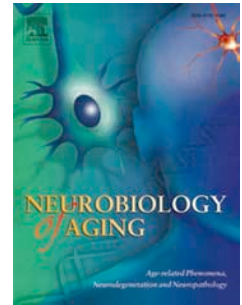


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Neurobiology of Aging

Apolipoprotein E and Lipid Homeostasis in the Etiology and Treatment of Sporadic Alzheimer's disease

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Abstract

The discovery that the apolipoprotein E4 (apoE4) allele is genetically linked to both sporadic and familial late onset Alzheimer's disease (AD) raises the possibility that a dysfunction of the lipid transport system could seriously affect lipid homeostasis in the brain of AD subjects. The presence of the E4 allele has been associated with lower levels of apoE in both serum and brain tissues of normal and AD subjects. In an attempt to reverse the apoE deficit in AD, we identified and characterized several apoE inducer agents using a low throughput in vitro screening assay. The most promising of these compounds is called probucol. Administration of probucol, an old cholesterol lowering drug, in a pilot trial in mild to moderate sporadic AD led to a significant increase in CSF apoE levels and a decrease of CSF in both phospho(181)-Tau and beta amyloid 1-42 concentrations without significant modifications of lipid hydroperoxides levels.

1. Introduction

In the last ten years or so, it has become obvious that the number of patients with Alzheimer's disease (AD) has increased noticeably around the world. To fully understand the situation, we need to take a look at life expectancy over the past few centuries. Figure 1A illustrates changes in human life expectancy since the beginning of the Christian era nearly two thousand years ago. Eighteen hundred years were necessary before a modest increase in life expectancy could be detected in European populations (Finch ,2012). However, life expectancy drastically changed these past 200 years where it nearly doubled at the turn of the current millennia (Wilmoth ,2000).

According to recent data from the World Health Organization (WHO), a new case of AD occurs every seven seconds worldwide. In the US, it is estimated that there are more than 5.1 million patients with Alzheimer's disease (Alzheimer's Association (US) ,2010) whereas it exceeds 6 million cases in Europe. Germany, Italy and France are at the top of the list, the latter currently having more than 900,000 cases. Recent research findings from Asia indicate that there are more than 6 million cases in China alone. Figure 1B gives the most cautious projections, based on US data, as to the number of cases expected by 2050(Alzheimer's Association (US) ,2010). Furthermore, roughly two-thirds of patients with diagnosed Alzheimer's disease are women.

Sporadic AD is now considered to be a multifactorial disease with a pre-eminent genetic component. The identification of specific mutations and polymorphisms in genes

associated with common AD has certainly changed our perception of the nature of the molecular changes controlling the pathophysiological process that characterizes the disease. The early onset familial, autosomal form of AD accounts for roughly 1-2 % of all cases worldwide, whereas the sporadic form of AD, representing 98-99% of the remaining cases, is generally believed to be of late onset, occurring after 65 years of age.

2. Vascular Changes and Alzheimer's disease

Converging evidences indicate a strong relationship between lipid homeostasis alterations and vascular changes in the brain of demented subjects. These associations include: recognition that apolipoproteins $\epsilon 4$ and J, as well as ABCA7 alleles (all being involved in cholesterol transport) are major genetic risk factors for vascular dementia, as well as familial and sporadic AD; epidemiological studies linking genetic and environmental vascular risk factors to dementia; awareness that small strokes do precipitate clinical dementia in cognitively normal elderly people with AD pathology; modulation of the degradation of the amyloid precursor protein and tau metabolism by pharmacological manipulations of cholesterol metabolism; association between hypercholesterolemia and amyloid deposition in young adults without symptoms of dementia and abnormal appearance of microvascular endothelial cells in brain areas affected by AD (Poirier, 2003a).

The precise mechanisms by which any or all of these lipid-related risk factors affect the pathophysiology of AD remain to be clarified. However, several independent

epidemiological and clinical studies examining the effect of cholesterol lowering drugs such as probucol, simvastatin and pravastatin on the incidence and/or progression of AD suggest a protective effect in subjects with varying risk of vascular diseases (Jick *et al.*,2000;Wolozin *et al.*,2000;Poirier ,2003a), particularly in the case of older statins such as pravastatin and simvastatin ({Wolozin, 2007 4205 /id;Bettermann, 2012 4573 /id}). Many of these studies support the notion of a subtle but significant interplay between cardiovascular (environmental and genetic) risk factors and the onset and/or progression of AD.

3. Genetics and Risk Levels

Although more than 695 genes (and 2973 different polymorphisms) have thus far been examined and many have been proposed as putative genetic determinants of sporadic AD, none (except ApoE) has yet been definitively accepted as such, in view of the lack of robustness of the associations observed between independent populations (Bertram *et al.*,2007) & (<http://www.alzgene.org>). Meta-analyses (n=320) of these genetic variants have been performed systematically in recent years. These studies have reached three conclusions: (i) except for the E4 polymorphism of the ApoE gene and other polymorphisms at this locus (promoter polymorphisms), very few genes are consistently associated with sporadic AD and they are all minor genetic determinants (Bertram *et al.*,2007); (ii) except for few genetic variants studied in detail, such as the insertion/deletion of the α 2-macroglobulin (59 publications), most genes have been studied by only one or two laboratories; (iii) most of the time, very few genetic variants

have been analyzed for each gene and they often differ from one study to another. To overcome some of these intrinsic problems, beyond the sharing of data using international databases, the study of AD genetics, like that of most multi-factorial diseases, has turned towards very high-throughput genotyping analyses. Populations exceeding several hundreds, even thousands of samples have been used to generate sufficient statistical power to characterize the polymorphisms in the genes involved with the disease among the hundreds of thousands of polymorphisms in each individual. This type of approach has been recently successful in AD with the characterization of the APOJ, CR1, PICALM, BIN1, ABCA7, and CD33 loci as new genetic determinants of AD (in addition to the well-established ApoE4 variant) (Lambert *et al.*,2009;Seshadri *et al.*,2010;Harold *et al.*,2009) with at least one independent GWAS replication for each major candidate. However, if the estimate that 60-80% of the AD risk in twin studies is due to genetic factors is correct, a non-negligible part of the additional genetic susceptibility loci remains to be identified.

Table 1 summarizes some of the top consensus candidate genes associated with i) the familial autosomal form of the disease and ii) the sporadic (common) form of the disease. The case of the butyrylcholinesterase (BuChE) gene is particular in that it was identified by GWAS using amyloid deposition imaging and not disease status as pairing association criteria (Ramanan *et al.*,2013). What is most interesting in this list of genetic risk factors is the high number of lipid-related genes. Figure 2 illustrates some of the known biological functions of the top 6 genes in Table 1 in the context of cholesterol homeostasis. ApoE and apoJ (also referred to as clusterin) were originally cloned in the

early 90s from a cDNA differential screening of mRNAs expressed in the AD hippocampus (May *et al.*,1990). Analysis of the expression of both mRNAs in experimentally deafferented hippocampus of rats revealed a time course profile that clearly indicate a strong association between apoE and apoJ overexpression and active compensatory synaptogenesis ((May *et al.*,1990;Poirier *et al.*,1991). ApoE and apoJ are normally synthesized and secreted by astrocytes and microglia in the brain and bind to high density lipoproteins (HDL) to facilitate cholesterol and phospholipids mobilization and transport toward cell surface receptors belonging to the LDL receptor family{Beffert, 2004 3665 /id}. As mentioned before, butyrylcholinesterase, which is best known for its ability to degrade acetylcholine both in the CNS and in periphery (Giacobini E.,2000), is also involved in lipoprotein remodeling (Annapurna *et al.*,1991;Iwasaki *et al.*,2007). Internalization of the apoE-HDL particles by members of the LDL receptor family occurs primarily in specific clathrin-coated pit structures in the plasma membrane where both BIN1 and PICALM gene products were shown to facilitate endocytosis of large complexes (McMahon and Boucrot ,2011). Once internalized via endocytic processes, the HDL complex is degraded and the cholesterol is released and esterified via the acyl-CoA:cholesterol acyltransferase (ACAT) for mid-term storage purpose (Figure 2). When intracellular concentration of cholesterol exceeds physiological requirements, a portion of the cholesterol is returned to the plasma membrane using the ABCA1/A7 reverse intracellular transport system for final transfer to surface-bound HDL complexes.

This molecular cascade involves six of the top ten genetic risk factors for AD identified by GWAS these past 5 years. In contrast to the disease-causing autosomal

dominant genes, which specifically target the amyloid precursor protein or its processing (via the presenilins), the so-called risk factors involved in sporadic AD are primarily related to the immune system and the lipid metabolism.

4. Apolipoprotein E and Cholesterol Transport in AD

The brain is a major site of apoE mRNA expression in humans, marmosets, rats and mice, exceeded only by the human liver. Transcripts for apoE are distributed throughout all regions of the brain and have been localized to astrocytes and microglia by *in situ* hybridization. Accordingly, apoE was shown to be synthesized and secreted mostly by glial cells (>95%) and to serve as a ligand for the members of the low density lipoprotein receptor family in the brain (Herz and Beffert, 2000). Primary cultures of hippocampal neurons from rat embryos and pro-simians have the capacity to internalize apoE-containing lipoproteins. Over the years, several explanations have been devised to address the pathophysiological role of apoE in the brain of ϵ 4 carriers. These working hypotheses can be divided on the grounds of their respective target metabolic cascades. The first one revolves around the concept that apoE4 directly and indirectly compromise amyloid metabolism and causes a toxic accumulation of the amyloid beta over time. This hypothesis has been reviewed extensively recently (Kim *et al.*, 2009) and will not be addressed in the present review. The second hypothesis implicates Tau protein metabolism and assumes that apoE is released in the cytoplasmic compartment, where it interferes directly with the cytoskeletal architecture of neurons (Brecht *et al.*, 2004). Finally, the third hypothesis, which stems in part from our understanding of the role of

apoE in the cardiovascular system, postulates that this core apolipoprotein acts as a key player in the maintenance of lipid homeostasis in the mature brain and that carriers of the apoE4 allele display reduced levels of apoE when compared to non-E4 carriers (Poirier, 2005).

The major physiological differences between apoE4 and -E3 are attributed to the amino acids at two key positions in the peptide chain, numbered 112 and 158, each of which can either be arginine (R) or cysteine (C). In periphery, the presence of R112 in apoE4 causes its preferential binding to triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins, VLDL), whereas apoE3 binds preferentially to high-density lipoproteins (HDL). These differences in lipoprotein binding by apoE3 and -E4 greatly influence lipoprotein clearance and LDL/HDL ratios in periphery, which are risk factors in cardiovascular disease. However, it should be noted that the brain is entirely devoid of LDL and highly dependent upon HDL to maintain cerebral lipid homeostasis. The semi-dominant nature of the association between the apoE4 allele and sporadic AD has been firmly established only recently (Genin *et al.*, 2011).

5. ApoE4: A Case of Evolutionary Underperformance

ApoE3 variant appears to have spread during later stages of human evolution after originating from the ancestral apoE4 gene. According to DNA sequences representing four distinct ethnic groups, apoE3 is estimated to have spread some 225,000 years ago. The depth of the tree is estimated at 311,000 years ago (range 0.176–0.579)(Fullerton *et*

al.,2000). Although these sequences analyses do not inform when E3 originated as a mutation, they imply that E3 arose before anatomically modern *Homo sapiens* first migrated from Africa about 100,000 years ago. This range also allows E3 to be present in Neanderthals (from 300,000 years ago) and in earlier ancestors of Africa or Europe from which *Homo sapiens* is thought to have diverged. Only one apoE genotype has been reported in chimpanzees that closely resembles human apoE4 with arginine (R) at positions 112 and 158 (Table 2)(Hanlon and Rubinsztein ,1995;Hanlon and Rubinsztein ,1995). All other primates examined so far have arginine at 112 and 158 (Finch and Sapolsky ,1999). Because of these similarities between human apoE4 and primate apoE and because of the sequence analysis of the genealogical depth of human apoE alleles, human apoE4 is considered the ancestral allele in primates (Hanlon and Rubinsztein ,1995). It should be noted that rodent apoE, as well as that of many other mammals belong to the apoE-type 4 family as well. Interestingly, apoE2 allele, which was shown to confer significant protection against sporadic AD, also happens to be over-represented in human centenarians (Blanche *et al.*,2001;Frisoni *et al.*,2001);clearly pointing toward a role in longevity and successful aging. These and other observations argue against the hypothesis that apoE4 allele exert it main effects in the AD brain through gain of toxic activity as proposed by some investigators.

6. Synaptic plasticity and integrity in AD as a function of apoE4 allele dose

In the nervous system, the importance of the polymorphic nature of apoE has recently been revealed, with regards to function in neuronal plasticity and with respect to other

pathologies such as dementia of the Alzheimer type (Poirier, 1994). Apolipoprotein $\epsilon 4$ allele was shown to be strongly associated with the familial and sporadic forms of Alzheimer disease (Poirier *et al.*, 1993; Strittmatter *et al.*, 1993). The apo $\epsilon 4$ allele can affect the rate of progression of the disease, the extent of the neuronal cell loss, cholinergic activity, accumulation of amyloid plaques in hippocampal and cortical areas, and total beta amyloid production and beta amyloid deposition in the brain of AD subjects. ApoE4 carriers were also shown to exhibit poor synaptic remodeling and defective compensatory plasticity in vulnerable brain areas in AD (Arendt *et al.*, 1997; Beffert *et al.*, 1998); particularly in cholinergic-rich region (Poirier *et al.*, 1995). Actually, the role of apoE in the maintenance of synaptic integrity and plasticity is so central to brain physiology that the ability of a subject to recover from traumatic brain injuries is highly dependent upon apo $\epsilon 4$ allele dose (Lichtman *et al.*, 2000; Friedman *et al.*, 1999).

The effect of apoE genotype on synaptic plasticity and recovery is not restricted to the AD condition. While the apoE4 allele is associated with poor clinical outcome in patients with Parkinson's (Li *et al.*, 2004), stroke (Nicoll *et al.*, 1996; Slioter *et al.*, 1997), ALS or other type of neurodegenerative disease (Hogh *et al.*, 2000; Sorbi *et al.*, 1995), apoE2 allele was found to be protective against several neurodegenerative diseases, including sporadic and familial late onset AD (Corder *et al.*, 1994).

7. ApoE Genotype versus ApoE Levels

The notion that human E4 allele carriers are unable to maintain effective apoE concentrations in blood, brain or CSF relatively to other isoform carriers has gained momentum in recent years. The original concept stems from observations made by Utermann and colleagues (Utermann *et al.*,1980) more than 30 years ago about the fact that humans expressing the apoE4/3 and apoE4/4 genotype display the lowest apoE blood levels of all living humans, whereas those with an apoE2/2 genotype (centenarian candidates) belong to a small group of humans with the highest blood concentration of apoE (for a review, see (Poirier ,2008)). This is true for blood (Utermann ,1985;Poirier ,2005;Panza *et al.*,2003;Gupta *et al.*,2011;Soares *et al.*,2012) , brain tissues (Bertrand *et al.*,1995;Beffert *et al.*,1999) (Glockner *et al.*,2002) and CSF (Cruchaga *et al.*,2012);GWAS study in ADNI) in humans and in fresh brain tissues from apoE4 knock-in mice {Sullivan, 2011 4439 /id;Bales, 2009 4440 /id} when using LS/MS/MS quantitative methodology. Figure 3B summarizes key published findings. Recently, cross sectional analysis of the subjects enrolled in ADNI revealed a progressive reduction of plasma apoE concentrations as a function of disease progression: i.e. normal cognitive controls > non converting MCI > converting MCI = AD (Figure 3C). Similar observations were recently reported in the CSF of ADNI subjects, with a highly significant decline in CSF apoE levels: CTL > MCI > AD.

The notion that compromised apoE levels in the CNS of apoE4 carriers contributes significantly to the pathophysiological process in AD was further extended by work performed in apoE knockout mice. The complete absence of apoE in the knockout mice is associated with progressive age-related cognitive deficit in the Morris swim maze

(Oitzl *et al.*,1997;Veinbergs *et al.*,1999;Champagne *et al.*,2002;Davignon *et al.*,1982), a marked loss of cholinergic innervation with age (Van Uden *et al.*,2000;Kleinfeld *et al.*,1998) and a pronounced loss of synaptic integrity after 10-12 months of age (Chapman *et al.*,2000;Veinbergs and Masliah ,1999). Furthermore, long-term potentiation (Krzywkowski *et al.*,1999), as well as synaptic plasticity and terminal proliferation (Veinbergs and Masliah ,1999;Champagne *et al.*,2005) are markedly compromised in apoE deficient mice in presence of abnormally high concentrations of tau phosphorylation (Gordon *et al.*,1996).

8. Apolipoprotein E as a Potential Therapeutic Target ?

The bulk of these observations led scientists to develop assays to identify potential apoE inducer agents that could be used *in vivo* for the treatment (and conceivably the prevention) of sporadic AD. Some of the most interesting apoE-inducing candidates identified so far include indomethacin (Aleong *et al.*,2003), a potent anti-inflammatory drug used in the past to treat mild-to-moderate AD (Rogers *et al.*,1993); estrogen, the controversial hormone that exhibits protective effect (Craig *et al.*,2005); probucol, the cholesterol lowering drug used to treat familial hypercholesterolemia (Champagne *et al.*,2003). More recently, the LXR agonist T0901317 (Riddell *et al.*,2007) and the RXR agonist bexarotene (Cramer *et al.*,2012) were both identified as modulators of the signaling cascade that regulate the acute synthesis of apoE, ABCA1/G1 and the LDL receptor family in the brain (for a review, (Leduc *et al.*,2010).

Supporting the notion that apoE induction might be beneficial for AD treatment; the potent apoE inducer bexarotene was shown to restore cognitive abilities in APP transgenic mice as does LXR agonist T0901317 in a different APP mouse model. While the bexarotene cognitive benefit was recently replicated (Fitz *et al.*, 2013), it is not clear that these effects have anything to do with resorption of fibrillary amyloid or the amyloid metabolism itself. The nuclear activators are used for the treatment of cancer, where moderate toxicity is tolerated, but are not safe enough for long-term use in either prevention or treatment of AD. Safer apoE inducers are needed and the lipid lowering drug probucol could be used as such an agent.

Probucol is an old cholesterol-lowering drug formerly given to treat hyperlipidemias and still used in many Asian countries. Added to rat and mouse diet (1% w/w), it achieves plasma concentrations that mimic those of high human doses (~1g/day), and induces cortical and hippocampal apoE synthesis (Champagne *et al.*, 2003). Probucol was shown recently to suppress enterocytic A β in the cerebral vessels of mice on a high fat diet (Pallebage-Gamarallage *et al.*, 2012) and to prevent cognitive and synaptic impairment resulting from intra-vascular A β injections (Santos *et al.*, 2012).

A few years ago, our team ran a small pilot proof-of-concept study of the then-standard dose of probucol (500 mg b.i.d.) in 12 people with mild-to-moderate AD who were not taking cognitive enhancers. We found a probucol-related increase in serum apoE (Poirier and Panisset, 2002) as reported earlier in cardiovascular trials (McPherson *et al.*, 1991; Quinet *et al.*, 1993) and saw a similar increase in CSF apoE after one month of

treatment. Testing probed the stabilization of scores on ADAS-Cog and MMSE, and improvement on the Disability Assessment of Dementia (DAD) scales over the 6-month trial. Cumulative probucol dosage (pill count) correlated in a dose-dependent fashion with CSF apoE levels (*Figure. 4A*). We also found that serum probucol levels measured by LC/MS/MC correlates well with changes in ADAS-Cog after 6 months (not shown). Cumulative dosage correlated similarly with ADAS-Cog change (*Figure. 4B*). *Figure 4C* shows correlation between change in CSF apoE levels after 1 month and 6-month improvement on the ADAS-Cog. Recently, we reassessed CSF levels of total *tau* (*Ttau*), 181P-*tau* and A β 42 using the widely used Innogenetics AlzBio3 kit on a Luminex apparatus. *Figure 4D* illustrates the result of a contrast analysis between apoE alteration and changes in P-*tau* concentrations in the brain, the latter serving as a marker of neuronal damage. These findings extended earlier results showing that apoE increase predicted decreased A β load, reflecting the amyloid scavenging properties of apoE-HDL complexes (Poirier, 2003b) and the removal of amyloid peptides from the brain to the CSF. Overall, improvement in CSF apoE concentration in probucol-treated subjects correlates well with cognitive performance, decline in P-*tau* and scavenging of total amyloid into the CSF.

9. Conclusion

Clinical trials with potential treatments for Alzheimer's disease (AD) have ended in repeated failures, without any new agents approved since 2003. Despite the obvious need, attempts to develop new drugs or especially prevention strategies have often encountered

safety concerns. To avoid such problems, the field has turned increasingly to safer lifestyle interventions. These have achieved some success in other applications, but usually require sustained behavioral interventions that may be of questionable “real world” utility. More typically, even strong evidence fails to dissuade most people from health-adverse behaviors. Pharmaco-prevention strategies may be more effective, but new drug development has been impeded by the enormous resources needed for discovery and testing of new agents (e.g., development times for new products often exceeding 13 years). Drug “re-purposing” may offer a more efficient alternative as suggested in this short review. Furthermore, familiar generic drugs have known safety profiles that can deter unexpected risks. Given the many efforts by others based on the amyloid cascade hypothesis, we believe that a rationally justified, gene-based, alternate approach seems timely.

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- Figure 1. A) Life expectancy over the century in the Western world. B) Projected prevalence of Alzheimer's disease in the next 40 years in the USA. Adapted from (Alzheimer Association, 2010)
- Figure 2. Schematic representation of the physiological compartmentalization of the most important proteins associated with the top 6 genetic risk factors identified by GWAS these past 5 years.
- Figure 3. ApoE levels in different regions according to apoE genotype or diagnosis. A) Human hippocampal and frontal cortex apoE levels as a function of disease status and APOE genotype (adapted from (Beffert *et al.*,1999)). Data represent average \pm S.E.M. Statistical analysis: $p < 0.05$ for both APOE genotype and pathology effect (ANOVA) in the hippocampus; not significant, $p = 0.12$ for APOE genotype (ANOVA) in the frontal cortex. B) Plasma apoE levels as a function of APOE genotype in mild to moderate AD cases ($p < 0.001$: adapted from (Poirier ,2005). C) Baseline plasma apoE levels as a function of disease progression and diagnosis ($p < 0.001$: adapted from (Soares *et al.*,2012)). The diagnosis was established at the follow-up, which was no later than 48 months after the first visit. ApoE levels are lower in patients with Alzheimer's disease and mild cognitive impairment (MCI) compared to healthy age-matched controls. Data represent means and 95 % confidence intervals. MCInp: MCI patients who were not diagnosed with dementia at the follow-up; MCIp:

patients with MCI who were diagnosed with dementia at the follow-up;
HCS: healthy control subjects.

Figure 4. Pilot study of probucol in mild-to-moderate AD. A) CSF apoE protein variation between 1 month and baseline correlated with cumulative dose of probucol (number of 250 mg pills consumed). B) Contrasting changes on ADAS-Cog (6 months vs baseline) as a function of cumulative probucol dose. C) ADAS-Cog change as a function of CSF apoE variation (1 month vs baseline). D) Reduction of P(181)-*Tau* concentration (standardized Innogenetic AlzBio3 X-MAP luminex bioassay) as a function of CSF apoE levels.

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Table 1: Genetics of Alzheimer's disease

Familial (autosomal dominant) form – 1-2 % of all AD cases			
Chromosome	Gene	Proportion of all AD cases	Function
1	PSEN2	Only a few families	APP processing
14	PSEN1	~2-3 %	APP processing
21	APP	Only a few families	Unknown
Common (sporadic) form – 98-99 % of all AD cases			
Chromosome	Gene	Proportion of all AD cases	Function
19	APOE (allele E4)	50-60 %	Lipid transport (extracellular)
8	CLU (APOJ)		Lipid transport (extracellular)
19	ABCA7		Lipid transport (intracellular)
2	BIN1		Lipid internalisation
11	PICALM		Lipid internalisation
3	BCHE		Amyloid Deposition/Lipid processing/neurotransmission
11	MS4A6A		Unknown
19	CD33		Immune reactions
21	TRPM2		Immune reactions

Table 2

Apolipoprotein E: Polymorphisms in human and primates			
ApoE Residue	Population Prevalence (%)	Site 112	Site 156
Human:			
ApoE2	8	Cysteine	Cysteine
ApoE3	78	Cysteine	Arginine
ApoE4	15	Arginine	Arginine
Chimpanzee	100	Arginine	Arginine
Gorilla	100	Arginine	Arginine
Orangutan	100	Arginine	Arginine

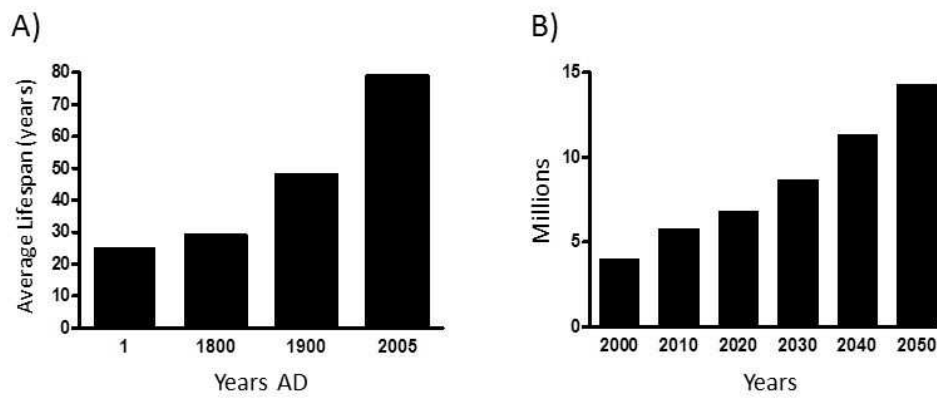


Figure 2

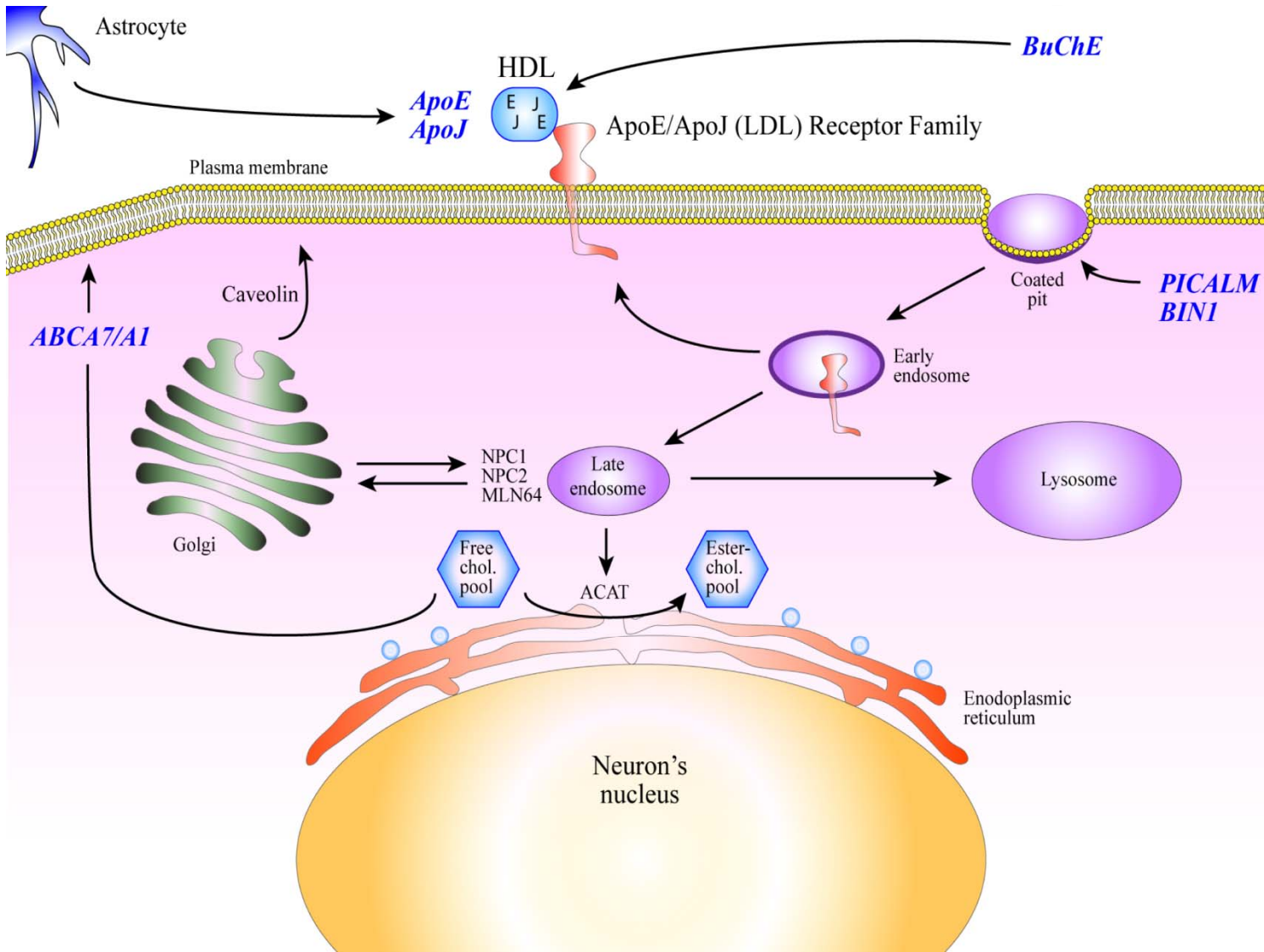


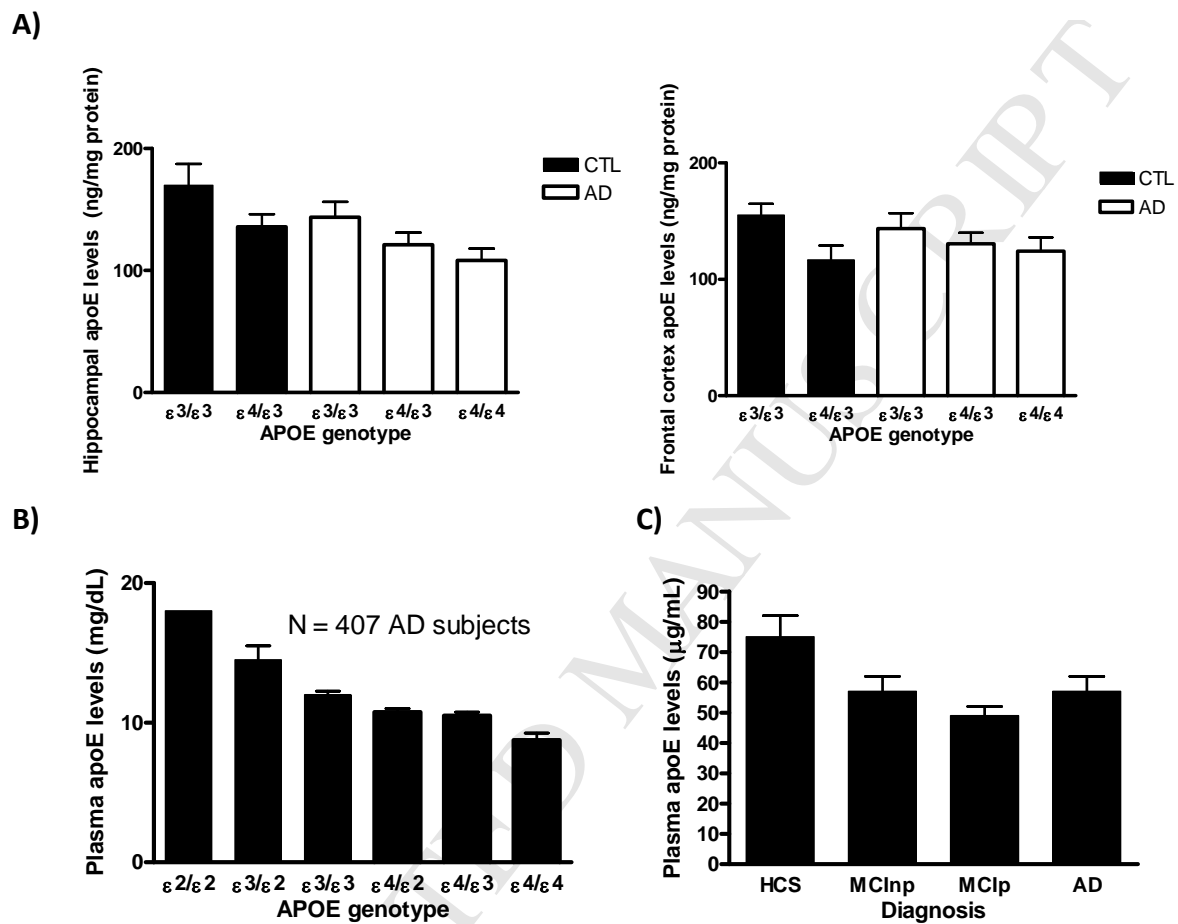
Figure 3

Figure 4

