if Applicable

'Index (1) is for HighGauss
If optFilter.Item(1) Then
    ret = IpFltConvolveKernel("HIGAUSS.7x7", 10, 1) 'HiGauss Filter Size:5x5; Strength:100%; Passes:1
End If

'Index (2) is for Lar's Amyloid Macro Filters
If optFilter.Item(2) Then
    ret = IpFltFlatten(0, 20)
    ret = IpWsConvertToGray()
    ret = IpFltConvolveKernel("HIGAUSS.7x7", 2, 1) 'HiGauss Filter Size:5x5; Strength:100%; Passes:1
    ret = IpHstEqualize(EQ_BESTFIT)
    ret = IpSegLoad(vstrSegmentationFile)
Else
    'loads segmentation file
    ret = IpSegLoad(vstrSegmentationFile)
    ret = IpSegShow(0)
    ret = IpSegSetAttr(SETCURSEL, 0)
    ret = IpSegSetAttr(Channel, 0)
    ret = IpSegPreview(ALL_C_T)
    ret = IpSegShow(0)
    ret = IpBlbShow(1)
End If
APPENDIX A (Continued)
End Function
'Allows user to create segmentation file from segmentation image if non existant
Private Sub cmdCreateSegmentationFile_Click()
ret = IpAppCloseAll
'Checks to see if user entered file path in background text box
If txtBackgroundImage = "" Then
MsgBox ("Please Enter Background Image")
Exit Sub
End If
'Checks to see if user entered file path in background text box
If txtSegmentationImage = "" Then
MsgBox ("Please Enter Segmentation Image")
Exit Sub
End If
If MsgBox("Are you using Lars-HiGauss Filter Option?", vbYesNoCancel,
"Create Sementation File") = vbYes Then
optFilter(2) = True
End If
'Load Background Image
vstrBackgroundImage = txtBackgroundImage.text
ret = IpWsLoad(vstrBackgroundImage, "tif")
'Load Segmentation Image
vstrSegmentationImage = txtSegmentationImage.text
ret = IpWsLoad(vstrSegmentationImage, "tif")
'Background Subtraction
ret = IpOpShow(3)
ret = IpOpBkgndSubtract(0, 0)
ret = IpOpShow(2)
If optFilter.Item(2) Then
ret = IpBlbShow(1)
ret = IpFltFlatten(0, 20)
ret = IpBlbShow(0)
ret = IpWsConvertImage(IMC_GRAY, CONV_SCALE, 0, 0, 0, 0)
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APPENDIX A (Continued)

ret = IpFltShow(1)
ret = IpFltConvolveKernel("HIGAUSS.7x7", 2, 1)
ret = IpFltShow(0)
ret = IpHstEqualize(EQ_BESTFIT)

ret = IpBlbShow(1)
ret = IpSegShow(1)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegSetAttr(Channel, 0)
ret = IpSegPreview(CURRENT_C_T)

ret = IpMacroStop("Adjust Segmentation and Press Continue to Save Segmentation File", 0)

With CommonDialog1
   .FileName = ""
   .Filter = "Color Segmentation Files (*.rge)|*.rge|All Files|*.*"
   .FilterIndex = 1
   .ShowSave
   vstrSegmentationFile = .FileName
   txtSegmentationFile = .FileName
End With

Else

ret = IpSegShow(1)
ret = IpSegSetAttr(SETCURSEL, 0)
ret = IpSegSetAttr(Channel, 0)
ret = IpSegPreview(ALL_C_T)
ret = IpMacroStop("Adjust Segmentation and Press OK to Save Segmentation File", 0)

With CommonDialog1
   .FileName = ""
   .Filter = "Color Segmentation Files (*.rge)|*.rge|All Files|*.*"
   .FilterIndex = 1
   .ShowSave
   vstrSegmentationFile = .FileName
   txtSegmentationFile = .FileName
End With
APPENDIX A (Continued)

End If

ret = IpSegSave(vstrSegmentationFile, 0)
ret = IpSegShow(0)
ret = IpAppCloseAll

End Sub

Private Sub cmdExit_Click()

Unload Me

End Sub

Private Sub cmdIcContinue_Click()

ret = fncExcelDataExport("% Area", vintExcelRow)

'Density Measurement
If chkDensity.value = vbChecked Then
    fncDensity
    ret = fncExcelDataExport("Density", vintExcelRow)
End If

'Area of Interest Total Area
fncAOI

'index for directory image
vintDirectoryIndex = vintDirectoryIndex + 1

'index for Excel Row
vintExcelRow = vintExcelRow + 1

'Reset Comment Box
txtIcComment.text = ""

ret = IpDocClose()

fncLoadDir
APPENDIX A (Continued)

If vintDirectoryStatus = 1 Then
    fncMeasure
Else
    ret = IpAppCloseAll()
End If

End Sub

Private Sub cmdIcDeselectItem_Click()
    ret = IpAoiShow(FRAME_NONE)
    ret = IpTemplateMode(1)
    ret = IpBlbHideObject(0, 0, 0)
    ret = IpTemplateMode(0)
    ret = IpAoiShow(FRAME_IRREGULAR)

End Sub

Private Sub cmdIcModifySegmentation_Click()
    ret = IpSegShow(1)
    ret = IpSegShow(1)
    ret = IpSegSetAttr(SETCURSEL, 0)
    ret = IpSegSetAttr(Channel, 0)
    ret = IpSegPreview(ALL_C_T)
    ret = IpSegShow(2)
    ret = IpSegSetAttr(SETCURSEL, 0)
    ret = IpSegPreview(ALL_C_T)
    ret = IpSegShow(1)
    ret = IpSegSetAttr(SETCURSEL, 0)
    ret = IpSegSetAttr(Channel, 0)
    ret = IpSegPreview(ALL_C_T)
    ret = IpSegShow(1)
    ret = IpSegSetAttr(SETCURSEL, 0)
    ret = IpSegSetAttr(Channel, 0)
    ret = IpSegPreview(ALL_C_T)
    ret = IpMacroStop("Adjust Segmentation and Press OK", 0)
    ret = IpSegShow(0)
    ret = IpBlbCount()
APPENDIX A (Continued)

ret = IpBlbUpdate(0)

End Sub

'Opens CommonDialog1 "Open File" dialog and sends file to appropriate textbox

Private Sub cmdOpenBackground_Click()
    With CommonDialog1
        .Filter = "TIF|*.tif|JPEG (.jpg)|*.jpg|All Files|*.*"
        .FilterIndex = 1
        .ShowOpen
        txtBackgroundImage = .FileName
    End With

End Sub

'Opens CommonDialog1 "Open File" dialog and sends file to appropriate textbox

Private Sub cmdOpenExcel_Click()
    With CommonDialog1
        .Filter = "Excel Spreadsheet File (.xls)|*.xls|All Files|*.*"
        .FilterIndex = 1
        .ShowOpen
        txtExcelFile = .FileName
    End With

End Sub

'Opens "Browse Directory Structure" dialog and allows one to choose directory and
'sends chosen directory to textbox

Private Sub cmdOpenImageDirectory_Click()
    Dim udtBrowseInfo As BROWSEINFO
    Dim lRet As Long
    Dim lPathID As Long
    Dim sPath As String
Dim nNullPos As Integer

    txtTargetImageDirectory.SetFocus

    'Specify the window handle for the owner of the dialog box
    udtBrowseInfo.hOwner = Me.hwnd

    'Specify the root to start browsing from;
    'if null, My Computer is the root
    udtBrowseInfo.pidlRoot = 0&

    'Specify a title. This is not the caption of the dialog. Useful for
    'adding any kind of additional information or instructions
    udtBrowseInfo.lpszTitle = "Select a folder"

    'Specify any flags; See Declarations section
    udtBrowseInfo.ulFlags = BIF_RETURNONLYFSDIRS

    'Call the function.
    'The return value is a pointer to an item identifier list that
    'specifies the location of the selected folder.
    'If the user cancels the dialog box, the return value is 0.
    lPathID = SHBrowseForFolder(udtBrowseInfo)

    sPath = Space$(512)
    lRet = SHGetPathFromIDList(lPathID, sPath)

    If lRet Then
        nNullPos = InStr(sPath, vbNullChar)
        txtTargetImageDirectory = Left(sPath, nNullPos - 1)
    End If

End Sub

'Opens CommonDialog1 "Open File" dialog and sends file to appropriate textbox

Private Sub cmdOpenSegmentationFile_Click()
    With CommonDialog1
        .Filter = "Color Segmentation Files (*.rge)|*.rge|All Files|*.*"
        .FilterIndex = 1
    End With
End Sub
APPENDIX A (Continued)

'.ShowOpen
    txtSegmentationFile = .FileName
End With

End Sub

'Opens CommonDialog1 "Open File" dialog and sends file to appropriate textbox
Private Sub cmdOpenSegmentationImage_Click()

    With CommonDialog1
        .Filter = "TIF|*.tif|JPEG (.jpg)|*.jpg|All Files|*.*"
        .FilterIndex = 1
        .ShowOpen
        txtSegmentationImage = .FileName
    End With

End Sub

Private Sub Exit_Click()
Unload Me
End Sub

Private Sub Form_Load()

    'Both "WinExec" and "SetWindowPos" require API calls - The real WinAPI file is too
    'large to be included in a VB executable, so a modified API file, DCmodAPI.rtf, is
    'used instead.

    'Loads IPP on start of program
    ret = WinExec("c:\ipwin4\ipwin32.exe", 1)

    'keeps VB controls on top of IPP
    '-1 = HWND_TOPMOST
    '&H2 = SWP_NOMOVE
    '&H1 = SWP_NOSIZE
    '4 Numbers between are screen coordinates
APPENDIX A (Continued)

ret = SetWindowPos(IPPMACRO hwnd, -1, 0, 0, 0, &H2 + &H1)

ret = IpAppCloseAll
chkArea.value = vbChecked

IPPMACRO.Height = 5840

'opens excel using user defined function
fncOpenExcel

End Sub
APPENDIX A (Continued)

'IpUtil32.bas

Function iprint(mystring As Variant)
    ' Replaces the print command in IP-Basic v. 3.0.
    ' Sends the string argument to the output window.
    IpOutput (mystring + Chr$(13) + Chr$(10))
End Function

Function IpTrim(mystring As String) As String
    ' Replaces RTrim$ command in IP-Basic v.3.0.
    ' The old command used to trim zeros as well as spaces.
    Dim Index%
    Index = InStr(mystring, Chr$(0))
    If Index > 0 Then IpTrim = Trim$(Left$(mystring, Index - 1)) Else IpTrim = Trim$(mystring)
End Function

Function IpDocActive() As Integer
    ' Returns the document Id of the active image
    ' Returns -1 if no image is displayed.
    Dim docid As Integer
    ret = IpDocGet(GETACTDOC, 0, docid)
    If ret < 0 Then IpDocActive = -1 Else IpDocActive = docid
End Function
'ModBrowse.bas

Option Explicit

'Function Declarations, type structure, and constants to use the 'Browse for Folder dialog box. For more information on these, 'consult the SDK, included with VB 4.0 Pro or Ent editions as 'part of the MSDN/VB Starter Kit.

Public Declare Function SHGetPathFromIDList Lib "shell32.dll" _
Alias "SHGetPathFromIDListA" (ByVal pidl As Long, _
ByVal pszPath As String) As Long

Public Type BROWSEINFO
    hOwner As Long
    pidlRoot As Long
    pszDisplayName As String
    lpszTitle As String
    ulFlags As Long
    lpfn As Long
    lParam As Long
    iImage As Long
End Type

Public Declare Function SHBrowseForFolder Lib "shell32.dll" _
Alias "SHBrowseForFolderA" (lpBrowseInfo As BROWSEINFO) As Long

'Below are the constants which can be specified in the ulFlags member 'of the BROWSEINFO structure.

'BIF_RETURNONLYFSDIRS = &H1
APPENDIX A (Continued)

'Does not include network folders below the domain level in the 'tree view control.
Public Const BIF_DONTGOBELOWDOMAIN = &H2

'Only returns file system ancestors. If the user selects anything 'other than a file system ancestor, the OK button is grayed.
Public Const BIF_RETURNFSANCESTORS = &H8

'Only returns computers. If the user selects anything other than 'a computer, the OK button is grayed.
Public Const BIF_BROWSEFORCOMPUTER = &H1000

'Only returns printers. If the user selects anything other than 'a printer, the OK button is grayed.
Public Const BIF_BROWSEFORPRINTER = &H2000

'Includes a status area in the dialog box. The callback function can set the status text by sending messages to the dialog box.
Const BIF_STATUSTEXT = &H4
'ModFileO.BAS

Option Explicit

'Function Declarations, type structure, and constants for the Open and Save 'common dialog boxes. For more information on these, consult the SDK, 'included with VB 4.0 Pro or Ent editions as part of the MSDN/VB Starter 'Kit.

Public Type OPENFILENAME
  lStructSize As Long
  hwndOwner As Long
  hInstance As Long
  lpstrFilter As String
  lpstrCustomFilter As String
  nMaxCustFilter As Long
  nFilterIndex As Long
  lpstrFile As String
  nMaxFile As Long
  lpstrFileTitle As String
  nMaxFileTitle As Long
  lpstrInitialDir As String
  lpstrTitle As String
  Flags As Long
  nFileOffset As Integer
  nFileExtension As Integer
  lpstrDefExt As String
  lCustData As Long
  lpfnHook As Long
  lpTemplateName As String
End Type

'Functions and constants for the common dialog boxes
APPENDIX A (Continued)

Public Declare Function GetOpenFileName Lib "comdlg32.dll" _
Alias "GetOpenFileNameA" (pOPENFILENAME As OPENFILENAME) As Long

Public Declare Function GetSaveFileName Lib "comdlg32.dll" _
Alias "GetSaveFileNameA" (pOPENFILENAME As OPENFILENAME) As Long

' These constants must be declared since the Common Dialog control is not
' part of the project and, therefore, the intrinsic constants are not
' defined.
Public Const OFN_READONLY = &H1
Public Const OFN_OVERWRITEPROMPT = &H2
Public Const OFN_HIDEREADONLY = &H4
Public Const OFN_NOCHANGEDIR = &H8
Public Const OFN_SHOWHELP = &H10
Public Const OFN_ENABLEHOOK = &H20
Public Const OFN_ENABLETEMPLATE = &H40
Public Const OFN_ENABLETEMPLATEHANDLE = &H80
Public Const OFN_NOVALIDATE = &H100
Public Const OFN_ALLOWMULTISELECT = &H200
Public Const OFN_EXTENSIONDIFFERENT = &H400
Public Const OFN_PATHMUSTEXIST = &H800
Public Const OFN_FILEMUSTEXIST = &H1000
Public Const OFN_CREATEPROMPT = &H2000
Public Const OFN_SHAREAWARE = &H4000
Public Const OFN_NOREADONLYRETURN = &H8000
Public Const OFN_NODEREFERENCELINKS = &H10000
Public Const OFN_LONGNAMES = &H20000
Public Const OFN_NOLONGNAMES = &H40000         ' force no long names for
4.x modules
Public Const OFN_EXPLORER = &H80000         ' new look comdlg
Public Const OFN_NODEREFERENCETELINKS = &H10000
Public Const OFN_LONGNAMES = &H20000         ' force long names for 3.x
modules
'DCmodAPI.rtf

Const STARTF_USESHOWWINDOW = &H1

Declare Function WinExec Lib "kernel32" (ByVal lpCmdLine As String, ByVal nCmdShow As Long) As Long

' error values for ShellExecute() beyond the regular WinExec() codes
Const SE_ERR_SHARE = 26
Const SE_ERR_ASSOCINCOMPLETE = 27
Const SE_ERR_DDETIMEOUT = 28
Const SE_ERR_DDEFAIL = 29
Const SE_ERR_DDEBUSY = 30
Const SE_ERR_NOASSOC = 31

' ShellExecute() and ShellExecuteEx() error codes

' regular WinExec() codes
Const SE_ERR_FNF = 2 ' file not found
Const SE_ERR_PNF = 3 ' path not found
Const SE_ERR_ACCESSDENIED = 5 ' access denied
Const SE_ERR_OOM = 8 ' out of memory
Const SE_ERR_DLLNOTFOUND = 32

' Note CLASSKEY overrides CLASSNAME
Const SEE_MASK_CLASSNAME = &H1
Const SEE_MASK_CLASSKEY = &H3
' Note INVOKEIDLIST overrides IDLIST
Const SEE_MASK_IDLIST = &H4
Const SEE_MASK_INVOKEIDLIST = &HC
Const SEE_MASK_ICON = &H10
Const SEE_MASK_HOTKEY = &H20
Const SEE_MASK_NOCLOSEPROCESS = &H40
Const SEE_MASK_CONNECTNETDRV = &H80
Const SEE_MASK_FLAG_DDEWAIT = &H100
Const SEE_MASK_DOENVSUBST = &H200
Const SEE_MASK_FLAG_NO_UI = &H400

Type SHELLEXECUTEINFO
    cbSize As Long
    fMask As Long
hwnd As Long
lpVerb As String
lpFile As String
lpParameters As String
lpDirectory As String
nShow As Long
hInstApp As Long
' Optional fields
lpIDList As Long
lpClass As String
hkeyClass As Long
dwHotKey As Long
hIcon As Long
hProcess As Long

End Type

Declare Sub WinExecError Lib "shell32.dll" Alias "WinExecErrorA" (ByVal hwnd As Long, ByVal error As Long, ByVal lpstrFileName As String, ByVal lpstrTitle As String)

Const SW_HIDE = 0
Const SW_SHOWNORMAL = 1
Const SW_NORMAL = 1
Const SW_SHOWMINIMIZED = 2
Const SW_SHOWMAXIMIZED = 3
Const SW_MAXIMIZE = 3
Const SW_SHOWNOACTIVATE = 4
Const SW_SHOW = 5
Const SW_MINIMIZE = 6
Const SW_SHOWMINNOACTIVE = 7
Const SW_SHOWNA = 8
Const SW_RESTORE = 9
Const SW_SHOWDEFAULT = 10
Const SW_MAX = 10

' Old ShowWindow() Commands
Const HIDE_WINDOW = 0
Const SHOW_OPENWINDOW = 1
Const SHOW_ICONWINDOW = 2
Const SHOW_FULLSCREEN = 3
Const SHOW_OPENNOACTIVATE = 4
APPENDIX A (Continued)

' Identifiers for the WM_SHOWWINDOW message
Const SW_PARENTCLOSING = 1
Const SW_OTHERZOOM = 2
Const SW_PARENTOPENING = 3
Const SW_OTHERUNZOOM = 4

Const WM_SHOWWINDOW = &H18
Declare Function ShowWindow Lib "user32" (ByVal hwnd As Long, ByVal nCmdShow As Long) As Long
Const SWP_SHOWWINDOW = &H40

Declare Function ShowWindowAsync Lib "user32" (ByVal hwnd As Long, ByVal nCmdShow As Long) As Long

'----------------------------------------------------
'Set Window Pos
Declare Function SetWindowPos Lib "user32" (ByVal hwnd As Long, ByVal hWndInsertAfter As Long, ByVal x As Long, ByVal y As Long, ByVal cx As Long, ByVal cy As Long, ByVal wFlags As Long) As Long

'SetWindowPos Flags
Const SWP_NOSIZE = &H1
Const SWP_NOMOVE = &H2
Const SWP_NOZORDER = &H4
Const SWP_NOREDRAW = &H8
Const SWP_NOACTIVATE = &H10
Const SWP_FRAMECHANGED = &H20        '  The frame changed: send WM_NCCALCSIZE
'Const SWP_SHOWWINDOW = &H40
Const SWP_HIDEWINDOW = &H80
Const SWP_NOCOPYBITS = &H100
Const SWP_NOOWNERZORDER = &H200      '  Don't do owner Z ordering

Const SWP_DRAWFRAME = SWP_FRAMECHANGED
Const SWP_NOREPOSITION = SWP_NOOWNERZORDER

'SetWindowPos() hwndInsertAfter values
Const HWND_TOP = 0
Const HWND_BOTTOM = 1
Const HWND_TOPMOST = -1
Const HWND_NOTOPMOST = -2

Type DLGTEMPLATE
APPENDIX A (Continued)

```vba
style As Long
dwExtendedStyle As Long
cdit As Integer
x As Integer
y As Integer
cx As Integer
cy As Integer
End Type

Type DLGITEMTEMPLATE
    style As Long
dwExtendedStyle As Long
    x As Integer
    y As Integer
cx As Integer
cy As Integer
    id As Integer
End Type
```
APPENDIX B

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Previously Published

Cognitive impairment in PDAPP mice depends on ApoE and ACT-catalyzed amyloid formation

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1. Abstract

Biochemical and genetic studies indicate that the inflammatory proteins, Apolipoprotein E (ApoE) and α1-Antichymotrypsin (ACT) are important in the pathogenesis of Alzheimer's disease (AD). Using several lines of multiply transgenic/knockout mice we show here that murine ApoE and human ACT separately and synergistically facilitate both diffuse Aβ immunoreactive and fibrillar amyloid deposition and thus also promote cognitive impairment in aged PDAPP(V717F) mice. The degree of cognitive impairment is highly correlated with the ApoE- and ACT-dependent hippocampal amyloid burden, with PDAPP mice lacking ApoE and ACT having little amyloid and little learning disability. Analysis of young mice before the onset of amyloid formation shows that steady state levels of monomeric Aβ peptide are unchanged by ApoE or ACT. These data suggest that the process or product of amyloid formation is more critical than monomeric Aβ for the neurological decline in AD, and that the risk factors ApoE and ACT participate primarily in disease processes downstream of APP processing.

Key words: α1-Antichymotrypsin, Apolipoprotein E, Alzheimer's disease, amyloid deposition, learning, memory, inflammation, transgenic mice, amyloid β-peptide
1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized psychologically by progressive mental decline and defined histopathologically by parenchymal amyloid deposits and neurofibrillary tangles. Mutations within the amyloid precursor protein (APP) gene that cause inherited AD place APP and the Aβ peptide at the center of the disease process [52, 26, 58]. Whether Aβ per se or the process or product of its conversion into amyloid underlies AD neurodegeneration remains unknown.

Insight into the role of APP and Aβ in AD may be provided by genetic and biochemical studies showing other proteins to be involved in the disease process. For example, Aβ amyloid deposits also contain α1-antichymotrypsin (ACT; [1]) and apolipoprotein E (ApoE; [43, 73]), which are over-expressed in affected regions of the AD brain as part of an inflammatory process [74, 1]. ACT levels are also increased in AD serum and CSF ([15]for data and discussion). It was proposed at the time of their discoveries that ApoE and ACT might function as amyloid promoters or “Pathological Chaperones”, and both in vitro and in vivo studies support this model [36, 56, 72, 8, 7, 28, 41, 45].

As with APP, the importance of inflammation and specifically ApoE and ACT in AD is supported by genetics. Inheritance of ApoE4 is the strongest risk factor for AD besides age [13, 59], and an ACT/A signal peptide variant that increases mature glycosylated ACT available for secretion [46] increases AD
susceptibility and pathology [30, 75] (for discussion see [44, 51]). Similarly, polymorphisms in the IL-1 promoter greatly increase the risk of AD [40]. These genetic variations increase IL-1 expression during inflammation and would therefore be expected to promote amyloid formation by increasing the production of both APP and ACT [24, 10, 22, 14, 46, 55].

Although genetic and biochemical studies have identified key proteins in the AD pathogenic pathway, it remains unclear what role Aβ, inflammation, and amyloid formation play in the cognitive dysfunction of AD. To answer this question, cognitive studies should be performed with AD mouse models in which the inflammatory proteins and the Aβ deposition they influence can be regulated without changing the level of monomeric Aβ peptide. To this end, we have generated and analyzed four mouse models of AD and find that 1) ApoE and ACT independently and synergistically promote Aβ immunoreactive and mature amyloid deposition without initially affecting Aβ levels and 2) cognitive impairment in aged AD mice depends on the amyloid promoting effect of ApoE and/or ACT.

2. Materials and methods

Construction of transgenic mice. A 1.5kbp full-length human ACT-cDNA-clone was subcloned into a modified GFAP-expression vector construct as previously described [45]. Heterozygous mice from one of these founder lines (#8784, FVB/N) were crossed with homozygous PDGF-hAPP(V717F) mice [Swiss-Webster x C57BL/6 x DBA/2] to generate mice with genotypes APP+/−.
APPENDIX B (Continued)

/ApoE<sup>+/+</sup>/ACT<sup>+/+</sup> and APP<sup>+</sup>/ApoE<sup>+/+</sup>/ACT<sup>+/+</sup>. Heterozygous ACT mice were also crossed with ApoE<sup>-/-</sup> mice [Swiss-Webster x C57BL/6 x DBA/2] to generate mice with genotype ApoE<sup>-/-</sup>/ACT<sup>+/+</sup>. These mice were then crossed with APP<sup>+</sup>/ApoE<sup>-/-</sup> mice [(Swiss-Webster x C57BL/6 x DBA/2) x C57BL/6] to generate mice with genotypes APP<sup>+</sup>/ApoE<sup>-/-</sup>/ACT<sup>+/+</sup> and APP<sup>+</sup>/ApoE<sup>-/-</sup>/ACT<sup>-/-</sup>. All offspring were screened by PCR for identity of the GFAP-ACT gene [45], the PDGF-hAPP gene [23], as well as the mouse ApoE gene and neo gene [8]. The basal ACT protein expression in the transgenic mice was 12 pmol/g wet weight in the cerebral cortex and 24 pmol/g wet weight in the hippocampus, which is at least ten-fold lower than the levels detected in human AD brain tissue [1, 32, 45]. The presence or absence of proper ACT protein expression in astrocytes was further verified by ACT-immunohistochemistry in all of the pathologically and behaviorally examined animals. The genetic background of the nontransgenic mice was Swiss-Webster. For the ease of reading, a single plus sign immediately following APP or ACT genotype refers to heterozygosity unless otherwise stated in the text. The presence of the normal complement of two murine ApoE genes in a strain is indicated in parenthesis as (mApoE+) and the absence of the human ACT transgene by (ACT-) for the sake of clarity. The four lines are therefore designated:

APP+(mApoE+), ACT+  (heterozyg PDAPP/homozyg murine ApoE/heterozyg ACT)

APP+(mApoE+)(ACT -)  (heterozyg PDAPP/homozyg murine ApoE/no ACT)

APP+, mApoE-KO, ACT+  (heterozyg PDAPP/murine ApoE knockout/heterozyg ACT)

APP+, mApoE-KO (ACT-)  (heterozyg PDAPP/murine ApoE knockout/no ACT)
ELISA Analysis of Aβ levels. At two months of age, animals of each genotype were sacrificed and their brains prepared for ELISA as previously described [18], with the modification that PBS rather than TBS was used to extract soluble Aβ.

General Protocol and Behavioral Analysis. The four groups of transgenic mice, along with three strains of non-transgenic mice (Swiss-Webster, B6D2/F1, and FVBJ) were maintained on a 12-hr light-dark cycle. The animals were provided free access to water and rodent chow, with behavioral testing always done during the light phase and by investigators unaware of animal genotypes. Beginning at 16 months of age, all animals were tested in a 6-week battery of sensorimotor, anxiety and cognitive tasks, which included the Morris water maze and the radial arm water maze (RAWM) as previously described [6, 38]. Two groups of animals, APP+ (mApoE+) (ACT-) and nontransgenic littermates, were behaviorally tested in the same battery of tasks at two months of age to determine whether animals destined to deposit amyloid were impaired at an early age before deposition began.

Briefly, the RAWM consisted of a circular pool, 1 meter in diameter, with six swim arms (19 cm wide) radiating from an open central area (40 cm in diameter). A variety of spatial cues were present on the walls and ceiling in the immediate vicinity of the pool for RAWM testing (as well as for Morris water maze testing), in order to spatially orient the animals while swimming. The
submerged escape platform was positioned near the end of a different arm for each of the 9 days of testing, thus forcing the animals to use spatial working memory to decrease their escape latency to find a given days “goal” arm over 5 trials per day. Four consecutive acquisition trials were done (T1-T4), followed by a retention trial (T5) 30 minutes later. The five arms not containing the platform were designated as start arms for each of the one-minute trials, in a varied semi-randomized sequence for each day of testing. For any given trial, the animal was placed in a start arm and allowed to make arm choices, with each incorrect choice resulting in the animal being gently pulled back to the start arm for that trial. The time required to locate the submerged platform was recorded and the animal was allowed to remain on the escape platform for 30 seconds. Animals that did not find the platform within any 60 sec trial were guided to the platform and the maximal 60 sec time was recorded. RAWM latency data were analyzed over three 3-day blocks. Because unimpaired animals generally require the first two blocks to learn this task’s procedural aspects, block 3 of testing (days 7-9) is most indicative of cognitive performance.

The Morris water maze consisted of an open circular pool, with a diameter of 1 meter, which was divided in four quadrants. An indiscernible 9-cm platform was positioned in quadrant 2 (Q2) 1.5cm below the water surface. Acquisition testing involved four trials per day for 9 days wherein an animal was placed successively into each of the four quadrants to initiate a 60 sec trial. The time to locate the platform was recorded for each trial and the
average latency to find the submerged platform was calculated for these four trials. Upon locating and ascending the platform (or after 60 sec) the animal was allowed a 30 sec inter-trial resting period on the platform.

Histology and Immunohistochemistry. At 18 months of age, brains were dissected from anesthetized and intracardially perfused mice and 25μm coronal tissue sections were processed as previously described [45]. The mounted sections were processed through antigen retrieval in prewarmed 25mM citrate buffer (pH 7.3) at +82°C for 5 min and further processed as previously described [45]. The sections were incubated with primary antibodies rabbit anti-ACT (AXL-145, dil 1:1000, Accurate) and mouse anti-Aβ (6E10, dil 1:5000, Senetek) overnight at +4°C. Secondary antibodies were anti-rabbit IgG (BA-1000, 1:300, Vector) or anti-mouse IgG and developed with NovaRED substrate kit (Vector). Congo Red-staining and Thioflavine S was performed according to well-established protocols[16].

Image analysis. Data were collected from five equally spaced coronal tissue sections for both dorsal hippocampus and overlying parietal cortex (Bregma -1.06 to -2.30mm; [45]. The sections were examined with a Nikon Eclipse E600 microscope at 100X (6E10 immunostaining) and 200X (Congo Red) magnification at a constant predefined light setting and video images captured with a color CCD-camera. All images were then processed through shading correction. The 6E10 immunostaining was converted to a grayscale image and
APPENDIX B (Continued)

segmented with an auto threshold function (Image Pro Plus, Media Cybernetics). Diffuse immunoreactive Aβ deposition (6E10) was estimated as area fraction (=stained area_{tot}/measured area_{tot}, expressed in %). Congo Red positive amyloid plaques were circled at 200X magnification, and their location noted on an anatomic atlas. The captured image was segmented with respect to threshold settings for RGB that had been specified prior to analysis so as to distinguish specific signals from background. The area occupied by amyloid within each circle, as defined by the image segmentation, was then quantitated and an area fraction calculated from the total measured area as previously described [45].

Statistical analysis. The histopathological results were analyzed with factorial ANOVA and post hoc Fisher LSD test. The behavioral data were evaluated with One-way ANOVA and post-hoc Fisher LSD test of each separate trial. Repeat-measures ANOVA across all trials yielded almost identical results. The correlations between histopathological and behavioral analysis were examined with linear regression. Discriminant analysis was performed to determine whether groups of mice with different strain backgrounds, different genotypes or varying amyloid depositing capacity, could be distinguished from one another behaviorally. The analysis, which was based on the performance of 7-19 measures from our behavioral test battery, was performed with the DISCRIM subroutine of the SYSTAT software package. The discriminant analysis was based on the following measures:
1. Open field activity (line crossings during a 5 minute trial),
2. Balance Beam (time before falling during 3 successive trials, in seconds),
3. String Agility (agility to put hindpaws and/or tail around a suspended string during a 1 minute trial),
4. Y-maze choices (number of arm choices in a single 5 minute trial),
5. Y-maze percent alternation (in a single 5 minute trial),
6. Morris maze acquisition (averaged daily latencies over 9 days of testing),
7. Morris maze acquisition (averaged latency for the last day of testing),
8. Morris maze retention (percent of time spend in former platform quadrant during a probe trial done the day following acquisition),
9. Circular platform errors (averaged over 8 days of testing),
10. Circular platform errors (last day of testing),
11. Circular platform escape latency (averaged over 8 days of testing),
12. Circular platform escape latency (last day of testing),
13. Platform recognition (averaged daily latencies over 4 days of testing),
14. Platform recognition (averaged latency for the last day of testing),
15. Radial arm water maze - Trial 4 latency over all 3 blocks of testing,
16. Radial arm water maze - Trial 5 latency over all 3 blocks of testing,
17. Radial arm water maze - Trial 1 latency for the last block of testing,
18. Radial arm water maze - Trial 4 latency for the last block of testing,
19. Radial arm water maze - Trial 5 latency for the last block of testing.

Discriminant analysis forms new variables called “discriminant functions”, which are linear composites of the multiple original measures that are used to evaluate the overall performance of groups of animals. The number of such functions generated always equals the number of groups analyzed minus one.
APPENDIX B (Continued)

Thus, if at least three groups are being compared, any two “discriminant functions” can be graphed to visually illustrate the presence or absence of group discrimination, as mathematically evaluated through the statistical analysis (Pillai’s trace).

3. Results

To study the effect of ApoE and ACT on Aβ immunoreactive and mature amyloid formation and cognitive decline, we have generated and analyzed four transgenic mouse lines: APP+(mApoE+)(ACT-) (n=11), APP+,(mApoE+),ACT+ (n=17), APP+,mApoE-KO,ACT- (n=8), and APP+,mApoE-KO,ACT+ (n=9). Animals from these heterozygous transgenic lines were behaviorally analyzed along with a group of nontransgenic mice (n=5) at 16 months of age and subsequently sacrificed at 18 months of age for histopathological evaluation. Other mice from these four lines were also sacrificed at two months of age to determine the effect(s) of ApoE and ACT on the steady state levels of monomeric Aβ before amyloid deposition begins. Some were also behaviorally analyzed to determine whether animals destined to deposit amyloid were impaired at this early age.

ApoE and ACT expression in the brain do not increase the steady state levels of Aβ_{1-40} or Aβ_{1-42} prior to amyloid deposition in PDAPP transgenic mice
All of the APP and presenilin mutations that have been found to cause familial Alzheimer's disease with an autosomal dominant mode of inheritance appear to act by increasing the amount of the Aβ(1-42) peptide, which displays a high propensity to polymerize into amyloid filaments [57, 20]. If ApoE or ACT were to similarly influence AD by affecting Aβ production or clearance, one would predict that steady state levels of monomeric Aβ should be altered in young PDAPP/ACT transgenic or PDAPP/mApoE-KO mice in comparison to unaltered PDAPP mice. In contrast, if the main role of ApoE and ACT is to promote the formation of amyloid deposits, the presence or absence of these genes would not necessarily affect monomeric Aβ levels.

To investigate the potential role of ApoE and ACT in the formation/clearance of Aβ, we first used ELISA to quantify both water soluble (defined as PBS-extractable) and membrane-bound (defined as guanidine-extractable) Aβ₁₄₀ and Aβ₁₄₂ in the hippocampus and cerebral cortex of young APP transgenic mice prior to the onset of amyloid deposition (2 month old transgenic mice). No consistent, statistically significant genotype-dependent difference in either water soluble or membrane-bound Aβ₁₄₀ or Aβ₁₄₂ were observed between our four lines of transgenic mice in the hippocampus. In general, transgenic mice expressing ApoE tended to display lower levels of water soluble Aβ, and mice expressing ACT tended to show reduced levels of membrane-bound Aβ in the hippocampus (Table 1 and Figure 1). Similar results and trends were observed in the cerebral cortex tissue extracts, where the level of water soluble Aβ was found to be more clearly reduced by ApoE
expression (Table 1). The ability of ApoE to reduce soluble Aβ levels in young APP mice has been reported in other studies [21, 18], and previous findings have shown that young APP/ACT mice have unchanged or slightly increased Aβ levels using different antibodies [41, 45].

In sum there is little evidence that either murine ApoE or human ACT increase Aβ levels in very young APP mice, but rather there is a trend toward ApoE and ACT reducing Aβ levels. Thus any increases in Aβ immunoreactive and mature amyloid deposition observed in the presence of ApoE or ACT in older mice is likely to be due to amyloid-promoting mechanisms other than merely increasing production or decreasing clearance of monomeric Aβ prior to amyloid deposition and the inflammation that it induces.

Young Adult (2 month old) APP transgenic mice are not impaired in either the Morris Water Maze or the RAWM

To determine whether animals genetically destined to deposit amyloid later in life show early behavioral deficits that could be attributed to expression of the transgene or the production of human Aβ peptide, we tested APP+ (mApoE+)(ACT-) and nontransgenic littermates in the same battery of tasks that would be used to assess the behavior of aged animals. The young animals showed no deficits in non-cognitive tasks. Furthermore, as shown in Figure 1e,f, the 2 month old APP-expressing and non-transgenic animals were also not significantly different in either Morris Maze acquisition or radial arm water maze (RAWM) working memory.
APPENDIX B (Continued)

ApoE and ACT separately and in combination increase Aβ deposition in PDAPP transgenic mice

Amyloid deposition is a complex process consisting of several stages. In order to develop the most effective therapies for Alzheimer’s disease, it is important to know which proteins contribute to which stages of the amyloid cascade and which stages cause cognitive decline. The histopathological analysis of our mice was designed to distinguish the individual and synergistic contributions of ApoE and ACT to both diffuse Aβ deposition and fibrillar amyloid formation and, together with the behavioral analysis described below, to distinguish the relative impact of these forms of Aβ deposition on cognitive decline.

Total Aβ deposition was measured as area (in %) occupied by Aβ immunoreactivity with the 6E10 antibody. Figure 2 shows that total Aβ deposition was markedly reduced in the hippocampus and absent in the cerebral cortex in PDAPP transgenic mice lacking ApoE expression [APP+, mApoE-KO, (ACT-)], consistent with previous findings [8]. Furthermore there was increased deposition in APP+(mApoE+),ACT+ transgenic mice, as compared to APP+,(mApoE+)(ACT-) transgenic mice, in the cerebral cortex (4.8±0.6% vs. 2.8±0.6%, P<0.05) and a modest elevation in the hippocampus (8.7±0.6% vs. 7.4±0.7%, n.s.), due to the presence of the ACT transgene (Figure 2a-b and 2e). The difference between APP+(mApoE+),ACT+ and APP+,(mApoE+)(ACT-) transgenic mice was greater in the lateral extension of the parietal cortex as compared to either the hippocampus or the retrosplenial.
APPENDIX B (Continued)
cortex, where immunoreactive Aβ deposition develops at an early age (Figure 2a-b). Furthermore there was an almost fourfold higher Aβ load in the hippocampus of the ACT-expressing/ApoE knockout (APP+,mApoE-KO,ACT+) transgenic mice as compared to the ACT minus/ApoE knockout (APP+,mApoE-KO,(ACT-)) mice (3.1±1.3% vs. 0.8±0.8%, P<0.05, Figure 2c-e).

In fact, only a single animal among the transgenic mice with genotype APP+,mApoE-KO,(ACT-) showed any immunoreactive Aβ deposition at 18 months of age.

**Mature, filamentous amyloid deposition is facilitated by either ApoE or ACT expression in the PDAPP transgenic mice**

In addition to forming diffuse, immunoreactive Aβ deposits, as quantified with the 6E10 antibody as above, the Aβ peptide also deposits as mature filamentous amyloid distinguishable by its strong beta sheet character. Congo Red staining, which measures such compact Aβ deposition, was about twofold elevated in both the hippocampus and cerebral cortex of the APP+,mApoE+,ACT+ mice (Figure 3a) as compared to APP+,mApoE+,(ACT-) transgenic mice (Figure 3b). This increase was statistically significant both in the cerebral cortex (0.028±0.004% vs. 0.014±0.0.004%, P<0.05) and the hippocampus (0.060±0.008% vs. 0.037±0.0.006%, P<0.05, Figure 3c). The ACT-induced increase in congophilic amyloid load was due to increased plaque density in both the cerebral cortex (3.12±0.46 vs. 1.44±0.44 plaques/mm², P<0.05) and the hippocampus (6.01±0.62 vs. 3.86±0.59 plaques/mm², P<0.05, Figure 3d). Furthermore ACT
antibodies immunostained Congo Red positive plaques in APP+,(mApoE+),ACT+, but not APP+,(mApoE+)(ACT-) mice (not shown). There were no congophilic Aβ deposits in the hippocampus and the cerebral cortex of either the APP+,(mApoE-KO),ACT+ or the APP+,(mApoE-KO),(ACT-) mice at 18 months of age (but see below).

ApoE-immunoreactivity has previously been demonstrated to be associated with a subset of Aβ immunoreactive plaques, which are essentially always Thioflavine S positive [7, 62]. However, using a more sensitive immunostaining protocol we now can show that ApoE is localized in essentially every Aβ immunoreactive plaque in an APP+,(mApoE+),ACT+ transgenic mouse (Fig 4a-b), while ACT-immunostaining is largely restricted to Thioflavine S positive amyloid plaques. Thioflavine S staining and ACT-immunostaining also correlate well in their relative intensity among individual amyloid plaques. These results suggest that ACT binds to Aβ peptide or an intermediate Aβ species, increases the extent of its beta sheet structure and thereby accelerates the formation of mature amyloid plaques (Fig 4c-d). In vitro binding and X-ray crystallography/structural modeling studies between ACT and Aβ support such a model of ACT function in AD [50, 70, 35, 34, 29].

At an advanced age (23 months) all (6/6) homozygous APP+,(mApoE-KO),ACT+ mice, but only some (6/10; 60%) age-matched APP+,(mApoE-KO),(ACT-) mice, developed Thioflavine S positive amyloid plaques in the hippocampus. The anatomical distribution of Aβ and Thioflavine S positive staining varied among individual animals, particularly among the
APPENDIX B (Continued)

APP+,mApoE-KO,(ACT-) mice. The Aβ-immunoreactive staining included the cerebral cortex in some of these mice, both with and without the ACT transgene. The Thioflavine S positive amyloid plaques in APP+,mApoE-KO,ACT+ mice were always Aβ-immunoreactive, and mostly ACT immunoreactive and were most frequently observed in the molecular layer of the dentate gyrus and the CA1 stratum lacunosum/moleulare, but were also present in the CA1 and CA3 stratum radiatum. ACT immunopositive staining was also observed in Aβ immunoreactive deposits that were not Thioflavine S positive in these aged APP+,mApoE-KO,ACT+ mice (Figure 4e-h). Thioflavine S was used in these experiments because we found it to be a more sensitive dye than Congo Red.

ApoE expression in aged PDAPP transgenic mice is associated with impaired learning ability in the Morris Water Maze

Gender and age-matched animals were randomly selected from the four transgenic groups and, along with a group of non-transgenic mice, were examined in a battery of standard behavioral tasks. As will be discussed in detail below, the non-cognitive tasks in the battery indicated that the mice in each of our lines were functioning well and could not be distinguished from each other in general non-cognitive abilities. For example, animals of all
genotypes performed at a similar level in open field activity and string agility tasks that estimate exploratory behavior and muscle strength and all exhibited similar swim speeds and were able to see.

Cognitive tasks, however, did reveal differences between the transgenic lines. The most common task for assessing cognition in mice is the standard Morris Water Maze which measures spatial learning ability. The escape latencies for the four experimental and the non-transgenic control mice during the first block of testing (Days 1-3) in the Morris Water Maze were the same ($F_{4,38} = 0.636; P=0.63$). This result reinforced the conclusion from the non-cognitive tasks that there is no essential difference between the four transgenic groups and the non-transgenic mice in ability to perform the non-cognitive aspects of the Morris Water Maze task. However in the last block of testing (Days 7-9), significantly higher escape latencies were recorded for the APP+,mApoE+(ACT-) mice ($41\pm5$ sec) as compared to the APP+,mApoE-KO,(ACT-) mice ($26\pm3$ sec, $P<0.05$) and the nontransgenic mice ($21\pm3$ sec, $P<0.01$). Similar inferior performance was displayed by the APP+,(mApoE+),ACT+ mice ($36\pm4$ sec) as compared to the APP+,(mApoE-KO,ACT+ mice ($23\pm4$ sec, $P<0.05$) and the nontransgenic mice ($21\pm3$ sec, $P<0.05$, Figure 5). Thus cognitive dysfunction as measured by the Morris Water Maze is associated with ApoE-catalyzed amyloid formation in APP-expressing mice.

ApoE knockout mice have been reported to exhibit cognitive deficits and thigmotaxic behavior (a tendency to swim in the peripheral annulus of the pool)
APPENDIX B (Continued)

in association with structural cytoskeletal and neurochemical deficits in the brain. However, the ApoE knockout mouse model we used shows no such defect [4, 3, 11, 64], and we did not observe any thigmotaxic behavior in our mice.

The Morris Water Maze was unable to reveal any difference between, for example, APP+,mApoE-KO,(ACT-) and APP+,mApoE-KO,ACT+ mice, although these groups of mice display different extents of Aβ immunoreactive histopathology. We surmised that a more demanding behavioral task would be more informative and sensitive in its ability to reveal subtle cognitive differences between the transgenic mice.

ApoE and ACT expression in aged PDAPP transgenic mice determine the extent of cognitive impairment in the Radial Arm Water Maze

The Radial Arm Water Maze (RAWM) is a more challenging version of the Morris Water Maze that is designed to test both visual-spatial and working memory in mice [6]. Previous work has shown that the RAWM provides a very sensitive behavioral measure that correlates well with the level of Aβ immunoreactive and mature amyloid load in transgenic mouse models of AD [6, 25, 38]. Therefore all of the behaviorally-analyzed mice were examined over three blocks of testing in the RAWM (Figure 6). Each of the three blocks consists of three days in which four acquisition trials (T1-4) and a delayed retention trial (T5) are performed on each day, with the location of the submerged platform being altered daily.
The escape latencies in the RAWM did not differ between the five groups of mice (four experimental and one control, non-transgenic) in the first trials (T1) of the first block of testing (Days 1-3), when the animals were naive to the task ($F_{4,31} = 0.60; P=0.67; \text{Figure 6a}$), indicating that there was no inherent difference between the groups in basic swimming and other non-cognitive performance (see further discriminant analysis below). However, over the full 3 blocks of testing, there developed group differences in both learning (Trial 1 compared to Trial 4 on each day) and memory (Trial 5). Of particular importance were group differences in average T5 performance across all three blocks, wherein APP+,(mApoE+) with or without ACT displayed substantially longer escape latencies ($47\pm4$ and $47\pm2$ sec.) compared to APP+,(mApoE-KO) mice with or without ACT ($36\pm4$ and $29\pm2$ sec., $P<0.05$, $P<0.001$) and nontransgenic controls ($29\pm3$ sec., $P<0.01$) (Figure 6b). In block 3, when cognitive learning processes are most distinguishable from the procedural learning that occurs during earlier testing blocks, APP+,(mApoE+) mice with or without ACT were impaired in their ability to reduce their escape latencies between trials T1 and T5. On both the final learning trial (T4) and the memory retention trial (T5), escape latencies for these two groups were significantly longer than those for APP+,(mApoE-KO),(ACT-) mice and nontransgenic mice.

Importantly, APP+,(mApoE-KO),(ACT+) mice displayed an intermediate RAWM performance. Although these mice were able to learn, as evidenced by their ability to reduce their escape latencies throughout the trials, their T4 escape latencies were significantly longer compared to both APP+,(mApoE-
KO,(ACT-) mice and nontransgenic mice. This result indicates that the ACT expression not only promoted amyloid formation, but also caused cognitive decline as measured in the RAWM.

ACT transgene expression in APP transgenic mice expressing ApoE further increased amyloid deposition but did not further reduce the already very poor spatial learning ability of APP+, (mApoE+) mice, which we interpret as a ceiling effect in this difficult behavioral task. Interestingly, one of the APP+, mApoE-KO, ACT+ transgenic mice lacking visible Aβ immunoreactive deposition performed poorly in the RAWM. It could be that biologically active Aβ intermediates that are detrimental to hippocampal function had already begun to form in this mouse, but had not yet reached the threshold level necessary to precipitate detectable Aβ immunoreactive deposits.

The extent of ApoE/ACT-catalyzed Aβ deposition correlates with cognitive performance.

The data clearly show that the process and/or the product of amyloid formation found in both groups of APP+, (mApoE+) transgenic mice is of prime importance for cognitive impairment in the RAWM. This conclusion is further reinforced by a strong correlation between the T5 retention trial escape latency over all three test blocks and the amount of both immunoreactive Aβ deposition (T5, r=0.68, P<0.001, Figure 6c) and mature congophilic amyloid (T5, r=0.60, P<0.001, Figure 6d) in the hippocampus.

Furthermore, the correlation between escape latency and immunoreactive Aβ deposition in the hippocampus remains strong when the
statistical analysis is restricted to the high-depositing APP+,(mApoE+) mice with or without ACT (T5, r=0.59, P<0.01). Extrapolating this linear correlation to its intercept on the y-axis (i.e. the extrapolated, theoretical, escape latency of an APP+,(mApoE+) transgenic mouse with zero Aβ deposition) yields a predicted latency of 31±12sec, which is close to the average recordings of the APP+,mApoE-KO mice (33±3sec). This extrapolation indicates that the main contribution of ApoE to cognitive decline is to promote Aβ immunoreactive and mature amyloid formation. Without such deposition, there would be no cognitive difference (i.e. difference in latency) between APP+,(mApoE+) mice and either APP+,(mApoE-KO mice or non-transgenic mice.

Diffuse Aβ deposition in the hippocampus is associated with cognitive impairment in PDAPP transgenic mice.

Thus far, the analysis has shown that ApoE and ACT catalyze Aβ immunoreactive and mature amyloid deposition in PDAPP mice and that the extent of these deposits correlates with the cognitive dysfunction of the mice, suggesting a cause and effect relationship. We then analyzed the data further to determine the relative impact of the two major forms of Aβ deposition, diffuse Aβ immunoreactivity and compact/fibrillar amyloid on cognitive performance.

All 18 months old PDAPP transgenic mice lacking ApoE and thus exhibiting only diffuse immunoreactive Aβ deposits with no compact Aβ amyloid deposition were stratified for the presence or absence of immunoreactive Aβ deposition in the hippocampus, and their behavior in the
RAWM was compared to that of nontransgenic mice (Fig. 6e). In the first trial (T1) of the first block of testing (Days 1-3), when the animals were naive to the RAWM, there were no differences between any of the experimental groups ($F_{2,18} = 3.05; P=0.072$). In block 3 (Days 7-9) it was found that working memory was worse in those APP+,mApoE-KO transgenic mice with diffuse hippocampal Aβ immunoreactive deposits as compared to APP+,mApoE-KO mice without diffuse Aβ deposits ($T_5, P<0.05$), which were indistinguishable from nontransgenic mice.

**Behavioral differences among the four groups of transgenic mice and the nontransgenic mice are not due to different genetic backgrounds**

Because of the multiple crosses during breeding that were necessary to generate our four lines of transgenic/knockout mice, it was not possible to assure that the strain backgrounds were identical short of many years of repeated backcrossing. Instead all of the mice have a uniform, mixed genetic background that should equally assure that any consistent difference between the lines must be due to the presence or absence of ApoE or ACT, which are the only genetic trait that the members of a particular line share.

To experimentally demonstrate that the differential behavior of the transgenic mice is largely due to the inheritance of the transgenic/knockout loci, we performed a comprehensive comparison of all of the behavioral data using discriminant function analysis. As previous studies have shown, this statistical tool allows for the comparison and discriminating analysis of characteristic
behavioral profiles of specific mouse strains [54]. We applied such discriminant analysis to the performance of our transgenic mice and of three lines of non-transgenic mice in the entire group of 19 different behavioral measures from multiple behavioral tasks in our test battery. There were consistent behavioral differences between the three groups of nontransgenic mice of different strain background, including the strains from which our transgenic/knockout mice were derived (P<0.025, Pillai’s trace, Figure 7a), indicating the ability of this technique to distinguish different mouse strains. In contrast, we were not able to behaviorally discriminate our control nontransgenic and the four transgenic groups of mice based on the same 19 behavioral measures (P>0.05, Pillai’s trace, Figure 7b). When the analysis was limited to 10 cognitive-based measures, the two APP+,(mApoE+) (high Aβ depositing) lines could be discriminated from the two APP+,mApoE-KO (low Aβ depositing) lines, confirming that cognitive-based behavioral measures are associated with the amyloid pathology. In contrast, the high depositing lines could not be distinguished from the low depositing lines on the basis of non-cognitive behavioral measures (P>0.05, Pillai’s trace). In sum, not only are the mice within each line that we have used more similar cognitively to each other than they are to mice in any other line, indicating that their differences are due only to ApoE or ACT, but no group can be distinguished by non-cognitive measures, indicating that they are all fundamentally similar in background.

4. Discussion
Previously, we and others had hypothesized and then demonstrated the ability of ApoE and ACT to catalyze the polymerization of Aβ into neurotoxic amyloid filaments [1, 73, 36, 72, 56]. Specific binding experiments, site directed mutagenesis and structural analysis then identified the molecular interactions between these pathological chaperones and Aβ and outlined the likely mechanism of action of the amyloid promoting reaction [50, 60, 35, 29]. The present study provides the first in vivo demonstration that the increased Aβ immunoreactive and mature plaque deposition caused by expression of the amyloid promoters ApoE and ACT, is associated with impaired spatial learning. Our results show 1) that ApoE and ACT separately and synergistically promote the development of both diffuse, immunoreactive Aβ deposits and mature amyloid plaques in Aβ overexpressing mice without affecting the levels of monomeric Aβ itself, 2) that impaired cognitive performance in two different spatial tasks requires both Aβ peptide and either ApoE or ACT as an amyloid promoter and 3) that both diffuse Aβ deposits and mature amyloid plaques are correlated with impaired spatial learning ability. Indeed, 18 month old APP mice that expressed neither ApoE nor ACT, deposited very little Aβ and displayed no cognitive deficits in the RAWM or Morris Water Maze, similar to nontransgenic mice. These findings imply that the process and/or product of ApoE or ACT-catalyzed amyloid formation is more critical for the cognitive decline in AD than is the amount of monomeric Aβ-peptide.

Although these experiments do not directly address which aspect or intermediate/product of amyloid formation causes cognitive decline, they
indicate that it must be ApoE and/or ACT-dependent and may be either some intermediate step in the process, the final, mature product, or both. Specifically, APP+,mApoE-KO,ACT+ animals accumulate diffuse, immunoreactive amyloid deposits and have impaired cognition. This finding suggests that diffuse Aβ immunoreactive deposition, or an even earlier, ApoE/ACT-dependent, step in the process of amyloid formation, may be equally important for the cognitive decline in these mouse models of AD as is mature congophilic amyloid. This interpretation of the data is supported by previous findings that Aβ oligomers and/or protofibrils appear to be more neurotoxic than monomeric or filamentous Aβ both in vitro [66, 67, 27, 31] and in vivo [68, 65].

The Morris water maze and the RAWM were chosen for these studies because the place learning that they assay depends on the hippocampus [39], which is pathologically and functionally affected early in AD. It has been suggested that there is some learning deficit of PDAPP mice in a working memory version of the Morris water maze that is age-independent and therefore unrelated to Aβ burden [12]. However, we observed no statistically significant cognitive deficits in young adult PDAPP mice (Figure 1e-f), indicating that most behavioral impairment in the PDAPP mice depends on later-developing Aβ deposition (and on the action of ApoE and ACT). Our data also indicate that the RAWM task is a more sensitive measure of Aβ deposition than is the traditional reference memory version of the Morris maze.
APPENDIX B (Continued)

Both PDAPP+,mApoE-KO and PDAPP+(mApoE+) mice have been reported to be impaired in an object recognition task as compared to nontransgenic mice. These findings were attributed to the high levels of soluble Aβ shared by the transgenic mice and seem to contrast with our results [19]. However, the object recognition task and the RAWM measure different aspects of cognition (identification vs. working memory) that may, to a differential extent, be dependent on certain brain regions (cerebral cortex vs. hippocampus) and be sensitive to different forms of Aβ deposition. Indeed, object recognition shows only weak correlation with Aβ-immunoreactive deposition or mature Thioflavine S positive Aβ deposition [19], while RAWM performance is strongly correlated with both diffuse and compact Aβ deposition, as demonstrated here and previously [5, 25]. In addition, clearance of soluble Aβ species from the brain through passive immunization conferred short-term cognitive benefits in the object recognition task [17]. However these were acute observations after an invasive procedure that alters the compartmentalization and equilibrium between soluble and insoluble Aβ concentrations and are thus difficult to compare to the present findings. It is also possible that acute cognitive benefits of passive vaccination derive from the resulting reduction in the vasoconstrictor activity of Aβ [63] or to alleviated hemodynamic responses [42], either of which could enhance cerebral blood flow and improve cognition without providing insight into the mechanism by which APP and Aβ normally cause cognitive decline.
APPENDIX B (Continued)

We have considered the possibility that a somewhat higher or lower proportion of the original strains in the various knockout/transgenic lines used in this study, and not differences in Aβ deposition, could have led to the observed differences in the cognitive test results. However such an explanation is highly unlikely because 1) Different behavioral characteristics of inbred mouse strains are often lost in F1 hybrid strains [48]. For example, the protocol for generating our mice required several crosses that assured that the mice of each line contained a random mixture of backgrounds, with the only common feature being the presence or absence of APP, ApoE or ACT. Therefore, if genetic traits attributable to differences in strain background had been the major determinant of cognitive function, the individual mice within each line would have been expected to show a widely variable performance on the cognitive tests, reflecting their different genetic backgrounds. Such variation would preclude us from being able to detect differences due to mApoE or the ACT transgene. Instead the mice within each line were very similar to each other and differed as a group from the mice of the other lines. 2) There is a strong correlation between escape latency and hippocampal Aβ-immunoreactive deposition among the APP+(mApoE+) (with or without ACT) mice which all have an identical strain background. 3) APP+, mApoE-KO (with or without ACT) mice with hippocampal Aβ-immunoreactive deposition are impaired as compared to APP+, mApoE-KO mice without Aβ-immunoreactive deposition. These latter two groups of mice also have an identical strain background showing that the ApoE effect on behavior cannot be due to
APPENDIX B (Continued)

background strain difference. 4) Discriminant analysis, which can compare and reveal differences among comprehensive behavioral profiles of different mouse strains, shows that the differences among the four groups of transgenic mice and the nontransgenic mice are restricted to those behavioral measures which are the most sensitive assays of cognitive performance. In contrast, different control mouse strains can be discriminated on the basis of both cognitive and non-cognitive behavioral tests.

Although ApoE and ACT clearly promote Aβ polymerization directly, these proteins likely have additional roles to play in AD and other brain diseases. For example, it has been shown that ACT inhibits Aβ degrading enzymes [76], and ApoE affects the level or location of APP processing so as to generate fewer Aβ peptides and more potentially toxic C-terminal fragments (in animals slightly older than ours and of an age to show initial stages of amyloid deposition; [18]). ApoE also affects cholesterol metabolism [49]. Particularly interesting is the finding that ApoE4 is less neuroprotective than ApoE3 both in vitro and in vivo [9].

It is also important to consider the possible direct and compensatory consequences of expressing or not expressing ApoE and/or ACT during development. For example, ApoE knockout mice develop hypercholesterolemia and atherosclerosis that could have an effect on amyloid formation. However, heterozygous PDAPP/ApoE+/- mice have normal serum cholesterol levels and develop amyloid deposits at a rate intermediate between full PDAPP/ApoE-KO and PDAPP/ApoE+/+ animals [7] (and Nilsson and
APPENDIX B (Continued)

Potter unpublished). This result suggest that the lower amyloid deposition induced by knocking out ApoE is not caused by high serum cholesterol due to ApoE deficiency. Similarly, the effect of long term expression of a protease inhibitor such as ACT could be deleterious or could be masked by developmental compensation that could itself have side effects. If present, such effects of ACT would affect development only late since the GFAP promoter of the ACT transgene does not become active until the very latest stages of fetal development. Furthermore, except for hypercholesterolemia in ApoE-KO mice, we have not observed gross differences in physiology or brain morphology due to knocking out ApoE or expressing ACT.

In sum, our findings of a strong association between cognitive decline and both diffuse Aβ deposits and mature amyloid plaques in transgenic mice indicate that a major pathological role of ApoE and ACT in Alzheimer’s disease is to promote Aβ polymerization and deposition, possibly with slightly different effects in terms of time course and anatomical distribution of the pathology. Furthermore, it may be the process of amyloid formation, or an intermediate formed there in, rather than the end product (mature plaques) that causes neuronal dysfunction and cognitive decline. These results showing the essential role of ApoE and ACT in both amyloid formation and cognitive decline, together with the finding that ApoE and ACT do not alter the steady state level of Aβ, suggest that inhibition of the Aβ/ApoE or Aβ/ACT interactions are prime targets for therapeutic intervention in AD. Previous experiments
APPENDIX B (Continued)

have shown that such interaction inhibitors effectively prevent ApoE and ACT-catalyzed polymerization in vitro [41].

Besides sharing the ability to promote Aβ polymerization, ApoE and ACT also share the feature of being inflammatory proteins whose mRNAs are overexpressed in affected areas of the AD and APP mouse brain. Thus, the present findings provide an explanation for the involvement of inflammation and the prophylactic benefit of NSAIDs in AD [37, 44, 2, 53] and point to novel targets for anti-inflammatory therapy. Specifically, inhibitors of glial overproduction and release of IL-1, ApoE, or ACT should prevent amyloid formation and cognitive decline. Both in vitro and in vivo experiments indicate the likely success of such approaches [14, 35, 33].

Finally, we note that the concept of pathological chaperones playing a catalytic role in diseases of protein conformation is potentially quite general, as it appears to extend to both other AD proteins and other amyloid disorders [61, 47, 69, 71, 16].
5. References


APPENDIX B (Continued)


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APPENDIX B (Continued)


[54] Rogers DC, Jones DN, Nelson PR, Jones CM, Quilter CA, Robinson TL and Hagan JJ. Use of SHIRPA and discriminant analysis to characterise


APPENDIX B (Continued)


APPENDIX B (Continued)


APPENDIX B (Continued)


Table 1. ELISA measurements of $\text{A}\beta(1-40)$ and $\text{A}\beta(1-42)$ levels in the hippocampus of 2 months old mice.

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<th>Guanidine-extractable (mean pg/mg tissue)</th>
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<th>$\text{A}\beta_{1-42}$</th>
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<td>27±3* 16±1**</td>
<td>133±11**</td>
<td>171±18</td>
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Values are mean±SEM; *P<0.05 as compared to PDAPP+ApoE+ACT+ transgenic mice for the hippocampal measurements. *P<0.05 and **P<0.01 as compared to PDAPP+ApoE+ACT+ as well as PDAPP+ApoE+ACT- transgenic mice for the measurements of the cerebral cortex.
6. Figure legends

Figure 1. ELISA measurements of Aβ(1-40) and Aβ(1-42) levels in hippocampus and behavior analysis of cognitive ability in 2 months old mice before amyloid deposition has begun.

(a-d) Quantitation of the levels of Aβ(1-40) and Aβ(1-42) in PDAPP+,(mApoE+),ACT+ (n=20, solid bar), PDAPP+(mApoE+)(ACT-) (n=12, open bar), PDAPP+,mApoE-KO,ACT+ (n=17, solid bar) and PDAPP+,mApoE-KO,(ACT-) (n=19, open bar). The quantitative measurements refer to soluble (PBS-extractable) and membrane-bound (guanidine extractable) Aβ levels. *P<0.05.

(e,f) Morris Water Maze and radial arm water maze performance of young adult APP+,(mApoE+)(ACT-) mice and non-transgenic littermates.

Figure 2. Total amyloid load, diffuse and compact, in four strains of 18 months old transgenic/knockout mice, as measured by 6E10 immunostaining. Representative animals with genotypes (a) PDAPP+,(mApoE+),ACT+, (b) PDAPP+,(mApoE+)(ACT-), (c) PDAPP+,mApoE-KO,ACT+ and (d) PDAPP+,mApoE-KO,(ACT-).

(e) Quantitative image analysis of total amyloid load from all investigated animals with genotype PDAPP+,(mApoE+),ACT+ (n=17, solid bar) and PDAPP+,(mApoE+)(ACT-) (n=11, open bar) in the hippocampus (left) and the cerebral cortex (middle), and PDAPP+,mApoE-KO,ACT+ (n=9, solid bar) and
APPENDIX B (Continued)

PDAPP+m,ApoE-, (ACT-) (n=8, open bar) in the hippocampus (right). *P<0.05. Scale bar measures 335 μm.

Figure 3. Compact amyloid deposition in four strains of 18 months old transgenic/ knockout mice as measured by Congo Red staining. Representative animals of the experimental groups (a) PDAPP+,(mApoE+),ACT+ and (b) PDAPP+,(mApoE+)(ACT-). Quantitative compact amyloid load from all investigated animals with genotypes PDAPP+,(mApoE+),ACT+ (n=17, solid bar) and PDAPP+,(mApoE+)(ACT-) (n=11, open bar) in the hippocampus and the cerebral cortex. Congo positive amyloid load is expressed as % compact Aβ load (c) and plaque density (d). *P<0.05. Scale bar measures 115 μm.

Figure 4. Amyloid pathology of an 18 months old heterozygous PDAPP+(mApoE+)ACT+ (a-d) and a 23 months old homozygous PDAPP+,mApoE-KO,ACT+ transgenic mouse (e-h). Aβ- (a and e), ApoE- (b and f), and ACT-immunostaining (d and h) and Thioflavine S staining (c and g).

Figure 5. Analysis of spatial learning in the Morris Water Maze. Average escape latency for four daily trials during block 1 (days 1-3), block 2 (days 4-6) and block 3 (days 7-9) of testing. PDAPP+,(mApoE+),ACT+ (n=11; yellow squares), PDAPP+,(mApoE+)(ACT-) (n=10; green squares), PDAPP+,(mApoE-KO,ACT+ (n=10; blue circles) and PDAPP+,(mApoE-
KO,(ACT-) (n=7; red circles) and nontransgenic mice (n=5; pink triangles).
Statistical significance refers to P<0.05 or higher level of significance as
compared to nontransgenic mice (*), PDAPP+,ApoE-,(ACT-) mice (#) or
PDAPP+,ApoE-,ACT+ mice (≠).

Figure 6. Analysis of working memory in the Radial Arm Water Maze
(a) Escape latency during block 1 (days 1-3), block 2 (days 4-6) and block 3
(days 7-9) of RAWM testing for the first and last acquisition trials (Trial 1, Trial
4), and the delayed memory retention trial (Trial 5).
(b) Combined average escape latencies of the memory retention (T5) trials
over all three blocks of testing.
(c) Correlation analysis of escape latency for Trial 5 over all three blocks and
total hippocampal amyloid deposition (6E10 immunoreactivity).
(d) Correlation analysis of escape latency for T5 over all three blocks and compact
amyloid deposition (Congo Red staining).
(e) Escape Latency during block 1 (days 1-3), block 2 (days 4-6) and block 3
(days 7-9) of RAWM testing for PDAPP+,mApoE-KO transgenic mice stratified
for the presence (n=5; green squares) or absence (n=11; blue squares) of
diffuse hippocampal amyloid deposition as well as nontransgenic mice (n=5;
pink triangles).
Statistical significance in panels (a)and e refers to P<0.05 or higher level of
significance as compared to PDAPP+,ApoE-,(ACT-) (#) or nontransgenic mice (*).
Statistical significance in panel (b) refers to $P<0.05$ (*) and $P<0.01$ (**). Dashed lines in panels (c) and (d) indicate 95% confidence interval of regression line.

*Figure 7. Discriminant analysis*

(a) Graphical demonstration of the ability of discriminant analysis to easily discriminate three different nontransgenic mice strains based on 19 measures of behavior.

(b) Lack of ability to behaviorally discriminate the four groups of APP transgenic mice and the nontransgenic mice based on the same 19 behavioral measures.
APPENDIX B (Continued)

Fig. 1

a) Soluble Aβ(1-40)

b) Soluble Aβ(1-42)

c) Membrane Bound Aβ(1-40)

d) Membrane Bound Aβ(1-42)

e) Morris Water Maze

f) Escape Latency (sec)
APPENDIX B (Continued)

Fig. 2

![Image of a figure showing different sections of the brain with amyloid load analysis.](image-url)

A bar graph is shown with the x-axis labeled as ACT+ and ACT- for Hippocampus and Cerebral Cortex. The y-axis is labeled as % 6E10 positive Amyloid load. The graph includes bars for APP+, ApoE+ and APP+, ApoE- conditions.
APPENDIX B (Continued)

Fig 3
APPENDIX B (Continued)

Fig. 4
Fig. 5
APPENDIX B (Continued)

Fig. 6

a. Radial Arm Water Maze

b. Escape Latency (sec)

<table>
<thead>
<tr>
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<th>Day 4-6</th>
<th>Day 7-9</th>
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<td>T1</td>
<td>T4</td>
<td>T5</td>
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<tr>
<td></td>
<td>T1</td>
<td>T4</td>
<td>T5</td>
</tr>
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</table>

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APP+, ApoE-KO, ACT-
APP+, ApoE-KO, ACT+
APP+, (mApoE), ACT-
APP+, (mApoE), ACT+
Nontransgenic

---

Escape latency (sec)

---

Congo Amyloid Load (%)

---

r = 0.68
P < 0.001

---

6E10 Amyloid Load (%)

---

r = 0.60
P < 0.001

---

Escape Latency (sec)

---

Diffuse amyloid
Nontransgenic
No amyloid
APPENDIX B (Continued)

Fig. 7

Previously Published
Environmental Enrichment Improves Cognition in Alzheimer’s Transgenic Mice Despite Stable β-Amyloid Deposition

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Date of Submission: 6/23/03
Rodents undergoing cognitive stimulation (CS) by being placed in an “enriched environment” (e.g., socially-housed in large cages containing toys, tunnels, running wheels, etc.) exhibit improved cognitive performance\(^1,2\), as well as increased hippocampal neurogenesis\(^3\), synaptogenesis\(^4\), growth factor levels\(^5\), and cognition-linked gene expression\(^6\). Although retrospective studies in humans have similarly found that a lifelong pattern of CS generally associated with educational/occupational attainment protects against Alzheimer’s Disease (AD)\(^7,8\), it is unclear to what extent long-term CS would benefit individuals who already have AD. Here we show that long-term environmental enrichment (EE) in aged AD transgenic mice results in global, overall improvement in cognitive function without decreasing brain beta-amyloid (A\(\beta\)) deposition, suggesting that long-term CS could provide cognitive stabilization or improvement to AD patients through mechanisms independent of A\(\beta\) deposition and clearance.

Recently, several cognitive stimulation programs have been developed in an attempt to slow or reverse the cognitive decline in AD patients\(^9,10\). These programs have generally involved a relatively short 4-12 week period of cognitive training classes and/or daily caregiver-directed mental activities, resulting in modest degrees of success. However, the therapeutic potential of intensive, “long-term” CS has not yet been evaluated in AD patients. Furthermore, retrospective/longitudinal studies cannot determine whether CS is a promotor or merely an indicator of intact cognition. Therefore, the present study sought to elucidate the cognitive-enhancing potential of long-term EE in
APPENDIX B (Continued)

aged APPsw transgenic mice, which overexpress the “Swedish” mutant amyloid precursor protein and bear moderate cortical/hippocampal Aβ deposition within mature AD-like neuritic plaques11.

Beginning at 16 months of age, APPsw mice were put into either EE (n = 5) or standard housing (SH; n = 4) for four months (Fig. 1). In addition to their enriched housing, EE mice were placed in novel complex environments 3 times weekly for several hours over the 4 month period. Beginning at 20 months of age, all mice were tested in four cognitive-based tasks as previously described11: Morris water maze (reference learning and memory), circular platform (reference memory), platform recognition (search/identification), and radial arm water maze (working memory). Following completion of the behavioral testing at 22 months of age, animals were euthanized and their brains processed for Aβ load determinations12. During the course of enrichment, 2 EE mice died.

Over 10 days of Morris maze acquisition (learning), EE mice performed significantly better than SH mice by having lower escape latencies (Fig. 2a). During the subsequent memory retention (probe) trial (Fig. 2b), EE mice showed an exclusive preference for the former platform-containing quadrant (p = 0.01, ANOVA), while SH mice showed nominal quadrant preference (p = n.s., ANOVA). In switching from the “reference” learning/memory strategy of the Morris maze to the search/identification strategy of platform recognition, SH mice showed obvious impairment, as evidenced by their high latencies over the first 3 days of platform recognition testing (Fig. 2c). By sharp contrast, EE
mice effectively and immediately changed strategies by exhibiting low escape latencies even on the first day of platform recognition testing (p<0.05; Fig. 2c). Indeed, a comparison of the improved behavior of EE mice in the above tasks indicates that they had reacquired the cognitive ability of previously-studied 16 month old non-transgenic mice with the same strain background\textsuperscript{11}.

To determine whether EE had an "overall" cognitive-enhancing effect encompassing all 4 tasks employed, we evaluated data from the two primary measures of each task. As shown in Fig. 3a, the means of EE mice on all 8 measures were better than those of SH mice. An analysis of these data showed that EE mice had significantly better "overall" cognitive performance spanning all 8 cognitive measures (p <0.005; Fisher Sign test). Moreover, discriminant function analysis (step-wise forward method), evaluating data from the same 8 behavioral measures, also revealed significantly better overall performance of EE vs. SH mice (F[4,2] = 33.57, p = 0.029; Wilks’ lambda = 0.0147); the Systat program found four measures from 3 of the 4 tasks to collectively provide maximal discrimination between EE and SH groups.

At the 22 month completion age of this study, total Aβ loads (diffuse + compact) in both parietal cortex and hippocampus of EE and SH mice were determined by immunohistochemistry with the 6E10 antibody. Moderate Aβ loads (5-10%) were evident in both brain areas, with no differences between EE and SH mice (Fig. 3b), indicating that the cognitive benefits of EE occurred in AD transgenic mice without an accompanying decrease in brain Aβ deposition.
These results indicate that a long-term period of EE in aged AD transgenic mice results in superior overall cognitive performance encompassing multiple cognitive domains (e.g., reference learning/memory, working memory, recognition, strategy switching). Although a reduction in brain Aβ deposition has been associated with behavioral benefit of Aβ immunotherapy in similar AD transgenic mice\textsuperscript{13}, the present results, together with our other study showing Aβ-independent cognitive benefit to AD transgenic mice given a blueberry-rich diet\textsuperscript{14}, indicate that mechanisms autonomous from Aβ deposition are sufficient for behavioral benefit. Along this line, EE-induced enhancements in neurogenesis\textsuperscript{3}, synaptogenesis\textsuperscript{4}, growth factor levels\textsuperscript{5}, and gene expression\textsuperscript{6} have all been seen in normal mice. In this first study to evaluate the potential of EE in AD transgenic mice, our results suggest that long-term intensive EE/CS (alone or in combination with cognition-stimulating drugs) could be useful in stabilizing or slowing the cognitive decline of AD and it’s predecessor, mild cognitive impairment (MCI), without needing to reduce Aβ load. Largely because of practical considerations, longitudinal interventional studies have not been done in humans to confirm controversial retrospective studies reporting AD risk reduction with “lifelong” education/occupation-related intellectual activity\textsuperscript{7,8}. A recent 5-year longitudinal study involving non-demented 75+ year olds did find participation in cognitively-stimulating leisure activities to be associated with a lower risk of dementia\textsuperscript{15}. However, a cause and effect relationship could not be established because the leisure activities were self chosen. Future studies
APPENDIX B (Continued)

in which EE is initiated early in adulthood of AD transgenic mice should more
definitively elucidate the potential protective effects of EE against development
of cognitive impairment and neuropathology.

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Research in Alzheimer’s Disease at the Suncoast Gerontology Center at USF.

Competing Interests statement

The authors declare that they have no competing financial interests.
APPENDIX B (Continued)

References

Figure Legends

Fig. 1: Standard housing of individual mice (a) and enriched housing of multiple mice in a large bin containing an inner cage (with platforms, passageways, lofts), running wheels, toys, and novel habitats (b).

Fig. 2: Environmental enrichment significantly improves reference learning, reference memory and recognition/identification abilities of AD transgenic mice. (a) Over 10 days of Morris maze acquisition (learning) at 20 months of age, EE transgenic mice had lower escape latencies compared to SH transgenic mice. Latencies to escape to a stationary submerged platform in a 100-cm diameter pool over 4 daily trials were averaged and are presented as five 2-day blocks. Group means were compared across blocks by Paired Design [ t(4) = 4.99; p=0.007] . (b) Spatial memory retention of EE transgenic mice during a 60 sec. probe trial on Day 11 was better than SH transgenic mice since EE mice had an exclusive preference for the former platform-containing quadrant (Q2) while SH mice showed only nominal quadrant preference. * = significantly lower than Q2 percentage at p<0.05 or higher level of significance (ANOVA). There was no difference in swim speed between EE and SH transgenic groups. (c) For 4 days of platform recognition testing at 21 months of age, transgenic mice were given 4 swim trials daily starting from the same position, with an elevated prominent ensigned escape platform moved to a different pool quadrant for each
trial. EE mice were immediately able to switch from the reference learning/memory strategy of Morris maze to the search/identification strategy of platform recognition, as evidenced by their low escape latencies even on the first day of testing. By contrast, SH mice had significantly higher escape latencies on the first 3 days of testing. * = significantly higher than enriched group at p<0.05 (Mann-Whitney U test).

Fig. 3: Environmental enrichment results in better “overall” cognitive performance in aged APPsw mice without affecting forebrain Aβ deposition. (a) EE mice had higher performance means vs. SH mice on two primary measures from each of four cognitive-based tasks evaluated between 20 - 22 months of age. Significantly better overall performance of EE mice across those 8 measures was evident from both the Fisher Sign test and discriminant function analysis (see text).

Abbreviations: WM acq. = Morris maze acquisition average escape latency; CP overall (x10) = circular platform average escape latency (1/10th scale); CP Final (x10) = circular platform escape latency on final day (1/10th scale); PR overall = platform recognition average escape latency; PR final = platform recognition escape latency on final day; RM overall (T5) = radial arm water maze average escape latency on the delayed retention trial; RM final (T5) = radial arm water maze escape latency on delayed retention trial in last block of testing; WM retention
deficit = % of time not spent in the former platform-containing quadrant during probe trial.  (b) At 22 months of age, total Aβ loads in both hippocampus and parietal cortex of APPsw mice were not affected by 4 months of EE.
APPENDIX B (Continued)

Figure 1
APPENDIX B (Continued)

Figure 2

a) Quadrant % Time in Quadrant

0 10 20 30 40 50 60

1 2 3 4

Standard Housing Enriched Environment

1 2 3 4

b) Standard Housing Enriched Environment

% Time in Quadrant

0 10 20 30 40 50 60

1 2 3 4

Latency (sec)

Standard Housing Enriched Environment

c) Latency (sec)

0 10 20 30 40 50 60

1 2 3 4

Days

Standard Housing Enriched Environment
APPENDIX B (Continued)

Figure 3

[Graph showing Escape Latency (sec) for Standard Housing and Enriched Environment, with data points for CP overall (x10), PR overall, RM overall (T5), and Total Ab load (% area) for Hippocampus and Parietal Cortex.]
ABOUT THE AUTHOR

David Costa was born in Connecticut, but has lived most of his life in Florida. He graduated, with honors, from the University of Florida in 1999, earning a Bachelor's degree in Microbiology. He earned his doctoral degree in medical sciences from the University of South Florida, College of Medicine, Department of Biochemistry and Molecular Biology in 2005. David is a member of the Society for Neuroscience, The Alzheimer's Association, and shares affiliations with the Johnnie B. Byrd, Sr. Alzheimer's Center & Research Institute and the Suncoast Gerontology Center. David currently resides in Brandon, Florida with his wife, Heather.