MODULATION OF LIPOPROTEIN METABOLISM IN RESPONSE TO BRAIN INJURY AND ALZHEIMER'S DISEASE: ROLES FOR APOLIPOPROTEIN E4 AND LIPOPROTEIN LIPASE

By

Jean-François Blain

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Department of Neurology and Neurosurgery
McGill University and
Douglas Hospital Research Center
Montreal, Canada

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ABSTRACT

From the association of the ε4 allele of apolipoprotein (apo) E with Alzheimer's disease (AD) to the more recent reports of reduced risks of developing the disease when taking cholesterol lowering agents, links seem to point for a central role for lipoprotein metabolism in the AD brain. While the association with apoE4 is the strongest and most reproduced risk factor for AD, roles for other proteins involved in lipoprotein metabolism in the periphery have not been thoroughly investigated.

Using the entorhinal cortex lesion (ECL) paradigm in human apoE-targeted replacement mice we examine whether apoE4 has effects on reactive synaptogenesis in the absence of the concentration bias observed in human and how these effects are mediated. In a second study, again using the ECL model, we investigate the role of lipoprotein lipase (LPL) in the brain in response to injury. Finally, we study the effect of intronic polymorphisms of the LPL on the risk and severity of AD.

The results show that mice expressing apoE4 have impaired astroglial organization resulting from an exacerbated inflammatory state and culminating into reactive synaptogenesis impairment. We also observe isoform-specific tau phosphorylation and beta-amyloid (Aβ) accumulation. Furthermore, we report that LPL plays a role in the degeneration phase following ECL. We propose that it is involved in the recycling of lipids together with the glial-specific proteoglycan syndecan-4. Finally we report associations between LPL polymorphisms and AD risk and severity. Since the polymorphisms associate with increased expression of LPL in AD brain as well as with increased senile plaque number, we propose that LPL may be involved in amyloid clearance and deposition.

Taken together these results confirm the importance of lipoprotein metabolism in AD as apoE and LPL are both involved in maintaining cholesterol homeostasis in the brain and also both participate in Aβ metabolism.

RÉSUMÉ

De l'association entre l'allèle ε4 de l'apolipoprotéine (apo) E avec la maladie d'Alzheimer (MA) jusqu'aux rapports plus récents montrant une réduction des risques de MA lorsque sous traitement avec des agents hypocholestérolémiants, plusieurs liens semblent démontrer que le métabolisme des lipoprotéines dans le cerveau joue un rôle prépondérant dans la MA. Bien que l'apoE4 soit le facteur de risque le plus fort et le plus reproduit dans la MA, le rôle d'autres protéines impliquées dans le métabolisme périphérique des lipoprotéines n'a pas été étudié de façon extensive dans le cerveau.

Nous avons utilisé le modèle de lésion du cortex entorhinal (LCE) chez des souris exprimant l'apoE humaine pour étudier l'effet de l'allèle ε4 sur la synaptogenèse réactive en l'absence du biais relatif à la concentration observé chez l'humain, ainsi que la cause de cet effet. Dans une deuxième étude, où nous utilisons encore le modèle LCE, nous avons étudié le rôle de la lipase lipoprotéique (LPL) dans le cerveau suite au dommage. Finalement, nous avons étudié l'effet de polymorphismes de la LPL sur le risque et la sévérité de la MA.

Les résultats obtenus montrent que l'apoE4 cause un déficit d'organisation astrogliale résultant d'un état inflammatoire exacerbé et qui culmine en un déficit de la synaptogenèse réactive. Nous observons aussi des effets spécifiques à l'isoforme quant à la phosphorylation de tau et l'accumulation de l'amyloid bêta (Aβ). De plus, nous rapportons un rôle pour la LPL dans la phase de dégénérescence suite à la LCE. Elle semble impliquée dans le recyclage des lipides en combinaison avec le protéoglycan glial syndecan-4. Finalement, comme les polymorphismes de la LPL sont en association avec le risque de MA, une expression élevé de LPL, ainsi qu'un nombre plus élevé de plaques séniles, nous suggérons que la LPL puisse être impliquée dans la clairance et/ou la déposition de l'amyloid.

Ces résultats confirment l'importance du métabolisme des lipoprotéines dans la MA puisque deux protéines y ayant un rôle prépondérant sont impliquées dans le maintien de l'homéostasie du cholestérol et participent aussi au métabolisme de l'Aβ.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Study 1: The results of this study further the understanding of the mechanism by which apoE4 mediates terminal sprouting impairment. They show that, following ECL, apoE4 causes an exacerbated inflammatory reaction that leads to impaired astroglial organization pattern and ultimately to the impaired neuronal sprouting.

Study 2: In this study we report a new role for LPL in the adult brain in response to injury. Our findings indicate that, during the degeneration phase observed following ECL, LPL is involved in the recycling of the lipids released by degenerating terminals and that this action involves the glia-specific proteoglycan syndecan-4.

Study 3: This study reports that intronic polymorphisms in LPL associate with the risk of AD in a pathologically-confirmed cohort of subjects. Moreover, this association extends to the severity of the disease as the polymorphism also associate with several biological markers of AD like a cholinergic marker (ChAT), neurofibrillary tangles number and senile plaques number. Our results suggest a role for LPL in amyloid clearance and deposition.

CONTRIBUTION OF AUTHORS

Study 1:

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Dr Judes Poirier supervised the research project, Dr Patrick M. Sullivan provided the mice and reviewed the manuscript. The author of this thesis carried out the work, analyzed the data and wrote the manuscript.

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Dr Judes Poirier supervised the research project. Nicole Aumont and Louise Théroux helped with DNA extraction, cholesterol measurements and performed preliminary genotyping. Doris Dae helped with mRNA extraction. The author of this thesis carried out the work, analyzed the data and wrote the manuscript.

LIST OF ABBREVIATIONS

24-OHC	24-hydroxycholesterol
ABCA1	ATP binding cassette A1
ACAT	Acyl-coenzyme A:cholesterol acyltransferase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADAS-Cog	Alzheimer's Disease Assessment Scale cognitive subunit
alv	Alveus
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APLP	APP-like protein
apo	Apolipoprotein
apoER2	Apolipoprotein E receptor 2
apoE-TR	Apolipoprotein E-targeted replacement
APP	Amyloid precursor protein
Aβ	Beta amyloid
BACE	Beta amyloid converting enzyme
BBB	Blood brain barrier
C/A	Commissural/associational fibers
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
ChAT	Choline acetyltransferase
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP46	Cholesterol 24-hydroxylase
Dab1	Disabled-1
DG	Dentate gyrus
DPL	Days post-lesion
ec	External capsule

EC	Entorhinal cortex
ECL	Entorhinal cortex lesion
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
FC	Free cholesterol
FH	Familial hypercholesterolemia
Fu	Fusiform gyrus
GFAP	Glial fibrillary acidic protein
GSK-3β	Glycogen synthase kinase 3β
HDL	High-density lipoprotein
HL	Hepatic lipase
HLP	Hyperlipoproteinemia
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPLC	High performance liquid chromatography
HSPG	Heparan sulfate proteoglycan
ICC	Immunocytochemistry
IL-1	Interleukin-1
IL-1ra	Interleukin-1receptor antagonist
IML	Inner molecular layer of the DG
ko	knockout
LCAT	Lecithin:cholesterol acyltransferase
LDLR	Low-density lipoprotein receptor
LOAD	Late onset Alzheimer's disease
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	LDL receptor-related protein
LTP	Long-term potentiation
LXR	Liver X receptor
MMSE	Mini mental state examination
MRI	Magnetic resonance imaging

NFT	Neurofibrillary tangle
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NPC	Niemann-Pick C
NSAID	Non-steroidal anti-inflammatory drug
NSE	Neuronal-specific enolase
OML	Outer molecular layer of the DG
PAGE	Polyacrylamide gel electrophoresis
PCx	Parietal cortex
PET	Positron emission tomography
PHF	Paired helical filaments
PL	Phospholipid
PNS	Peripheral nervous system
PP2A	Protein phophatase 2A
PS	Presenilin
RAP	Receptor-associated protein
RT-PCR	Reverse transcriptase polymerase chain reaction
SCAP	SREBP cleavage-activating protein
SNP	Single nucleotide polymorphism
SP	Senile plaque
SR-BI	Scavenger receptor B type I
SRE	Sterol response element
SREBP	SRE binding protein
Tg	Triglyceride
VLDLR	Very-low-density lipoprotein receptor

1.0 GENERAL INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 Historical and clinical aspects

In 1907 Alois Alzheimer published a study entitled "*A characteristic serious disease of the cerebral cortex*" (Alzheimer, 1907) in which he reported for the first time clinical symptoms and histopathological findings of a dementia that would, 3 years later, be named after him. Today, Alzheimer's disease (AD) is recognized as the most common form of dementia affecting the elderly. In 2000, there were approximately 18 million persons affected by dementia worldwide with more than half suffering from Alzheimer's disease (AD). It is estimated that the prevalence of AD doubles every 5 years or so and is expected to affect 1% of 60-year-olds and about 30% of 85-year-olds (Cummings, 2004).

Over the years, the development of more specific diagnostic tools has led to a diagnosis accuracy of approximately 90%. Cognitive functions are usually assessed using the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) and the cognitive subunit of the Alzheimer's Disease Assessment Scale (ADAS-Cog) (Rosen et al., 1984). Functional assessment can be performed using a combination of different tests like the Activities of Daily Living Questionnaire (Johnson et al., 2004), the Physical Self-Maintenance test and the Instrumental Activities of Daily Living (Lawton et al., 1969). The Global Deterioration Scale is also widely used (Reisberg et al., 1982). Imaging techniques such as positron emission tomography (PET) and magnetic resonance imaging (MRI) are also recognized to be superior to cognitive testing for early diagnosis of AD (Zamrini et al., 2004).

AD is primarily characterized by the progressive onset of symptoms that will initially affect episodic (short-term) memory. It then progresses by affecting cognitive processes causing language deficits (e.g. speech and comprehension), visuo-spatial functions alterations (e.g. spatial thinking and orientation), executive functions deficits (e.g. planning, decision-making, judgement) and finally impairment in activities of daily living (e.g. eating, dressing, washing). Awareness of this decline in the beginning of the disease often leads to depression and while insight gradually fades, it will be replaced by

denial and rationalisation (Lindeboom et al., 2004). Patients survive an average of 8 years following clinical onset (Barclay et al., 1985) and most often die of bronchitis/pneumonia (Beard et al., 1996).

1.1.2 Pathological features

Since clinical diagnostic tools are not 100% accurate, post-mortem analysis of the brain is necessary to confirm the nature of dementia. Alois Alzheimer described "*very peculiar changes of the neurofibrils*" leading to "*...only tangles of fibrils indicate where a nerve cell had been previously located.*" He also noted the "*disappearance of nerve cells especially in the upper cortical layers*" and the presence of "*miliar foci, which are caused by the deposition of a peculiar substance in the cortex*" (Graeber et al., 1999). Today, the neuronal loss, neurofibrillary tangles (NFT) and senile plaques (SP) are still considered the necessary pathological markers for confirming an AD diagnosis.

1.1.2.1 Neuronal death and synaptic loss

One of the major pathological hallmark of AD are the widespread neuronal and synaptic losses (Bowen et al., 1979; Brun et al., 1981; Hamos et al., 1989; Scheff et al., 1993; Brun et al., 1995). Included among the most affected areas in AD are the hippocampus (Ball, 1977), the entorhinal cortex (Gomez-Isla et al., 1996), the basal forebrain (Whitehouse et al., 1982), the nucleus basalis of Meynert (Whitehouse et al., 1981; Tagliavini et al., 1983; Arendt et al., 1985; Etienne et al., 1986), the locus coeruleus (Bondareff et al., 1987; Burke et al., 1988; Zarow et al., 2003) and to a lower extent the dorsal raphe nucleus (Lyness et al., 2003). This leads to major deficits in cholinergic (Bowen et al., 1976) and noradrenergic (Zarow et al., 2003) neurotransmission. Serotonergic and glutamatergic neurotransmission is also affected but to a lesser extent (Hardy et al., 1987; Procter et al., 1988; Lyness et al., 2003).

The extensive and specific cholinergic neuronal loss (Davies et al., 1976) combined to the memory impairments caused by cholinergic blockers (Drachman et al., 1974) lead to the hypothesis that modulation of the cholinergic system might be beneficial for AD patients. Inhibitors of acetylcholinesterase (AChE) were developed to prevent the degradation of the transmitter acetylcholine (ACh). Three AChE inhibitors are widely used for the symptomatic treatment of AD patient, namely donepezil

(Aricept®), rivastigmine (Exelon®) and galantamine (Reminyl®). They alleviate some of the symptoms for a period of time however, their efficacy is lost as neurons continue to die. Moreover, response to treatment is dependent on apolipoprotein E genotype (see section 1.2.4) (Poirier et al., 1995a).

More recently, glutamatergic transmission was also targeted. Memantine (Namenda®), an antagonist of the glutamate *N*-methyl-D-aspartate (NMDA) receptor, has now been approved by the Food and Drug Administration as a treatment for AD. It alleviates some of the cognitive deficits associated with AD either by interfering with glutamate toxicity or improving hippocampal neuronal function (Parsons et al., 1999). Development of AMPA receptor potentiators is also currently under way (O'Neill et al., 2004) as glutamatergic transmission through these receptors is responsible for long-term potentiation (LTP) which is thought to be the molecular mechanism underlying learning and memory formation (Lynch, 2004).

1.1.2.2 The amyloid cascade

Another typical marker of AD is the presence of SP in the brain which are mainly composed of deposited β-amyloid (Aβ) (Glenner et al., 1984a; Masters et al., 1985). This Aβ is derived from a precursor called the amyloid precursor protein (APP) that belongs to a type I transmembrane protein family that also includes APP-like protein-1 and 2 (APLP1, APLP2) in mammals (Tanzi et al., 1987; Wasco et al., 1992; Wasco et al., 1993). In the nervous system, APP was shown to preferentially localize at synaptic sites (Schubert et al., 1991) and more specifically on post-synaptic densities (Shigematsu et al., 1992), suggesting a role in synaptic activity. The recent finding of neuromuscular junction synapse defect in APP/APLP2 double knockout mice confirms that the APP family of proteins plays a crucial role in synaptic integrity (Wang et al., 2005a).

APP can undergo proteolysis through the α-secretase/ADAM10 or the β-secretase (BACE) pathway, followed by an intramembrane cleavage by the enzymatic complex γ-secretase (composed of APH-1, PEN-2, nicastrin and presenilin) to generate either the non-amyloidogenic peptide p3 or Aβ (40 or 42 amino acids) respectively (Haass, 2004). The predominant form of Aβ found in the cerobrospinal fluid (CSF) is $A\beta_{40}$ which

represents 90% of the total concentration in humans. However, this proportion is changed to about 50% for both species in AD patients (Mehta et al., 2001). The more fibrilogenic $A\beta_{42}$ is the first species to deposit, forming what is known as diffuse plaques (Iwatsubo et al., 1994) and $A\beta_{40}$ gradually accumulates to form the mature plaques (Iwatsubo et al., 1994; Gravina et al., 1995).

According to the "amyloid hypothesis of AD", the oligomerization and deposition of $A\beta_{42}$ would lead to progressive synaptic injury, development of tangles and ultimately cell death and dementia (Hardy et al., 2002). Based on this hypothesis, therapeutic strategies consisting at interfering with Aβ formation and/or accumulation were considered. Two different strategies directly target Aβ metabolism. The first one consists in the inhibition of the secretases responsible for Aβ formation. For BACE inhibitors, research is under way to identify potent small molecules that could cross the BBB and have a high affinity for the enzyme (Cumming et al., 2004; Hom et al., 2004; Lefranc-Jullien et al., 2005). In the case of γ-secretase, many potent inhibitors are already available but there is concern about their safety because γ-secretase is also involved in Notch signaling (De Strooper et al., 1999) and could provoke undesirable effects (Lewis et al., 2003; Wong et al., 2004). Development of inhibitors that discriminate between Aβ and Notch cleavage might prove useful in the treatment of AD (Netzer et al., 2003; Takahashi et al., 2003). The second strategy uses active or passive immunization against Aβ to prevent its oligomerization and the subsequent deposition or enhance its clearance (Schenk et al., 1999; Bard et al., 2000; DeMattos et al., 2001a). However, the clinical trial involving Aβ immunization (AN-1792; Elan and Wyeth-Ayerst) had to be halted because some patients started to develop symptoms of inflammation in the CNS (Munch et al., 2002a; Munch et al., 2002b; Robinson et al., 2004).

It is significant to note that Aβ was shown to have neurotrophic effects on neuronal cells *in vitro* (Whitson et al., 1989; Yankner et al., 1990) and that its production was critical for the viability of neurons (Plant et al., 2003). However another form of Aβ, the Aβ-derived diffusible ligands or ADDLs were shown to be highly toxic for neuronal

cells (Lambert et al., 1998a; Hartley et al., 1999) and could be a better potential target than soluble Aβ.

1.1.2.3 Neurofibrillary tangles

As opposed to the senile plaques, neurofibrillary tangles (NFTs) are better markers of AD duration and severity (Arriagada et al., 1992) and they also parallel neuronal loss (Gomez-Isla et al., 1997). Moreover, their consistent distribution lead Braak and Braak to the definition of six neuropathological stages of AD (Braak et al., 1991). NFTs are composed of the cytoskeletal protein tau (Kondo et al., 1988; Goedert et al., 1988; Wischik et al., 1988) (Lee et al., 1991) which is found in a hyperphosphorylated state compared to normal tau (Ksiezak-Reding et al., 1992). In the normal brain tau is specifically expressed in neurons and localized in axons (Binder et al., 1985; Migheli et al., 1988) where it promotes microtubule assembly and stabilization of the cytoskeleton. On the other hand, AD brains display aberrant neuronal tau immunoreactivity (Kowall et al., 1987). The highly phosphorylated state of tau favors its assembly into a structure called paired helical filaments (PHFs) and greatly affects its ability to bind microtubules (Bramblett et al., 1993). This destabilization of microtubules interferes with axonal transport and ultimately leads to neurodegeneration.

A balance between kinase and phosphatase activities regulates the phosphorylation state of tau. Fetal and adult CNS tau are phosphorylated on the same sites than PHF-tau suggesting that a decreased phosphatase activity could lead to abnormal phosphorylation (Matsuo et al., 1994). It was in fact shown that protein phosphatase (PP)2A can regulate tau phosphorylation state (Sontag et al., 1996; Planel et al., 2001) and that its mRNA and protein levels are reduced in AD brain (Vogelsberg-Ragaglia et al., 2001; Sontag et al., 2004a; Sontag et al., 2004b).

In light of these evidences tau-based therapies were suggested. It is suggested that inhibiting specific kinases or activating PP2A could help reduce phosphorylation thereby inhibiting PHFs formation (Iqbal et al., 2004; Bhat et al., 2004). Microtubule stabilization is also considered as a therapeutic avenue and recently, one such approach was successfully tested in a mouse model of tauopathy in which deficient fast axonal transport and motor impairments were ameliorated (Zhang et al., 2005).

1.1.3 The entorhino-hippocampal pathway

Memory loss is the major clinical feature of AD and is the result of hippocampal dysfunction (Milner et al., 1955; Penfield et al., 1958; Milner, 1959). More than a century ago, Ramòn y Cajal described a massive fiber input to the hippocampus that was coming from the entorhinal cortex (EC). He termed it 'perforant pathway' and evidenced it as the major source of cortical input to the hippocampus (Ramòn y Cajal, 1995). Indeed, the EC receives afferents from association areas of the cortex that provide somatosensory information (Van Hoesen et al., 1972; Van Hoesen et al., 1975a; Van Hoesen et al., 1975b) and it also receives input from the presubiculum and parasubiculum which are both important relays for limbic circuits as well as from the subiculum (Shipley, 1974; Shipley, 1975; Kohler, 1985). In turn, layers II and III of the EC send projections (via the perforant path) to the outer two third of the molecular layer of the dentate gyrus as well as to the CA1 and CA3 neurons in the Ammon's horn (Van Hoesen et al., 1975c; Witter et al., 1991) which also sends projections back to the EC and association cortices (Hjorth-Simonsen, 1971; Rosene et al., 1977). The EC is thus involved in memory consolidation as it does not only act as an information relay but also as an integrator of the flow of information directed to the hippocampus (Lavenex et al., 2000).

1.1.3.1 Implication for Alzheimer's disease

Considering that the EC relays the major cortical inputs to the hippocampus, it is not surprising to find that it is one of the first and most affected brain structure in AD. Neurofibrillary tangles were shown to be most severely found in the EC (Ball, 1978) and APP immunoreactivity is higher in EC neurons (Roberts et al., 1993). Moreover, neurons in the layers II and IV of the EC are specifically affected in AD, a process that isolates the hippocampus from a lot of its neuronal input and output, thus leading to the memory deficits observed (Hyman et al., 1984). Atrophy of the EC using imaging techniques is also reported and in some cases correlated to the magnitude of cognitive impairment (Kesslak et al., 1991; Narkiewicz et al., 1993; Krasuski et al., 1998; Xu et al., 2000; Cardenas et al., 2003; deToledo-Morrell et al., 2004; Jack, Jr. et al., 2004).

1.1.3.2 The entorhinal cortex lesion paradigm in rodents

Neuronal death in the EC isolates the hippocampus and leads to memory impairments. Based on this evidence, the entorhinal cortex lesion (ECL) paradigm that had been developed in the rat to study reactive sprouting in the hippocampus (Lynch et al., 1972) was used to compare markers of plasticity in human AD and ECL rats brains (Geddes et al., 1985). The similarity between both models lead the authors to propose that the AD brain was also capable of a reactive growth response to degeneration (Geddes et al., 1985). This similarity with AD, the extensive characterization of the paradigm in the rodent (Lynch et al., 1972; Lynch et al., 1976; Matthews et al., 1976a; Matthews et al., 1976b; Cotman et al., 1977; Gall et al., 1979; Hoff et al., 1982a; Hoff et al., 1982b; Steward et al., 1988; Gehrmann et al., 1991a) and the availability of transgenic animal models make the ECL model an attractive one to study the course of the changes that could be observed in a human AD brain.

1.1.4 Genetics of Alzheimer's disease (Familial vs. Sporadic)

Alzheimer's disease can either be classified as familial or sporadic. The 'familial' form represents a small fraction (~10%) of all AD cases and is characterized by the presence of many affected siblings over many generations of a family. On the other hand, the 'sporadic' form can appear in individuals with no familial history of the disease. It is now clear that specific genetic mutations in the amyloid precursor protein and presenilins are responsible for the rare but aggressive early-onset familial form of the disease whereas the sporadic form doesn't appear to have a definitive genetic cause but rather a series of genetic susceptibility factors that affect either the age of onset or the rate of progression.

1.1.4.1 Amyloid Precursor Protein and Presenilins

Autosomal dominant familial Alzheimer's disease (FAD) can be caused by mutations in either one of these three genes: the amyloid precursor protein (APP), the presenilin (PS)-1 or the PS-2, that result in an aggressive early-onset (< 65 years old) form of the disease (**Figure 1**).

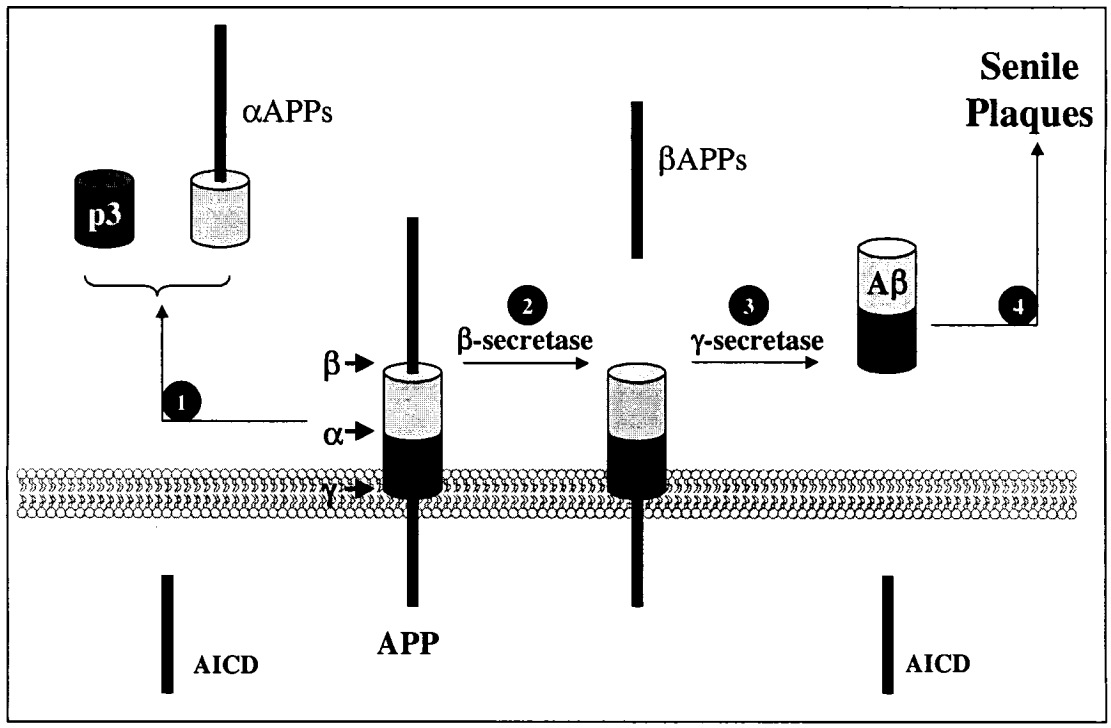

Figure 1. The amyloid cascade.

The amyloid precursor protein (APP) can either be cleaved by the α-secretase to enter a non-amyloidogenic pathway (1) or the β-secretase to enter the amyloidogenic pathway (2) both giving rise to an α or β soluble fragment of APP (APPs) respectively. Subsequent intramembranous cleavage by the γ-secretase gives rise either to the peptide p3 and the APP intracellular domain (AICD) in the case of α cleavage (1) or beta amyloid (Aβ) and AICD in the case of β cleavage (3). Accumulation of Aβ, in combination with other proteins, form the senile plaques (4).

Amyloid deposits similar to those in AD also accumulate in Down's syndrome (trisomy 21) (Glenner et al., 1984b). Linkage analysis of DNA markers on chromosome 21 of patients with early-onset FAD revealed that it was linked to the disease and mapped to sequence for APP (Tanzi et al., 1987; Goldgaber et al., 1987; George-Hyslop et al., 1987). Mutations were found in APP from FAD patients (Chartier-Harlin et al., 1991; Murrell et al., 1991; Goate et al., 1991) and patients with hereditary cerebral hemorrhage (Levy et al., 1990; van Broeckhoven et al., 1990) and today there are 18 (out of 23 known) pathogenic mutations reported, with most of them present in exon 17 of APP, the region coding for Aβ. Some of these mutations were shown to result in either an increased production of Aβ (Citron et al., 1992; Cai et al., 1993) or an increased ratio of $A\beta_{42}/A\beta_{40}$ (Suzuki et al., 1994; Tamaoka et al., 1994). A relatively new mutation called the 'Arctic mutation' increases the amyloid protofibril formation instead of modulating Aβ levels and ratios (Nilsberth et al., 2001).

Two other genetic loci with strong linkage to FAD were found on chromosomes 1 and 14 (St George-Hyslop et al., 1992; Mullan et al., 1992; van Broeckhoven et al., 1992; Schellenberg et al., 1992; Li et al., 1995; Levy-Lahad et al., 1995). The gene products are the homologous proteins PS-1 (chr.14) and PS-2 (chr.1). They are part of the γ-secretase enzymatic complex (mostly PS-1) which is the final step in Aβ production. There are now 142 and 10 mutations reported for PS-1 and PS-2 respectively in FAD with the majority resulting in an increased $A\beta_{42}$ production (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996; Citron et al., 1997; Walker et al., 2005).

1.1.4.2 Apolipoprotein E

As linkage to chromosome 21 failed to reproduce in familial late-onset AD (LOAD; > 65 years old), the search for other possible genetic markers lead to the identification of a new locus on chromosome 19 (Pericak-Vance et al., 1991) and in 1993, a genetic association between apolipoprotein (apo)E and AD was established. ApoE is a cholesterol transporter expressed in three different isoforms in humans referred to as apoE2, apoE3 and apoE4 (see section 1.2.4). The inheritance of a specific allele of apoE has profound effects on the brain's response to injury. For instance, the ε4 allele of apoE associates with a higher risk of developing AD, a phenomenon observed for

familial (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993a) and sporadic (Saunders et al., 1993; Poirier et al., 1993a) LOAD as well as for early-onset forms (van Duijn et al., 1994; Chartier-Harlin et al., 1994). On the other hand, the ε2 allele protects against AD (Corder et al., 1994; Chartier-Harlin et al., 1994) and delays its onset by nearly 20 years making it a longevity marker (Schachter et al., 1994; Frisoni et al., 2001). As opposed to the causative genes associated with FAD, apoE4 is rather referred to as a risk factor since its inheritance increases AD susceptibility and its presence is not a predictor of the disease development.

1.1.4.3 Cholesterol-related genetic factors

Following the discovery of the genetic association between apoE4 and LOAD, the hunt for other genetic factors underlying the etiology of AD started. There is now over a hundred genes that are reported to associate with AD and around 50 that turned out reproducible (Finckh, 2003). In addition to apoE, it is interesting to note that there is a cluster of genes also relating to cholesterol homeostasis. It includes apoCI (Drigalenko et al., 1998; Petit-Turcotte et al., 2001), the VLDL receptor (Okuizumi et al., 1995; Helbecque et al., 1998; Yamanaka et al., 1998; McIlroy et al., 1999), the ATP-Binding Cassette (ABC) A1 and A2 (Chen et al., 2004; Katzov et al., 2004), the cholesterol 24-hydroxylase (CYP46) (Kolsch et al., 2002; Desai et al., 2002; Papassotiropoulos et al., 2003) and, even though there is conflicting reports about its association, the LDL receptor related-protein (LRP) (Fallin et al., 1997; Clatworthy et al., 1997; Kang et al., 1997; Wavrant-DeVrieze et al., 1997; Lendon et al., 1997; Scott et al., 1998; Kamboh et al., 1998; Hollenbach et al., 1998; Lambert et al., 1998b; Lambert et al., 1999; Beffert et al., 1999a). Finally, genetic variants of the LDL receptor (LDLR) were recently shown to modulate AD susceptibility (Gopalraj et al., 2005). These associations suggest that cholesterol homeostasis must have a prominent role in AD etiology.

1.2 Lipoproteins in Peripheral and Central Cholesterol Homeostasis

Lipoproteins are macromolecular complexes composed of proteins and lipids and are responsible for the transport of these lipids between tissues . They are normally spherical in shape and are composed of a neutral lipid core made of cholesteryl esters

(CE) and triglycerides (Tg) surrounded by a shell of phospholipids (PL) and free cholesterol (FC). The protein moiety of lipoproteins called apolipoprotein is exposed at the surface and mediates targeting of the particles to specific receptor and/or modulation of enzymes (see section 1.2.1 and **Figure 2**).

Lipoproteins can be divided into four major classes depending upon their density following ultracentrifugation: chylomicrons, very low (VLDL), low (LDL) and high density lipoproteins (HDL). Chylomicrons are Tg-rich particles synthesized in the intestine that transport dietary lipids into the circulation which, following the action of the lipoprotein lipase (LPL), release fatty acids and glycerol from Tg which are then used as fuel or stored in adipocytes. The cholesterol-enriched 'remnant' particles are then taken up by the liver via the LDLR or LRP (Veniant et al., 1998). When excess dietary fat is absorbed, the other Tg-rich lipoproteins VLDL are synthesized in the liver and, similarly to chylomicrons, transport Tg to muscles and adipose tissue and their remnants are finally removed from the circulation via receptor-mediated uptake by the liver (Veniant et al., 1998). Some VLDL particles are converted to LDL by the action of lipases (mostly LPL but also hepatic lipase (HL)) and LDL supply extrahepatic tissues with cholesterol for membrane or steroid hormone synthesis. LDL are the major cholesterol transporters in periphery.

On the other hand, the HDL are mostly involved in reverse cholesterol transport. Indeed, HDL particles facilitate cholesterol removal from peripheral tissues by targeting it to the liver for disposal (Davis et al., 1982). Due to their low lipid to protein ratio, the subclass of particles with the highest density, HDL_3 and pre-β-HDL are the most efficient cholesterol acceptors (Castro et al., 1988; Miida et al., 1990).

A major difference between brain and peripheral lipoproteins is the existence of a unique particle of the HDL type in the former (LaDu et al., 1998; Fagan et al., 1999). These lipoproteins, secreted by astrocytes, are composed of PL and FC and contain apoE or apoJ (see section 1.2.1). They are devoid of neutral lipid core and are discoidal in shape (Fagan et al., 1999). Unlike the extended knowledge we have for peripheral lipoproteins, less is known about their exact role in the CNS. They participate in the redistribution of lipids and maintenance of cholesterol homeostasis but as opposed to

peripheral HDL, their role in mediating efflux of cholesterol to the periphery is limited (Pitas et al., 1987a; Pitas et al., 1987b; Bjorkhem et al., 1997).

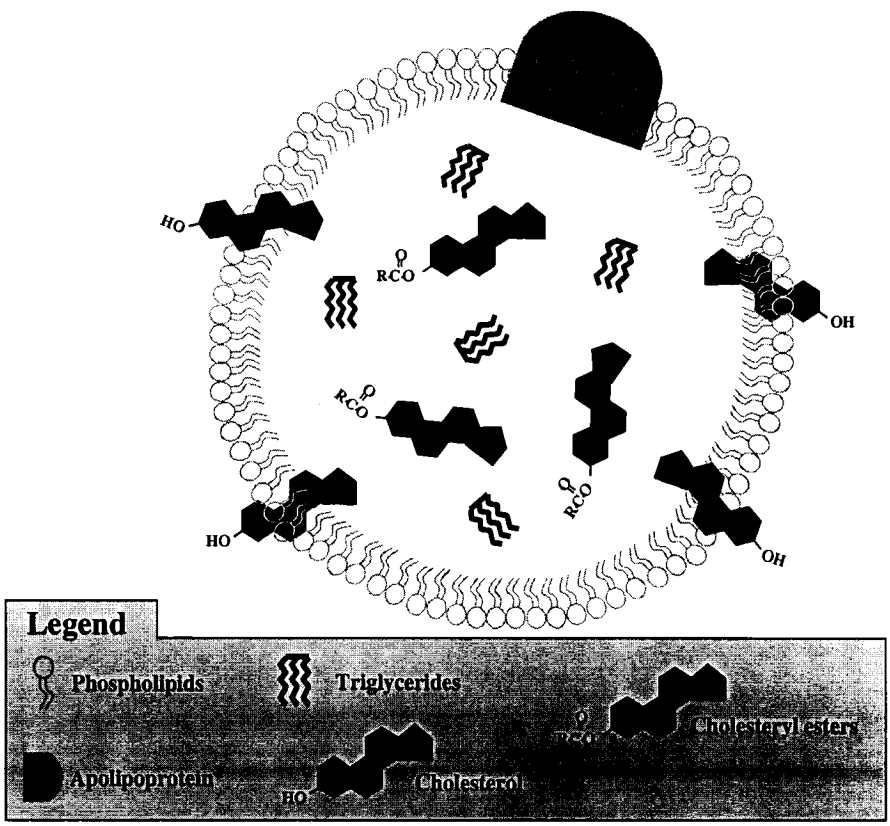

Figure 2. Structure of a lipoprotein.

1.2.1 Apolipoproteins, receptors and their functions

The protein component of lipoproteins is called apolipoprotein. Its major function is to transport lipids in the vascular space and tissues but depending on the type of apolipoprotein it also exerts modulation of enzymes mostly involved in lipid homeostasis. All classes of apolipoproteins (A through J) were isolated from plasma but only apoA-I, apoA-IV, apoC-I, apoD, apoE and apoJ were shown to be locally produced in the brain (Elshourbagy et al., 1985; Drayna et al., 1986; Lauer et al., 1988; Weiler-Guttler et al., 1990; de Silva et al., 1990a; Liu et al., 2001).

1.2.1.1 Apolipoprotein A

There are three proteins of the apoA type, namely apoA-I, A-II and A-IV. In periphery apoA-I is the major HDL protein (Alaupovic et al., 1972) and is involved in targeting cholesterol to the liver for elimination, a phenomenon known as 'reverse cholesterol transport', making it an anti-atherogenic apolipoprotein. Its major site of synthesis is the liver but it is also synthesized in the intestine. It binds to a receptor called scavenger receptor class B type I (SR-BI) and mediates the transfer of CE to the liver for disposal and also to steroidogenic tissues (Acton et al., 1996; Williams et al., 2000; Silver et al., 2001; Temel et al., 2002). ApoA-I is also an activator of the enzyme lecithin:cholesterol acyltransferase (LCAT) which is present on the surface of nascent HDL particles (Yokoyama et al., 1980) as well as of cholesteryl ester transfer protein (CETP) (Guyard-Dangremont et al., 1994). LCAT catalyzes the esterification of cholesterol that precedes its packaging in the core of the lipoprotein particle while CETP promotes the transfer of CE between LDL and HDL particles. ApoA-I is found centrally in the CSF where it could originate from local production (Weiler-Guttler et al., 1990) or filtration at the blood-brain barrier (BBB) (Roheim et al., 1979).

Liver and intestine also produce apoA-II which is found on HDL particles, but its precise role remains unclear. It seems to have an antagonistic effect on the reverse cholesterol transport (Barbaras et al., 1987; Lagrost et al., 1995), negatively modulate LCAT and CETP which are involved in the remodeling of HDL particles (Lagrost et al., 1994; Labeur et al., 1998; Durbin et al., 1999) and even though it binds to SR-BI (Pilon

et al., 2000), it is less efficient than apoAI at delivering CE (Huang et al., 1995; Rinninger et al., 1998a).

Finally, peripheral apoA-IV is synthesized solely by the small intestine in humans where it is found associated with chylomicrons and VLDL. In fact, its expression is modulated by long chain fatty acids and chylomicron assembly (Weinberg et al., 1990). It is also found associated with HDL and evidences suggest that its primary role is in intestinal lipid absorption (Kalogeris et al., 1997). More recently it was shown to be synthesized in the hypothalamus (Liu et al., 2001) and to act centrally in the control of satiety (Fujimoto et al., 1992; Woods et al., 1998).

1.2.1.2 Apolipoprotein B

ApoB exists in two forms, apoB-48 and apoB-100 (Kane, 1983). ApoB-48 is a truncated version of apoB-100 (Powell et al., 1987; Chen et al., 1987) that is synthesized in the small intestine where it is found associated with chylomicrons. On the other hand, apoB-100 is primarily synthesized in the liver and is necessary for the formation of VLDL. Upon action of LPL and HL, VLDL are converted to LDL which are mainly targeted to a cell-surface receptor called apoB/E or LDLR, thereby delivering cholesterol to peripheral tissues and regulating cholesterol homeostasis (Brown et al., 1986). Megalin (a.k.a. gp330/LRP2) is also a receptor of the LDLR family that was shown to bind apoB-100 containing particles (Stefansson et al., 1995).

1.2.1.3 Apolipoprotein C

ApoC consists of three proteins with different properties namely apoC-I, C-II and C-III. ApoC-I and C-II are located in a gene cluster with apoE on chromosome 19 (Myklebost et al., 1986; Lauer et al., 1988; Smit et al., 1988) while apoC-III is located in another gene cluster with apoA-I and A-IV on chromosome 11 (Karathanasis, 1985). ApoC-I is primarily synthesized in the liver but also in the brain (Lauer et al., 1988; Petit-Turcotte et al., 2001) whereas apoC-II and C-III are synthesized in the liver and intestine (Protter et al., 1984; Wei et al., 1985). They are all found in association with chylomicrons, VLDL and HDL and share the ability of inhibiting lipoprotein binding to LDLR, LRP and VLDLR (Weisgraber et al., 1990; Kowal et al., 1990; Sehayek et al.,

1991; Clavey et al., 1995), even though they have distinct roles in lipid homeostasis. For instance, apoC-II is an essential activator of LPL while apoC-I and C-III are inhibitors (LaRosa et al., 1970; Brown et al., 1972; Ekman et al., 1975; Wang et al., 1985). They also affect LCAT and CETP activities in opposite manners (Soutar et al., 1975; Nishida et al., 1986; Steyrer et al., 1988; Sparks et al., 1989; Kushwaha et al., 1993).

1.2.1.4 Apolipoprotein D

ApoD is associated with HDL particles (McConathy et al., 1973) but its precise role is still unclear. It is weakly expressed in intestine and liver whereas kidney, adrenals, spleen and brain express high levels (Drayna et al., 1986). There is no report of binding to any receptor suggesting it has a limited role in cholesterol homeostasis. Evidences suggest that it could form a complex with LCAT thus favoring reverse cholesterol transport (Fielding et al., 1980; Steyrer et al., 1988). However, its affinity for cholesterol and CE is very low (Peitsch et al., 1990; Patel et al., 1997). It can bind arachidonic acid, steroids and bilirubin suggesting that it is a multi-ligand transporter (Pearlman et al., 1973; Peitsch et al., 1990; Dilley et al., 1990; Simard et al., 1991; Morais Cabral et al., 1995). It also has been hypothesized to have a role in certain types of cancer (Balbin et al., 1990; Sanchez et al., 1992; Aspinall et al., 1995) and in central and peripheral nervous system repair (Boyles et al., 1990; Goodrum et al., 1995; Ong et al., 1997; Terrisse et al., 1998; Terrisse et al., 1999).

1.2.1.5 Apolipoprotein E

ApoE will be discussed in more details in Section 1.3 but briefly, it is a polymorphic protein that is a component of HDL, VLDL, and it is also acquired by intestinal chylomicrons for transporting dietary lipids. It is primarily synthesized in the liver but is also found in numerous tissues including adrenals, testes, kidney and brain (Zannis et al., 1985; Newman et al., 1985; van et al., 1996). Interestingly, the brain is the second major site of apoE mRNA synthesis after the liver (van et al., 1996). It binds the LDLR with high affinity thus delivering cholesterol to the tissues (Innerarity et al., 1978). There is now a whole family of receptors similar to the LDLR reported to bind apoE namely LRP (Beisiegel et al., 1989; Kowal et al., 1989), megalin/gp330 (Willnow et al., 1992), VLDLR (Takahashi et al., 1992; Sakai et al., 1994), apoER2 (Kim et al., 1996;

Novak et al., 1996) and LR11/SorLA-1 (Taira et al., 2001). ApoE also binds to heparan sulfate proteoglycans (HSPG) that can act as receptors or co-receptors for LRP (Ji et al., 1993). ApoE thus plays a central role in vascular biology but is also involved in neurobiology and immunology as it will be discussed in section 1.3.

1.2.1.6 Apolipoprotein J

ApoJ is also known as clusterin because it was discovered as a protein capable of eliciting cell clustering (Blaschuk et al., 1983). It is expressed in almost all tissues with high levels in the brain, ovaries, testes and liver (de Silva et al., 1990a). In the plasma it is found associated with HDL that also contain apoA-I and CETP activity (de Silva et al., 1990b). It is also found on astrocytes-derived HDL particles (LaDu et al., 1998; Fagan et al., 1999; DeMattos et al., 2001b). It binds to the megalin/gp330 receptor which mediates its internalization and degradation (Kounnas et al., 1995a). Its precise function has yet to be determined but it has been shown to be involved in a number of diverse processes such as sperm maturation (Bettuzzi et al., 1989; Mattmueller et al., 1991; Veeramachaneni et al., 1991; Cyr et al., 1992), complement activation and apoptosis (controversial) (Jenne et al., 1989; Kirszbaum et al., 1989; Choi et al., 1990; Garden et al., 1991), lipid transport (de Silva et al., 1990a; de Silva et al., 1990b; Jenne et al., 1991) and amyloid metabolism (Zlokovic et al., 1994; Zlokovic et al., 1996; Hammad et al., 1997; DeMattos et al., 2002; DeMattos et al., 2004).

1.2.1.7 Other minor apolipoproteins

Some minor apolipoprotein were also isolated and following the ABC nomenclature were named apoF, apoG, apoH and apoM.

ApoF was first isolated from HDL particles (Olofsson et al., 1978) but was later shown to be almost exclusively associated with LDL particles (Wang et al., 1999a). It is homologous to the previously described lipid transfer inhibitory protein (LTIP) which is an inhibitor of CETP activity (Wang et al., 1999a).

ApoG was isolated from HDL particles (Ayrault-Jarrier et al., 1978) but there is no information on its specific function.

ApoH, also known as beta 2-glycoprotein-I, is synthesized in the liver (Steinkasserer et al., 1991) and is an important auto-antigen in patients with

antiphospholipid syndrome (McNeil et al., 1990; Galli et al., 1990). In relation to lipid metabolism, it is found in all the major lipoprotein fractions but 70% is located under the 1.21 g/ml density fraction (Polz et al., 1979). It is suggested to have anti-atherogenic properties as evidenced by its capacity to activate LPL (Nakaya et al., 1980) and mediate triglyceride clearance (Wurm et al., 1982), as well as inhibiting oxidized LDL internalization (Hasunuma et al., 1997).

Finally, apoM was isolated from Tg-rich lipoproteins (Xu et al., 1999) and was later shown to be synthesized exclusively by the liver and kidneys (Zhang et al., 2003). It is mostly associated with HDL and to a lesser extent Tg-rich lipoproteins and LDL (Zhang et al., 2003). It was recently shown that apoM plays an important role in reverse cholesterol transport as it is required for the formation of lipid-poor HDL as well as cholesterol efflux to HDL (Wolfrum et al., 2005).

1.2.2 Cholesterol homeostasis

Cholesterol homeostasis is regulated through sophisticated mechanisms involving its *de novo* synthesis, absorption from the diet and elimination. There is however a major difference between extrahepatic tissues and the brain as the BBB leaves the CNS to rely solely on *de novo* cholesterol synthesis whereas in periphery a contribution from the diet also comes into play (**Figure 3**).

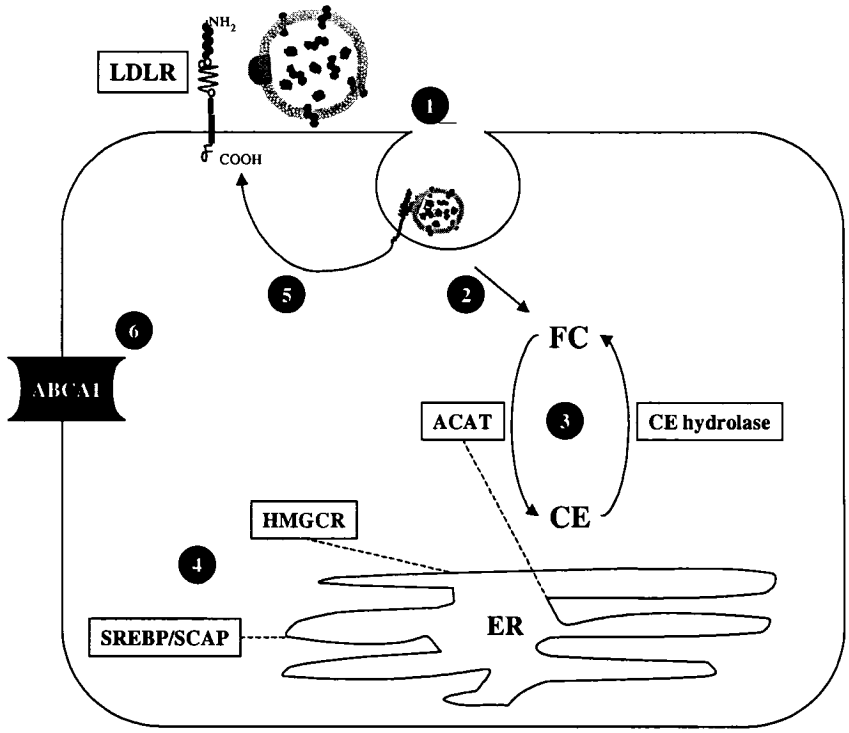

Figure 3. Cellular pathway regulating cholesterol homeostasis.

A lipoprotein interacts with a cell-surface receptor of the LDLR family and gets internalized via clathrin-coated vesicles or caveolae (1). After passage through endosomes and lysosomes (2), cholesterol is released inside the cell and stored in its ester form following the action of ER-localized enzyme ACAT (3). Cholesterol build-up in the cell causes the retention of SREBP in the ER and repression of LDLR and HMGCR expression (4). LDLR can escape the lysosomal pathway and endosomes are recycled to the cell surface with intact receptor (5). ABCA1 is involved in the transfer of cholesterol to lipid-poor lipoproteins (mostly HDL) (6). **Abbreviations**: **LDLR**—low-density lipoprotein receptor; **FC**—free cholesterol; **CE**—cholesteryl ester; **ER**—endoplasmic reticulum; **ACAT**—acylCoA:cholesterol acyltransferase; **HMGCR**—3-hydroxy 3-methylglutaryl-CoA reductase; **SREBP**—sterol response element binding protein; **SCAP**—SREBP cleavage-activating protein; **ABCA1**—ATP binding cassette type A1.

1.2.2.1 Regulation in periphery

Diet contributes in part to the body pools of cholesterol. Indeed, dietary cholesterol is first absorbed from the intestine by a transporter called Niemann-Pick C1 Like 1 (NPC1L1) (Davis, Jr. et al., 2004; Altmann et al., 2004) and then esterified in enterocytes by acyl-CoA:cholesterol acyltransferase type 2 (ACAT2) (Buhman et al., 2000) before being delivered to the liver via the chylomicron pathway. Even though all cells require cholesterol for their normal functions, it is not an essential component of the diet since it can be synthesized from simple precursors with the liver being the major contributor. It is normally synthesized following a cascade of enzymatic reactions from which the reduction of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) to mevalonate by the enzyme HMG-CoA reductase (HMGCR) is the limiting step (Qureshi et al., 1976). It can also enter the cell, associated with lipoproteins, through receptor-mediated endocytosis (Anderson et al., 1977). The endocytosed vesicles containing lipoproteins bound to their receptors fuse with endosomes where the complex dissociates, the receptors are recycled to the plasma membrane and the lipoproteins targeted to lysosomes (Anderson et al., 1977; Basu et al., 1981). Cholesterol is then stored in its ester form following the action of ACAT1 (Brown et al., 1975a).

The uptake of cholesterol triggers negative feedback regulation of its *de novo* synthesis (Siperstein et al., 1960; Shapiro et al., 1971; Brown et al., 1973). Most of the mechanisms involved in this feedback regulation were brought to light by the group of Nobel laureates Brown & Goldstein. By unraveling the genetic defect underlying familial hypercholesterolemia (FH) they revealed the intimate relationship existing between cholesterol internalization and synthesis (Goldstein et al., 1974a; Brown et al., 1974a). The presence of serum (or LDL) suppresses HMGCR activity (Brown et al., 1973; Goldstein et al., 1974a), increases cholesterol esterification (Goldstein et al., 1974b) and leads to a decreased expression and synthesis of the LDLR (Brown et al., 1975b). Furthermore, the limited binding capacity of FH cells for apoB-containing lipoproteins is associated with an inability to suppress HMGCR activity, while cholesterol in a non-lipoprotein form is able to mediate feedback regulation (Brown et al., 1974b).

Cholesterol regulates the expression of HMGCR and LDLR at the molecular level as evidenced by the presence of sterol regulatory elements (SRE) in their promoters (Sudhof et al., 1987; Osborne et al., 1988). This DNA motif binds a transcription factor called sterol regulatory element binding protein (SREBP) which is a transmembrane protein embedded in the endoplasmic reticulum (ER) membrane (Briggs et al., 1993; Wang et al., 1993). Following a double proteolytic cleavage, SREBP translocates to the nucleus where it binds to SRE and promotes the transcription of HMGCR and LDLR (Hua et al., 1993; Yokoyama et al., 1993; Briggs et al., 1993; Wang et al., 1993; Wang et al., 1994). The proteolytic processing is the step dependent on intracellular sterol concentration. A protein called SREBP cleavage-activating protein (SCAP) can form complexes with SREBP in the ER membrane and also directly bind cholesterol (Hua et al., 1996; Radhakrishnan et al., 2004). When cells are depleted in sterols, SCAP escorts SREBP from the ER to the Golgi where it can be cleaved (Hua et al., 1996; Nohturfft et al., 2000; Radhakrishnan et al., 2004). On the other hand, when there is a sterol build up inside the cell, SCAP undergoes a conformational change that allows its binding to INSIG-1 or 2 resulting in a retention of SREBP in the ER and repression of HMGCR and LDLR expression (Yabe et al., 2002; Yang et al., 2002) thus mediating the negative feedback regulation. It is interesting to note that NPC1L1 also contains a sterol sensing domain and is down-regulated following ingestion of high cholesterolemic meals (Davis, Jr. et al., 2004).

Finally, body pools of cholesterol are also dependent on its elimination through reverse cholesterol transport. It is generally agreed that HDL is the transporter for excess peripheral cholesterol to the liver and that apoA-I and SR-BI are the major apolipoprotein and receptor involved in HDL-cholesterol excretion (Temel et al., 2002). However, modification of the level or activity of the different components involved in reverse cholesterol transport do not increase its excretion (Jolley et al., 1998; Alam et al., 2001). In the recent years, members of the ATP-binding cassette (ABC) transporters family have been found to mediate an important role in reverse cholesterol transport. The analysis of two sterol-related disorders led to the discovery of mutations in three genes that have now been shown to influence in some way reverse cholesterol transport.

Tangier disease is a genetic disorder of cholesterol transport partially characterized by decreased apoA-I, A-II and HDL levels as well as acumulation of tissue cholesterol (Fredrickson, 1964; Hoffman et al., 1965; Engel et al., 1967; Kocen et al., 1967) in which mutations in the gene coding for ABCA1 have been identified (Rust et al., 1999; Bodzioch et al., 1999; Brooks-Wilson et al., 1999). It seems that ABCA1 is essential for cholesterol efflux towards apoA-I and HDL (Remaley et al., 1997; von Eckardstein et al., 1998) but studies in ABCA1 null mice showed that reverse cholesterol transport was unaffected as measured by the unchanged sterol excretion (Groen et al., 2001; Drobnik et al., 2001).

Mutations in ABCG5 and ABCG8 (Berge et al., 2000; Lee et al., 2001) were found to underlie the genetic defect sitosterolemia, a disorder in which plant sterols are absorbed more than normal (Bhattacharyya et al., 1974). They are both half transporters that need to heterodimerize to move to the cell surface (Graf et al., 2004) and they are both essential for sterol secretion into the bile as demonstrated by the modulation of biliary cholesterol secretion in mice either overexpressing or knock-out for *ABCG5/G8* (Yu et al., 2002a; Yu et al., 2002b).

1.2.2.2 Regulation in the brain

Because of the impermeability of the BBB to plasma lipoproteins, the contribution of dietary intake is minimized, thus cholesterol has to be synthesized *in situ* to meet brain requirements (Chobanian et al., 1962). Cells of the central nervous system are capable of *de novo* cholesterol synthesis and can also take up lipoproteins from their local environment. Astrocytes synthesize 2 to 3-fold more cholesterol than neurons but the myelin-producing oligodendrocytes produce even more than astrocytes (Saito et al., 1987). Synthesis occurs at a very low rate and cholesterol's estimated half-life in the brain is 2-4 months (Bjorkhem et al., 1997) and even reaches 1 year in myelin (Ando et al., 2003). As observed in periphery, biosynthesis is regulated by the activity of HMGCR (Sudjic et al., 1976) which is dependent on lipoprotein uptake by the LDLR and the sterol concentration of the cell (Poirier et al., 1993b). *In vitro* studies showed that LRP also appears to modulate the internalization of cholesterol rich lipoproteins in neurons (Holtzman et al., 1995; Veinbergs et al., 2001).

One of the major difference between brain and peripheral cholesterol is that brain cholesterol is almost completely unesterifed (>99.5%) and resides either in myelin sheaths or plasma membranes (Bjorkhem et al., 2004). Another major difference is the excretion mechanism used by the brain to eliminate excess cholesterol. As opposed to the reverse cholesterol transport that requires lipoproteins to eliminate cholesterol in periphery, the brain relies on two different strategies: the excretion of apoE-bound cholesterol in the CSF (Pitas et al., 1987b) and the quantitatively more important oxidation of cholesterol into 24-hydroxycholesterol (cerebrosterol, 24-OHC) (Lutjohann et al., 1996; Bjorkhem et al., 1997). The enzyme responsible for this chemical modification is the cholesterol 24-hydroxylase (CYP46) which is a member of the cytochrome P450 family expressed in the brain at 100-fold higher levels than in the liver (Lund et al., 1999; Lund et al., 2003). Interestingly it is localized in neurons which have the lowest cholesterol synthesis capacity among brain cells (Saito et al., 1987; Bogdanovic et al., 2001). As opposed to the BBB insolubility of cholesterol, 24-OHC is very soluble. It has a half-life of 0.5 days and is rapidly expelled from the brain by direct efflux through the BBB into the blood (Bjorkhem et al., 1997). Most of the circulating 24-OHC is thought to originate from the brain (Lutjohann et al., 1996; Meaney et al., 2000). A very tight balance is maintained between cholesterol synthesis and excretion in the brain as revealed by the 40% decrease in synthesis observed in CYP46 knock-out mice (Lund et al., 2003).

1.2.2.3 Cholesterol in relation to amyloid metabolism

The first study reporting an association between cholesterol and amyloid metabolism in the brain was originally published by Sparks *et al.* (Sparks et al., 1994). They showed that rabbits fed with a 2% cholesterol diet for 8 weeks exhibited a marked accumulation of intracellular Aβ immunoreactivity in hippocampal and cortical areas. The study was repeated in a primate model exposed to a high saturated fat diet for 5 years and Aβ accumulations were observed in cortical and hippocampal areas (Schmechel et al., 2002). Manipulation of cholesterol diet in APP transgenic mice was shown to significantly enhance Aβ accumulation in the brain and to accelerate and increase the amyloid deposition normally seen in these animals(Refolo et al., 2000; Shie et al., 2002).

Several i*n vitro* experiments have clearly demonstrated that cholesterol greatly influence Aβ metabolism and conversely, that $A\beta_{40}$ and $A\beta_{42}$ markedly alter cholesterol transport and internalization. For instance, the cholesterol content of neurons regulates APP processing (Simons et al., 1998) by directly acting on the secretases in cholesterol-enriched microdomains such as lipid rafts and caveolae (Nishiyama et al., 1999). Increases in membrane cholesterol tend to favor the beta site cleavage of APP whereas pharmacological depletion of brain cholesterol using methyl-β-cyclodextrin or HMGCR inhibitors such as the statins favor the balance toward the non-amyloidogenic pathway (i.e. the alpha site cleavage) (Kojro et al., 2001). It is thought that a high membrane cholesterol content decreases fluidity and increases viscosity, allowing more effective interactions between APP, BACE and γ-secretase. It was also shown that BACE's presence is enriched in lipid rafts (Marlow et al., 2003) and that γ-secretase activity is cholesterol-dependent (Wahrle et al., 2002). It is interesting to note that an increase in caveolin-1 expression in AD brains (Gaudreault et al., 2004) and a higher cholesterol content of tangle-bearing neurons (Distl et al., 2001) are completely consistent with a cholesterol-mediated alteration in Aβ production (Puglielli et al., 2001).

Numerous studies have shown that Aβ can either increase or decrease hippocampal synaptic plasma membrane fluidity by changing the membrane cholesterol content (Gibson et al., 2003). Aβ was also shown to affect cholesterol homeostasis by altering its cellular distribution (Igbavboa et al., 2003) and decreasing its esterification (Liu et al., 1998). These mechanisms could be seen as regulatory feedback mechanism so that Aβ would control its production by acting on cholesterol homeostasis. There is also evidence that amyloid directly interacts with lipoprotein complexes and promotes apoE-mediated cholesterol-rich lipoprotein internalization in a dose-dependent fashion in primary neurons (Beffert et al., 1998). Recently it was reported that middle-aged hypercholoesterolemic subjects who died in their 40-50ies display marked accumulation of amyloid plaques in the hippocampal and cortical areas when compared to age-matched normocholesterolemic subjects (Pappolla et al., 2003), consistent with the notion of a tightly regulated interaction between cholesterol and amyloid metabolism in the human brain.

1.2.2.4 Therapeutic avenues

A promising approach for the treatment of common AD is the use of pharmacological agents that modulate cholesterol homeostasis. Evidence obtained from epidemiological studies indicates that treatment with statins that were used to stabilize high blood cholesterol also reduce the risk of developing AD by more than 65% (Rockwood et al., 2002). However these early retrospective analyses have been recently challenged by a prospective epidemiological study that failed to see a reduction of risk in statin users (Li et al., 2004). Treatments with statins are known to cause an up-regulation of LDLR at cell surface, reduction in blood LDL and a marked increase in HDL. At the moment, it is unclear whether statins need to enter the brain to confer protection or if the effect is indirect. The hydrophilic nature of pravastatin prevents its penetration in the brain but it was shown to reduce AD prevalence to the same extent as lovastatin which crosses more readily the BBB (Wolozin et al., 2000).

Several studies have shown that cholesterol-lowering agents were able to modify Aβ metabolism in the brain. For instance, guinea pigs treated with high doses (200-fold those used in humans) of the HMGCR inhibitor simvastatin show reduced plasma cholesterol levels as well as decreased Aβ production in the brain and CSF, even though brain cholesterol levels remained unaffected (Fassbender et al., 2001). APP-PS1 double transgenic mice treated with the cholesterol-lowering drug BM15.766 also show reduced blood cholesterol as well as brain Aβ production but in this case, the treatment also produced a slight significant brain cholesterol reduction (Refolo et al., 2001). A recent study using 6 different statins in humans reported marked reductions in cholesterol and its metabolites in the CSF but no change in Aβ42 concentration after 6 months (Fassbender et al., 2002). The cholesterol-lowering compound Probucol was shown to increase apoE and LRP expression in the brain, changes that might indirectly favor Aβ clearance from the brain (Champagne et al., 2003).

Results from the first prospective, proof-of-concept, double blind, placebo-controlled, randomized clinical trial of a potent statin in mild-to-moderate AD have been made public recently (Sparks et al., 2005). The placebo-controlled investigation of atorvastatin effect in mild to moderate AD individuals indicate a stabilization or mild improvement of the clinical outcome measures. Significance was not reached in most of

the parameters measured. However, it was expected as this was designed as a pilot proof-of-concept study with a small number of patients with results that support the trial's rationale (Sparks et al., 2005). Larger cohorts of patients will be necessary to extend and confirm theses results.

The effect of statins on the progression of AD is consistent with another report on the beneficial effect of Probucol, a cholesterol-lowering agent that stimulates brain apoE production without affecting cholesterol synthesis. In this study, mild to moderate AD patients treated with Probucol for 6 month revealed a relative stabilization of symptoms according to the Alzheimer's disease Assessment Scale-Cognition (ADAS-Cog) and on the Disability Assessment Scale (Poirier et al., 2002). Benefits on the ADAS-Cog correlated with increase in CSF apoE levels and an inverse relationship between apoE and total Aβ in the CSF was found for all the patients (Poirier, 2003).

Overall, the results of these different studies suggest that it is possible to affect the onset and/or progression of the disease by interfering with brain cholesterol homeostasis prior to or shortly after a diagnosis of AD. It is still unclear if the beneficial effect of lowering cholesterol directly targets brain metabolism or if it is an indirect effect caused by a reduction of the cardiovascular risks. In secondary stroke prevention studies with statins in humans, the risk reduction was not correlated with the cholesterol-lowering effect of the drug, suggesting that alternative modes of action may be at play in the central nervous system (Milani, 2004).

1.3 Apolipoprotein E: Genetic Risk factor and Therapeutic Target

ApoE is a 34 kD glycoprotein composed of 299 amino acids (Rall, Jr. et al., 1982). Three isoforms (ε2, ε3, ε4) with a single unit of net charge difference are revealed by isoelectric focusing (Utermann et al., 1977; Utermann et al., 1978). They arise from multiple alleles at a single genetic locus found on chromosome 19 (Zannis et al., 1981) that result in a single amino acid switch (Cys ↔ Arg) at the protein level (apoE2, apoE3, apoE4) (Weisgraber et al., 1981). Thus apoE3 expresses a cysteine at position 112 and an arginine at position 158, apoE2 expresses cysteine at both sites and apoE4, arginine at both sites (Rall, Jr. et al., 1982). Six genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4)

and the corresponding phenotypes (E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, E4/E4) are found in the eastern Canadian population with an allelic distribution of approximately 8, 77 and 15% for ε2, ε3 and ε4 respectively (Davignon et al., 1987), a distribution that is similar to other populations (Mahley et al., 2000).

1.3.1 Apolipoprotein E isoforms

As mentioned in section 1.2.1.5, apoE is a major player in cholesterol homeostasis by targeting lipoprotein particles to receptors for internalization. The existence of different isoforms in humans has physiological consequences affecting the vascular biology and neurobiology of the protein.

1.3.1.1 Vascular biology

ApoE measurements in the plasma reveal a genotype-dependent concentration where apoE2 > apoE3 > apoE4 (Utermann et al., 1980; Boerwinkle et al., 1988). This is due to the different rates of metabolism for each isoform. Indeed, apoE4 has a plasma residency which is half of that for apoE3 (Gregg et al., 1986) while apoE2 accumulates due to its defective binding to the LDLR (Weisgraber et al., 1982). Cholesterol and apoB concentrations are also modulated by apoE genotype but in a reverse fashion to apoE concentration (apoE4 > apoE3 > apoE2) (Boerwinkle et al., 1988). Moreover, lipoprotein interaction is also affected with respect to isoform. Due to its structure, apoE4 binds preferentially to VLDL particles when compared to apoE3 which is mostly found on HDL (Gregg et al., 1986; Steinmetz et al., 1989; Dong et al., 1994; Dong et al., 1996a).

Since apoE3 contributes very little to lipid variations, it is regarded as the "normal" form. On the other hand, apoE2 and apoE4 modulate lipemia in opposite fashion and with different mechanisms.

ApoE2 is associated with a lipid disorder called type III hyperlipoproteinemia (HLP) or dysbetalipoproteinemia (Utermann et al., 1977; Weisgraber et al., 1981). All the patients exhibiting this disorder are carriers of at least one ε2 allele (Ghiselli et al., 1982a). ApoE2 has a defective capacity at binding the LDLR (Weisgraber et al., 1982; Rall, Jr. et al., 1983; Dong et al., 1996b) which, when combined with secondary factors, leads to accumulation of lipoprotein remnants or β-VLDL (Utermann et al., 1975; Havel

et al., 1980). Even though apoE2 associates with higher cholesterol levels in type III HLP, more than 90% of apoE2 carriers are either normo- or hypolipedemic, a phenomenon due to an impairment of LPL-mediated lipolysis of VLDL triglycerides that result in a decrease in LDL particles in the blood (Ehnholm et al., 1984; Huang et al., 1998).

ApoE4 is, on the other hand, associated with higher plasma and LDL cholesterol concentrations (Bouthillier et al., 1983; Utermann et al., 1984; Assmann et al., 1984), type V HLP (Ghiselli et al., 1982b) and a higher risk of cardiovascular diseases (Davignon et al., 1987; Kuusi et al., 1989; van Bockxmeer et al., 1992). The mechanism by which these effects are mediated is not fully understood. Hypotheses suggest that it could either result from: 1) increased clearance of dietary lipids and a more rapid remnant catabolism, leading to decreased LDLR expression and accumulation of LDL-cholesterol (Gregg et al., 1986; Weintraub et al., 1987) or 2) prolonged VLDL residence time leading to increased conversion to LDL (Demant et al., 1991; Bergeron et al., 1996; Knouff et al., 1999). These conflicting hypotheses have yet to be resolved, however, they are both in agreement on the end result that is increased LDL-cholesterol concentration of about 15%.

1.3.1.2 Neurobiology

The importance of apoE in neurobiology emerged when it was first shown that its expression was modulated during peripheral nerve degeneration and regeneration (Ignatius et al., 1986; Dawson et al., 1986; Snipes et al., 1986; Boyles et al., 1990; LeBlanc et al., 1990; Goodrum, 1991). It is also expressed in the CNS (Boyles et al., 1985; Pitas et al., 1987a; Pitas et al., 1987b) and is modulated following a lesion (Poirier et al., 1991a; Poirier et al., 1991b). These findings set the stage for the discovery of the association between AD and the ε4 allele of apoE (Corder et al., 1993; Saunders et al., 1993; Poirier et al., 1993a; Strittmatter et al., 1993a). To date, it is the only risk factor that has been repeatedly shown to associate with the disease. It is also associated with poor clinical outcome following head injury (Nicoll et al., 1995; Jordan et al., 1997; Friedman et al., 1999) and stroke (Pedro-Botet et al., 1992; Slooter et al., 1997; Sheng et al., 1998). On the other hand, apoE2 confers protection against AD and is also associated

with successful aging and longevity (Corder et al., 1994; Schachter et al., 1994; Frisoni et al., 2001).

The exact mechanism by which apoE4 confers AD susceptibility is not yet known but many hypotheses have been proposed involving its role in Aβ metabolism (see section 1.3.2.3) (Strittmatter et al., 1993a; LaDu et al., 1994; Holtzman et al., 1999; Beffert et al., 1999b), tau phosphorylation (Strittmatter et al., 1994a; Strittmatter et al., 1994b), neurite outgrowth (Nathan et al., 1994; Nathan et al., 1995; Holtzman et al., 1995), synaptic density and plasticity (Poirier, 1994; Teter et al., 1999a; Wang et al., 2005b) and oxidative stress (Ramassamy et al., 1999; Ramassamy et al., 2000). As observed in plasma, ε4 carriers have lower concentrations of apoE in the brain (Bertrand et al., 1995; Beffert et al., 1999c) which could also limit the recycling and redistribution of cholesterol following injury.

A distortion in apoE's allelic expression is observed, which influences its expression. Indeed, when measured in ε3/ε4 carriers, the ε4 mRNA expression is always lower than the ε3 mRNA, even though it is significantly increased in AD brains (Lambert et al., 1997). This suggests that non-coding polymorphisms in regulatory regions might be involved in expression levels. Polymorphisms in the promoter region of apoE were found and shown to associate with AD risk (Bullido et al., 1998; Artiga et al., 1998a; Artiga et al., 1998b; Lambert et al., 1998c; Lambert et al., 1998d). The effect of the promoter polymorphisms is to modulate the expression of apoE mRNA expression which, combined with differences in protein levels, highlights the importance of apoE concentration in the brain.

1.3.2 ApoE, its receptors, and their functions in the brain

The identification of apoE (Elshourbagy et al., 1985; Boyles et al., 1985) and its receptors (Pitas et al., 1987a; Herz et al., 1988; Takahashi et al., 1992; Kim et al., 1996) in the brain suggested that they might be involved in maintaining cholesterol homeostasis in this organ. It is now clear that apoE has functions that extend outside its cholesterol transporter role and that the existence of isoforms modulates its capacity to accomplish these functions. The creation of genetically modified animals also helped a lot in the understanding of how these proteins were involved in brain functions.

1.3.2.1 Synaptic plasticity

The expression of LDLR and apoE containing lipoproteins in the brain suggests that regulation of cholesterol homeostasis is similar to what is observed for other organs (Pitas et al., 1987a). What is interesting is that, as first observed in periphery (Skene et al., 1983; Muller et al., 1985; Ignatius et al., 1986; Muller et al., 1986), apoE expression increases following damage to the CNS (Poirier et al., 1991a; Poirier et al., 1991b) and while there is no regeneration occurring in the CNS, it coincides with hippocampal synaptogenesis and terminal proliferation (Poirier et al., 1991a). The increase in apoE expression also parallels a decrease in HMGCR activity and an increased binding of LDLR in the hippocampus of lesioned animals (Poirier et al., 1993b). These results all suggest a role for apoE in cholesterol redistribution following injury to the CNS in order to support neuronal remodeling and synaptic plasticity (Poirier, 1994; Poirier et al., 1995b).

In view of the role attributed to apoE, it is not surprising to see that ε4 carriers, who are at higher risk of developing AD, have lower levels of apoE in the brain (Bertrand et al., 1995; Beffert et al., 1999c) as well as impaired plastic neuronal remodeling (Arendt et al., 1997; Ji et al., 2003). In the same line of evidence, APOE knock-out (ko) mice show a reduced capacity for neuronal plasticity (Masliah et al., 1995; Champagne et al., 2005), LDLRko mice have a decreased synaptic density (Mulder et al., 2004), human apoE4-targeted replacement (TR) mice show synaptic deficits (Wang et al., 2005b) and two different lines of transgenic mice expressing human apoE4 have compromised plasticity compared to apoE3 mice following a lesion (Buttini et al., 1999; White et al., 2001a). These human apoE4 transgenic mice also develop an age-dependent synaptic loss observable after 19 to 20 months of age (Cambon et al., 2000). Except for the study using human apoE4-TR mice that express levels similar to human apoE3-TR mice (Sullivan et al., 2004), these studies all support the hypothesis that lower apoE levels are causing plasticity and neurite outgrowth defects.

In vitro studies also show that apoE supports neurite outgrowth and neuronal sprouting (Teter et al., 1999b; Posse De Chaves et al., 2000; Vance et al., 2000), that this effect is isoform-specific with apoE3 > apoE4 (Nathan et al., 1994; Teter et al., 1999a) and that it is dependent on the presence of LRP (Holtzman et al., 1995) and LDLR (Posse

De Chaves et al., 2000). However, instead of being the result of differences in levels, the poorer ability of apoE4 to mediate neuronal sprouting seems to be due to a gain of negative function compared to apoE3 (Teter et al., 2002a).

Another hypothesis regarding apoE's role in neuronal remodeling involves its interaction with the cytoskeletal protein tau. *In vitro* studies report differential effects for apoE3 and E4 on cellular microtubules where apoE3 but not apoE4 binds the cytoskeletal protein tau therefore possibly slowing the initial rate of its phosphorylation and self-assembly into PHFs (Strittmatter et al., 1994b; Nathan et al., 1995; Fleming et al., 1996). Since tau is known to promote microtubule assembly, its hyperphosphorylation would lead to destabilization of the cytoskeleton and ultimately to cell death. However, *in vivo* data is less clear as tau phosphorylation is reported to be unaffected (Mercken et al., 1995) or increased (Genis et al., 1995) in APOEko mice and increased (Buttini et al., 1999; Tesseur et al., 2000a) or decreased at some epitopes (Kobayashi et al., 2003) in different models of transgenic mice expressing human apoE4. Two apoE receptors, apoER2 and VLDLR, also modulate tau phosphorylation state (Hiesberger et al., 1999). Ligand binding to these receptors induces the phosphorylation of the adaptor protein Disabled-1 (Dab1) ultimately leading to the inhibition of glycogen synthase kinase 3 beta (GSK-3β) (Beffert et al., 2002) which is thought to promote PHF formation in AD (Mandelkow et al., 1992; Hanger et al., 1992).

1.3.2.2 Learning and memory

ApoE seems to play a role in maintaining neuronal integrity in the brain during aging and in response to insults (Poirier et al., 1993b; Masliah et al., 1995). It is thus not surprising to see APOEko and LDLRko animals exhibiting cognitive deficits (Gordon et al., 1995; Gordon et al., 1996; Oitzl et al., 1997; Krzywkowski et al., 1999; Champagne et al., 2002; Mulder et al., 2004), deficits that are reversible by apoE infusion in the case of APOEko mice (Masliah et al., 1997). Moreover, there is also an isoform-specific effect of apoE on cognitive performances in humans (Cole et al., 1995; O'Hara et al., 1998; Caselli et al., 1999; Dik et al., 2000) and transgenic animals (Raber et al., 2002; Levi et al., 2003; Grootendorst et al., 2005). As discussed earlier, memory and learning can be molecularly assessed by measuring LTP. Electrophysiological recordings in

brains of APOEko animals reveal that LTP is altered (Veinbergs et al., 1998; Krzywkowski et al., 1999; Valastro et al., 2001), a phenomenon also observed in human apoE4-TR mice (Trommer et al., 2004). Finally, a report on the role of VLDLR, apoER2 and one of their ligands, reelin, shows that activation of the receptors enhances synaptic plasticity and learning (Weeber et al., 2002). ApoE4 alteration of learning and memory could be the result of competition with reelin for the receptors as apoE interferes with this interaction in an isoform-specific manner with apoE4 > apoE3 > apoE2 (D'Arcangelo et al., 1999).

1.3.2.3 Amyloid metabolism

ApoE's role in AD goes beyond its function as a lipid transporter as highlighted by its role in amyloid metabolism. It was first observed that amyloid deposits in AD and Creutzfeldt-Jakob disease brains exhibited immunoreactivity for apoE (Namba et al., 1991) and that apoE associated with cerebral and systemic amyloid (Wisniewski et al., 1992). Following the report of the association between the ε4 allele of apoE and the increased risk of developing AD, it was found that ε4 carriers also had increased amyloid load compared to ε3 carriers (Schmechel et al., 1993; Rebeck et al., 1993; Beffert et al., 1996). There is a gene-dose association between apoE4 and $A\beta_{40}$ levels in the hippocampus and cortex of AD patients whereas $A\beta_{42}$ levels do not correlate (Ishii et al., 1997; Beffert et al., 1999c). Moreover, amyloid deposition following head injury associates with the presence of apoE4 (Nicoll et al., 1995).

In vivo studies using genetically modified mice provided much insight on the role of apoE in Aβ metabolism. For instance, the PDAPP mouse overexpresses human APP and develops plaques by the age of 8 months (Games et al., 1995). Crossing this mouse with the APOEko mouse results in complete abolition of plaque deposition in mice as old as 22 months of age (Bales et al., 1999) suggesting a role for apoE in Aβ deposition. On the other hand, when PDAPP mice are crossed with transgenic mice expressing human apoE3 or E4, amyloid deposition is markedly suppressed at 9 months of age compared to animals expressing murine apoE (Holtzman et al., 1999) whereas, by 15 months of age, the apoE4 animals had 10-fold more fibrillar Aβ deposits than apoE3 animals (Holtzman et al., 2000) suggesting that apoE facilitates fibrillar Aβ deposition (Fagan et al., 2002;

Fryer et al., 2003). These findings, although seemingly contradictory, are consistent with the emerging view that apoE (and apoJ) act as extracellular chaperone molecules that regulate the metabolic fate of extracellular soluble Aβ. Indeed, PDAPP mice crossed with apoE knockout or apoE/apoJ double knockout progressively accumulate soluble Aβ in the CSF because of impaired clearance of the potentially toxic peptides (DeMattos et al., 2004). This clearance property of apoE is also exemplified in two studies: 1) high dietary cholesterol diet leads to decreased processing of APP in gene-targeted APP mice (genetically humanized in the Aβ domain and expressing the Swedish familial AD mutations) with reductions in both Aβ peptides inversely correlated with apoE concentration in the brain (Howland et al., 1998) and 2) an alteration of Aβ 40/42 ratio in brain and CSF of human apoE4-TR mice crossed with Tg2576 mice that develop plaques and cerebral amyloid angiopathy (Fryer et al., 2005).

Numerous studies attempted to explain how apoE could modulate amyloid metabolism in an isoform-specific manner. Using purified apoE it was shown that amyloid bound apoE4 with higher avidity than apoE3 *in vitro* (Strittmatter et al., 1993b). In contrast, cell secreted apoE3 showed a higher preference for Aβ compared to apoE4 (LaDu et al., 1994; Zhou et al., 1996; Yang et al., 1997; Morikawa et al., 2005). This discrepancy is explained by the fact that purification of apoE results in delipidation and denaturation of the protein that abolishes the isoform specificity of the binding (LaDu et al., 1995). As observed *in vivo*, *in vitro* studies also show that apoE can promote Aβ fibrillogenesis (Castano et al., 1995). Moreover, it is also involved in its internalization through receptor-mediated endocytosis (Beffert et al., 1998; Beffert et al., 1999b). Interestingly, for internalization to occur, apoE needs to be part of a reconstituted lipoprotein, as purified apoE could not bind its receptor (Beffert et al., 1998; Beffert et al., 1999b). In this lipidated configuration, apoE4 was less effective than apoE3 at clearing extracellular Aβ in primary hippocampal neurons cultures (Beffert et al., 1999b).

Data from *in vivo* and *in vitro* studies all point to a role for apoE in Aβ clearance. Thus one might think that modulating apoE expression with the non-statin cholesterol-lowering drug Probucol (Champagne et al., 2003) or liver X receptor (LXR) agonists (Laffitte et al., 2001; Mak et al., 2002; Sun et al., 2003a) could lead to a decrease of Aβ

accumulation. Indeed a proof-of-principle study with Probucol report a correlation between increased apoE expression and lower Aβ levels (Poirier et al., 2002; Poirier, 2003). Modulating apoE levels should however be made with care as it was shown that increasing apoE4 could lead to a gain of negative functions (Teter et al., 2002a).

1.3.2.4 Immune functions

Immunohistochemical evidences point to an inflammatory state in the AD brain (McGeer et al., 1987; Rogers et al., 1988; McGeer et al., 1989a; McGeer et al., 1989b). Aβ can activate glial cells and trigger the expression of inflammatory mediators such as interleukin(IL)-1 and 6 (Lorton, 1997; Hu et al., 1998a; Apelt et al., 2001), nitric oxide (NO) (Vitek et al., 1997; Hu et al., 1998a; Akama et al., 2000), reactive oxygen species (El Khoury et al., 1996) and activation of complement (Rogers et al., 1992). This Aβ-mediated glial activation requires LRP (LaDu et al., 2000a). On the other hand, apoE attenuates Aβ activity both in glial cells and neurons (Whitson et al., 1994; Miyata et al., 1996; Puttfarcken et al., 1997; Jordan et al., 1998; Hu et al., 1998b) whereas the inflammatory response to Aβ or LPS is exacerbated in glial cells isolated from APOEko animals (Laskowitz et al., 1997; Laskowitz et al., 1998; LaDu et al., 2001). This anti-inflammatory property of apoE is isoform-specific as apoE3 (as compared to apoE4) does not potentiate complement activation (McGeer et al., 1997), suppresses brain inflammation following LPS treatment (Lynch et al., 2003), induces higher levels of IL-1 receptor antagonist expression (Grocott et al., 2001), does not induce PGE2 nor IL-1β expression in microglia (Chen et al., 2005), reduces the release of NO from microglia (Brown et al., 2002) and is inversely correlated in a gene-dose fashion with microglial activation in AD brains (Egensperger et al., 1998). Moreover, serum from AD patients carriers of the ε4 allele contains factors that can induce microglia activation *in vitro* (Lombardi et al., 1998).

One might be tempted to take advantage of this anti-inflammatory property of apoE for the treatment of AD. Considering that the use of non-steroidal anti-inflammatory drugs (NSAID) might have protective effects against AD development (McGeer et al., 1996; Szekely et al., 2004) it is interesting to note that molecules of the

NSAID family are effective at inducing apoE expression in primary glial cell cultures even at nanomolar concentrations (Aleong et al., 2003).

1.4 LIPOPROTEIN LIPASE

It seems obvious that cholesterol homeostasis appears to be at the center of AD pathophysiology. The numerous reports associating apoE to the disease are good examples of its importance. There is however more to the brain cholesterol homeostasis than its major transporter apoE. A number of other proteins are studied in the cardiovascular field that could prove to be of importance in AD. One of these proteins, the lipoprotein lipase (LPL; EC 3.1.1.34), is a member of a lipase gene family that also includes the hepatic lipase, endothelial lipase and pancreatic lipase.

1.4.1 Functions of lipoprotein lipase and its associated proteins in lipid metabolism

LPL is a protein with a dual role in lipid homeostasis. It is primarily an enzyme involved in the hydrolysis of triglycerides on the surface of the Tg-rich lipoproteins VLDL and chylomicrons (Korn, 1955a; Havel et al., 1960) but it also has a role in bridging lipoprotein particles to the cell surface (Felts et al., 1975; Beisiegel et al., 1991; Saxena et al., 1992).

1.4.1.1 Lipolysis and cholesterol homeostasis

It was first noted that injection of heparin in dogs induced a decrease in postprandial lipemia by releasing a factor called "clearing factor" because of its property to clear lipemic serums or fat emulsions *in vitro* (Hahn, 1943). This factor was later isolated from the rat heart and found to be a lipoprotein lipase (Korn, 1954; Korn, 1955a; Korn, 1955b). Subsequent studies showed that post-heparin plasma from humans with hyperchylomicronemia could not hydrolyze chylomicrons in vitro, clearly linking LPL to this disorder and highlighting its essential role in chylomicron catabolism (Havel et al., 1960; Angervall et al., 1962; Fredrickson et al., 1963; Kuo et al., 1965). LPL is also responsible for the metabolism of VLDL which become the Tg-poor and cholesterol-rich particles known as VLDL remnants (or IDL) following its action (Shore et al., 1962; Nichols et al., 1968; Bilheimer et al., 1972).

LPL activity is dependent on the presence of an essential co-factor, apoC-II (LaRosa et al., 1970; Havel et al., 1973; Ekman et al., 1975). Interestingly, a genetic deficiency of apoC-II also results in hyperchylomicronemia (Breckenridge et al., 1978). Other apolipoproteins also have the capacity to modulate LPL activity, namely apoC-I (Ekman et al., 1975; Shachter et al., 1996), apoC-III (Brown et al., 1972; Ekman et al., 1975; Fielding et al., 1980; Aalto-Setala et al., 1992) and apoE (Ganesan et al., 1976; Yamada et al., 1980) which were all shown to inhibit LPL enzymatic activity with diverse efficiencies.

Considering the relationship between LPL and apolipoproteins it is not surprising that LPL, as well as playing a central role in lipolysis, also modulates cholesterol homeostasis. Indeed, modification of LDL particles by LPL induces an enhancement of cholesterol uptake by the cells (Aviram et al., 1988). Moreover LPL is able to mediate the uptake and degradation of lipoprotein particles of different classes like chylomicrons (Mann et al., 1995), VLDL (Ishibashi et al., 1990; Eisenberg et al., 1992; Medh et al., 1996; Merkel et al., 1998; de Beer et al., 1999; Tacken et al., 2000), LDL and its oxidized form (Rumsey et al., 1992; Obunike et al., 1994; Hendriks et al., 1996; Wang et al., 1999b; Seo et al., 2000; Zimmermann et al., 2001; Boren et al., 2001; Makoveichuk et al., 2004) as well as of HDL-associated cholesteryl esters (Schorsch et al., 1997; Panzenboeck et al., 1997; Rinninger et al., 1998b; Merkel et al., 2002a). Of course, this involves binding of LPL to the cell surface via different interactions.

1.4.1.2 Cell-surface binding

Following hydrolysis of Tg, chylomicron remnants stay associated with LPL, at the cell surface (Felts et al., 1975). Interestingly, it is 15 years after this report that LPL was first shown to bind to a receptor of the LDLR family, LRP, which also mediates its catabolism (Beisiegel et al., 1991; Chappell et al., 1992). In their study, Beisiegel et al. demonstrate that LPL is able to bind directly to LRP and also to increase by several folds the binding of apoE-liposomes to the receptor. This binding property is independent of the lipolytic activity of LPL as demonstrated by a mutation study (Merkel et al., 1998).

All the other major receptors of the LDLR family namely the LDLR itself (Medh et al., 1996), gp330 (Kounnas et al., 1993), apoER2 (Tacken et al., 2000) and the VLDLR

(Takahashi et al., 1995) were subsequently shown to have binding capacity for LPL. However, the cell-surface molecules having the most affinity for LPL are the heparan sulfate proteoglycans (HSPG) (Saxena et al., 1991; Eisenberg et al., 1992; Mulder et al., 1992; Mulder et al., 1993). These molecules consist of a core protein to which are attached long chains of negatively charged glycosaminoglycans. The major core proteins either belong to the perlecan, glypican or syndecan family. There are no evidence yet on the role of glypicans in lipoprotein metabolism. This might be due to their globular structure and the placement of their HS chains close to the plasma membrane which would hinder the binding of large particles (Williams et al., 1997). On the other hand, the other cell-surface HSPG syndecan can bind and internalize lipoproteins, and LPL seems to act as a natural ligand (Fuki et al., 1997; Fuki et al., 2000a). The basement membrane perlecan is also capable of binding and catabolizing lipoprotein particles (Fuki et al., 2000b).

Apolipoproteins, together with LPL, modulate the binding properties of lipoprotein particles to the cell surface. For instance, apoCs were shown to reduce lipoprotein binding to LPL-HSPG complexes (van et al., 1996) whereas enrichment in apoE increases this interaction (van et al., 1996; van Barlingen et al., 1997). This is consistent with the observation that apoC inhibits binding of particle to the cell surface (Windler et al., 1985; Weisgraber et al., 1990) while apoE, which has a high affinity for HSPG, increases it (Ji et al., 1993; Ji et al., 1994).

Even though LPL can directly bind to receptors of the LDLR family and HSPG can mediate lipoprotein internalization independent of the coated-pit pathway, the classical view is that LPL acts as a bridging molecule between lipoprotein particles and HSPG (Mulder et al., 1992) thus favoring the interaction between the lipoproteins and their receptors (Mulder et al., 1993).

1.4.2 Lipoprotein lipase in the central nervous system

Lipoprotein lipase is expressed in many tissues and its sequence contains tissue-specific transcriptional elements (Gimble et al., 1995). It is present and functional in the brain (Eckel et al., 1984; Shirai et al., 1986; Goldberg et al., 1989; Vilaro et al., 1990; Nunez et al., 1995) and also developmentally regulated (Tavangar et al., 1992; Nunez et

al., 1995). LPL activity in the brain peaks a few days after birth and then declines in the adulthood (Nunez et al., 1995). This is consistent with the absence of its activator apoC-II in the brain parenchyma (Zannis et al., 1985; Datta et al., 1987; Lenich et al., 1988; Hoffer et al., 1993) and the fact that the brain contains virtually no triglycerides (LaDu et al., 1998). In the CNS, the spinal cord expresses the highest levels of LPL (Bessesen et al., 1993; Cole et al., 1995) and it is also expressed at high levels in the hippocampus (Ben Zeev et al., 1990; Paradis et al., 2004a). Its precise function has however yet to be determined but based on its functions in periphery, it is suggested to play a role in vitamin E transfer to tissues (Traber et al., 1985), internalization of HDL-cholesteryl esters (Schorsch et al., 1997; Panzenboeck et al., 1997; Rinninger et al., 1998b) and cholesterol recycling (Huey et al., 2002). In vitro experiments have shown that it could induce neuronal differentiation (Paradis et al., 2004b), neurite outgrowth of sympathetic neurons (Postuma et al., 1998) and that it protects neurons from oxidative insults (Paradis et al., 2003). Finally, its association with amyloid in senile plaques suggests that it could also be involved in Aβ metabolism and the development of AD (Rebeck et al., 1995; Lorent et al., 1995).

1.4.3 Lipoprotein lipase polymorphisms

Approximately 100 naturally occurring mutations or single nucleotide polymorphisms (SNPs) have been described for LPL. They almost all result in decreased LPL activity with the majority causing hyperchylomicronemia and some causing hypertriglyceridemia and increased coronary artery disease (CAD) risk (Merkel et al., 2002b). Ironically, the Ser447stop mutation, which is the most common among the population (20% bears it), is the only variant resulting in lower Tg levels and reduced CAD risk (Humphries et al., 1998). It is interesting to note that the French Canadian population exhibits a very high rate of LPL mutations probably due to the genetic 'founder effect' (Minnich et al., 1995). Meta-analyses reveal that carriers of the most common LPL mutations namely Gly188Glu, Asp9Asn and Asn291Ser have an atherogenic lipoprotein profile whereas Ser447stop carriers have an anti-atherogenic lipoprotein profile (Wittrup et al., 1999). The Ser447stop variant was shown to decrease the risk of brain infarction (Myllykangas et al., 2001) and AD in clinically-diagnosed

patients (Baum et al., 1999). On the other hand, the Asn291Ser SNP was shown to associate with increased AD risk however both associations with AD risk were lost in pathologically-confirmed subjects (Baum et al., 1999). Moreover, four other independent studies failed to report association between these SNPs and AD risk (Myllykangas et al., 2000; Retz et al., 2001; Martin-Rehrmann et al., 2002; Fidani et al., 2002).

While the majority of mutations and SNPs occur in the coding region for LPL, some of them are also found in introns. The most common non-coding forms are probably the intron 6 *Pvu*II and intron 8 *Hind*III SNPs which were also reported to associate with triglyceride and cholesterol levels as well as with CAD risk and diabetes (Chamberlain et al., 1989; Thorn et al., 1990; Peacock et al., 1992; Ahn et al., 1993; Jemaa et al., 1995; Gerdes et al., 1995; Vohl et al., 1995; Wang et al., 1996; Chen et al., 1996; Anderson et al., 1999). Finally, the *Hind*III SNP in intron 8 was recently shown to associate with AD risk in a clinically-diagnosed cohort of patients (Scacchi et al., 2004).

1.5 Specific Aims

Numerous evidences now link cholesterol homeostasis to the pathogenesis of AD. While it has been known for a number of years now that a polymorphism in the major cerebral cholesterol transporter apoE confers higher risk of developing the disease (Corder et al., 1993; Poirier et al., 1993a), more recent findings suggest that the use of cholesterol lowering agents (e.g. statins, probucol) could be protective against AD (Jick et al., 2000; Wolozin et al., 2000; Poirier et al., 2002). The majority of the studies looked at relations between apoE, amyloid metabolism and cholesterol homeostasis. However, lessons from the cardiovascular field of research tell us that there are numerous proteins involved in cholesterol homeostasis that are also known to interact with apoE in periphery and that are present in the brain. One of these proteins, lipoprotein lipase, has not been extensively studied in the CNS.

In the first study, we take advantage of a new mouse model expressing human apoE3 and apoE4 under the control of the host's regulatory sequences to evaluate the isoform-specificity of the changes following a lesion of the entorhinal cortex. While the ε4 allele of apoE has repeatedly been reported to increase the risk of developing AD, the

mechanism by which it confers such susceptibility remains unknown. In humans the ε4 allele associates with lower levels of apoE in the brain that could lead to reduced cholesterol redistribution and lack of neuronal remodeling. Since CNS apoE expression is identical between both strains, they provide an excellent model for studying isoform-specific effects post-injury.

Because of the virtual absence of triglycerides in the brain, enzymatic LPL activity did not appear as relevant as its lipoprotein-bridging role. Thus, in the second study, we monitor the expression of LPL following hippocampal deafferentation as well as that of receptors known to promote its cell-surface binding. Relevance to synaptic remodeling and AD is discussed.

Finally, the results from the second study combined with reports of genetic associations (more or less significant) between LPL polymorphisms and AD risk motivated the third study. In this last one, we evaluate the association of LPL intronic polymorphisms known to modulate lipid homeostasis in periphery with the disease risk and severity as measured by characteristic biological markers. A role for LPL in the pathogenesis of AD is proposed.

2.0 STUDY 1

A DEFICIT IN ASTROGLIAL ORGANIZATION CAUSES THE IMPAIRED REACTIVE SPROUTING IN HUMAN APOLIPOPROTEIN E4 TARGETED REPLACEMENT MICE

Jean-François Blain, Patrick M. Sullivan and Judes Poirier

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2.1 FOREWORD

In AD, hippocampus is isolated by the destruction of afferents originating from the entorhinal cortex (Hyman et al., 1984). Following a lesion to the entorhinal cortex (ECL) in rodents reactive synaptogenesis in the denervated zone is observed (Lynch et al., 1972; Lynch et al., 1976; Cotman et al., 1977). The increased expression of apoE in the hippocampus (Poirier et al., 1991a) supports this reactive synaptogenesis by delivering cholesterol to sprouting neurons (Poirier et al., 1993b) a process supported by the sprouting impairment observed in APOEko mice (Champagne et al., 2005).

The ε4 allele of APOE is a major risk factor for AD development (Corder et al., 1993; Poirier et al., 1993a). Moreover ε4 carriers exhibit neuronal remodeling impairments compared to ε2 and ε3 carriers (Arendt et al., 1997). While lower brain concentrations of apoE (Bertrand et al., 1995; Beffert et al., 1999c), isoform-specific effects on amyloid metabolism (Strittmatter et al., 1993a; LaDu et al., 1994; Holtzman et al., 1999; Beffert et al., 1999b) or tau phosphorylation (Strittmatter et al., 1994b; Nathan et al., 1995; Fleming et al., 1996) have been proposed, the exact mechanism by which apoE4 mediates its effect is currently unknown. Recent evidence obtained in APOEko mice suggest that apoE might also be involved in the glial reactivity observed following ECL (Champagne et al., 2005).

To gain further insight on the mechanism responsible for the detrimental effect of apoE4 we used human apoE-targeted replacement mice on which we performed a lesion of the entorhinal cortex and observed apoE expression, glial reactivity, inflammatory response, tau phosphorylation an amyloid metabolism.

2.2 ABSTRACT

The ε4 allele of apolipoprotein (apo)E associates with an increased risk of developing Alzheimer's disease (AD) as well as an earlier age of onset. However, the exact mechanisms by which apoE4 confers such susceptibility is currently unknown. We used a human apoE targeted replacement (hE-TR) mouse model to investigate the allele-specific response to entorhinal cortex lesion (ECL). We observed a marked impairment in reactive sprouting in hE4 mice compared to hE3 mice. ApoE expression was similar between genotypes at day post-lesion (DPL) 2 and 14. Thirty days post-lesion hE4 mice had more reactive astrocytes as well as a defective outward migration pattern of the astrocytes in the dentate gyrus. The expression of the anti-inflammatory cytokine IL-1ra was delayed in hE4 mice compared to hE3 mice. ApoE and beta-amyloid (Aβ) 1-40 accumulated at 30 DPL in hE4 mice. These results suggest that the presence of apoE4 delays the astroglial repair process and indirectly compromises synaptic remodeling.

2.3 INTRODUCTION

Cholesterol homeostasis is tightly regulated in the brain and relatively independent from the periphery. Lipid transport and distribution is mediated predominantly by apolipoprotein (apo) E, the most prevalent apolipoprotein in the brain. ApoE is a 34 kD protein expressed as three different isoforms in humans referred to as apoE2, E3 and E4. ApoE2 which is associated with type III hyperlipoproteinemia (Ghiselli et al., 1982) is also a marker of longevity (Frisoni et al., 2001; Schachter et al., 1994). ApoE2 reduces the risk of Alzheimer's disease (AD) (Corder et al., 1994) and delays its onset by nearly 20 years. In contrast, apoE4 is associated with increased risk of developing both familial and sporadic AD (Poirier et al., 1993; Strittmatter et al., 1993).

Studies from human AD brains have shown that apoE4 carriers exhibit a gene-dose decrease in apoE concentration compared to other genotypes (Beffert et al., 1999a; Bertrand et al., 1995; Glockner et al., 2002; Yamada et al., 1995) and that neuronal dendritic and synaptic remodeling are markedly impaired in E4 carriers (Arendt et al., 1997). β-amyloid (Aβ) levels and deposition were shown to be apoE4 gene-dose dependent (Gearing et al., 1996; Ishii et al., 1997; Schmechel et al., 1993) and inversely correlated with local apoE concentration (Beffert et al., 1999a). ApoE isoform-specific effects are also observed with neurite outgrowth (Holtzman et al., 1995; Nathan et al., 1994; Nathan et al., 1995), Aβ binding (LaDu et al., 1994; Strittmatter et al., 1993), Aβ clearance (Beffert et al., 1999b; Holtzman et al., 1999), synaptic density (Teter et al., 1999; Wang et al., 2005) and oxidative stress (Ramassamy et al., 1999; Ramassamy et al., 2000). In animal models of hippocampal deafferentation, apoE regulates lipid redistribution during the active phase of neuronal dendritic and terminal remodeling (Poirier et al., 1991b; Poirier et al., 1991a). Studies in apoE-knockout mice suggest that apoE is critical for remodeling of neuronal networks (Masliah et al., 1995). More recently, studies in human apoE4 transgenic mice showed compromised synaptic plasticity and terminal remodeling as well as a deficient response to environmental stimulation of synaptogenesis and memory compared to apoE3 transgenic animals (Levi et al., 2003; White et al., 2001).

For post-lesion reinnervation to occur in the CNS, a number of well characterized events must take place. The first event in the deafferented hippocampus is the proliferation of microglial cells in the denervated zone, followed shortly by astrocyte activation and proliferation (Gehrmann et al., 1991);(Gall et al., 1979; Hailer et al., 1999). Astrocytes secrete growth factors such as nerve growth factor (NGF) which is essential for survival and terminal remodeling of the surviving neurons (Lindsay, 1979; Tarris et al., 1986; Van der Zee et al., 1992; Whittemore et al., 1987). Microglia migrate to the deafferented site and release interleukin (IL)-1, a pro-inflammatory mediator that promotes the recruitment of astrocytes (Fagan and Gage, 1990; Gage et al., 1988; Giulian et al., 1986; Giulian et al., 1988a). Both, microglia and astrocytes progressively engage in active phagocytosis and internalization of terminal debris (Bechmann and Nitsch, 1997). Removal of myelin debris is critical for reinnervation otherwise it blocks neurite outgrowth and repair mechanisms (Schnell and Schwab, 1990).

We chose hE3 and hE4-TR mice to study the apoE isoform-specific effects on ECL because this model uses the host's (mouse) regulatory sequences to control human apoE expression. The human apoE-TR (knock-in) mice were created via gene targeting by replacing the mouse genomic (coding) sequences with homologous human sequences. Thus, the apoE3 and E4-TR mice express apoE at identical levels in the same spatial and temporal gene expression pattern (Knouff et al., 1999; Sullivan et al., 1997). Since CNS apoE expression is identical between both strains (Sullivan et al., 2004), they provide an excellent model for studying isoform-specific effects post-injury. We present evidence for apoE genotype-specific effects on tau phosphorylation, beta-amyloid clearance, astroglial response to hippocampal deafferentation, as well as interleukin-1 receptor antagonist response. Our results show that expression of apoE4 results in impaired reinnervation of the deafferented hippocampus and a delayed astroglial response, compared to apoE3.

2.4 METHODS

2.4.1 Animals

Human apoE3 and E4 targeted replacement (TR) mice were generated as previously described (Sullivan et al., 1997). All experiments were performed on age (average age of 40 weeks) and sex (male) matched animals for each genotype. All animals were singly housed in an enriched environment throughout their life span with *ad libitum* access to food and water. All procedures were carried out in accordance with the Canadian Guidelines for Use and Care of Laboratory Animals and were approved by the Animal Care Committee of McGill University.

2.4.2 Entorhinal Cortex Lesion (ECL)

Unilateral electrolytic ECL was performed as described previously (Blain et al., 2004). Briefly, the lambda 0 was taken by aligning the electrode with the suture lines. The skull was drilled to allow the electrode to pass through the four different coordinates. The electrode is inserted at a 6° angle in the right side of the brain following these coordinates: RC (+0, +0, +0.5, +1.0); L (-3.0, -3.5, -4.0, -4.0); DV (-3.0, -4.0 at each point). A 1mA current was then applied for 10 sec at each coordinate. Animals were sacrificed 2, 14 or 30 days following the lesion. The contralateral side of the brain was used as an internal negative control. Lesion size was assessed as described previously (Champagne et al., 2005) and was equivalent between both genotypes (data not shown).

2.4.3 Tissue homogenization

Left and right hippocampi were dissected and sonicated on ice in a phosphate buffered solution containing proteases inhibitors (AEBSF, leupeptin, bestatin, aprotinin, E-64, pepstatin A; Sigma-Aldrich Canada Ltd, Oakville, ON). Protein concentration was assessed using the BCA technique (Pierce Biotechnology Inc, Rockford, IL). Whole tissue homogenate was routinely used for subsequent analyses. Phospho-Tau detection required the use of soluble tissue fraction. Samples were thus centrifuged at 14000 rpm for 90 min at 4°C and protein concentration was determined in the supernatant.

2.4.4 PAGE and Immunoblot analysis

Polyacrylamide gel electrophoresis for apoE (25 μg), Glial Fibrillary Acidic Protein (GFAP; 5μg) and PP2A (25 μg) were performed using hippocampal total extracts under reducing conditions. For PHF-Tau detection, 15 μg of the soluble fraction was separated by PAGE. Antibodies were used at 1:1000 unless mentioned otherwise: anti-human apoE (Calbiochem, SanDiego, CA), anti-GFAP (1:2500; Dako Diagnostics Canada Inc, Mississauga, ON), anti-PHF-Tau (clone AT8; Pierce Biotechnology Inc., Rockford, IL), anti-Protein Phosphatase 2A (PP2A; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Chemiluminescent detection was done using an ECL kit (Perkin-Elmer, Boston, MA) on an Image Station 440CF (Eastman Kodak Co., Rochester, NY). Density analyses were performed using a MCID-II image analyzer.

2.4.5 Brain perfusion and slide preparation

Thirty days after the lesion, mice were sacrificed by a lethal injection of anesthetic solution and perfused via the heart for 2 min with ice cold saline followed by 10 min with a solution of 4% paraformaldehyde (PFA). Brains were removed and placed in 4% PFA containing 30% sucrose overnight at 4°C before being frozen. Tissues were kept at –80°C. 30 μm sections were cut and mounted onto poly-Lysine coated slides then air dried and left in a dessicator (under vacuum) at 4°C overnight.

2.4.6 Acetylcholinesterase (AChE) histochemistry

Synaptic and dendritic remodeling was assessed using sections stained for AChE activity as previously described (Hedreen et al., 1985). All products were from Sigma (Sigma-Aldrich, St-Louis, MO). Briefly, slides were incubated at room temperature in the substrate solution (0.0072 % ethopropazine; 0.075% glycine; 0.05% cupric sulfate; 0.12% acetyl thiocholine iodide; 0.68% sodium acetate; pH 5.0) for 1 h. Slides were washed three times in water then incubated 6 min in the developer solution (0.38% sodium sulfide; pH 7.8). Three other washes were done prior to the silver intensification step (1 min in 1% silver nitrate). Slides were then air dried, dehydrated and coverslipped for quantitative analysis.

2.4.7 Assessment of cholinergic sprouting and layer reorganization in the dentate gyrus

Cholinergic sprouting is assessed by measuring the relative density of AChE staining in the outer molecular layer (OML) of the dentate gyrus (DG). Using a MCID-II image analyzer, density measurements were taken at 6 different positions in the OML on 3-4 sections per animal. Results are reported as a ratio of the density between ipsi- and contralateral DG and corrected for tissue shrinkage of the deafferented OML as reported previously (Fagan et al., 1994). Measures of the inner molecular layer (IML) width of the DG were obtained by measuring the distance between the lower boundary of the AChE staining and the supragranular border of the granule cells at 6 different positions on 3-4 sections per animal.

2.4.8 Immunocytochemistry (ICC)

Free-floating sections were incubated overnight at 4°C with anti-GFAP (1:5000, Dako Diagnostics Canada Inc, Mississauga, ON), then incubated 2h at room temperature (RT) in the biotinylated secondary antibody followed by avidin-biotin-peroxidase complex using the Vectastain Elite kit (Vectorlabs, Burlington, ON). Peroxidase reactions consisted of 1.4 mM diaminobenzidine with 0.03% hydrogen peroxide in PBS for exactly 2 min and 0.5% nickelous ammonium sulfate was included in the peroxidase reaction rendering positive stain a dark blue color.

2.4.9 β-Amyloid ELISA

Aβ concentration was determined using 30 and 75 μg of protein from total hippocampal homogenates for $A\beta_{1-42}$ and $A\beta_{1-40}$ respectively. Coating antibodies used were R163 and R165 (Mehta et al., 1998) for $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively (generous gift from Dr P.D. Mehta, New York Institute for Basic Research, Staten Island, New York). Plates were coated overnight at 4°C and then blocked with PBS-BSA 0.1% for 2h at room temperature (RT). Following 5 washes with TBS-T, samples were incubated for 2h at RT with biotinylated 4G8 (Signet Laboratories Inc., Dedham, MA) for 1h at RT with agitation. The plates were washed 5X and streptavidin-alkaline phosphatase complex was added for 1h at RT. Plates were finally washed 5X with TBS-T and once

with water and AttoPhos reagent (Calbiochem, San Diego, CA) was added for 30-60 min and a reading was taken on a Bio-Tek FL600 fluorescence microplate reader.

2.4.10 IL-1 receptor antagonist ELISA

Tissue IL-1ra levels were measured in whole hippocampal homogenates using 100 μg of protein. Detection was performed using Mouse IL-1ra/IL-1F3 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) using the manufacturer's protocol.

2.4.11 Statistical analysis

ANOVA was used to compare the different strains for Western analyses and ELISAs. Two-tailed unpaired t-test was used to compare AChE staining and IML width (p was set at 0.05). They were performed using Datasim software.

2.5 RESULTS

2.5.1 ApoE4 levels remain high post ECL

Western blot analysis was used to measure hippocampal apoE levels in response to deafferentation and reinnervation following ECL (**Fig. 4A**). **Fig. 4B** shows that, at DPL 2, hE3 (-12%) and hE4 (-14%) mice showed only slight decreases in apoE levels. During the early phase of reinnervation at 14 DPL, both lines of mice showed robust increases in apoE levels that reached 65% ($p<0.0001$). At DPL 30, in the late phase of the reinnervation process, apoE levels returned to basal levels in the hE3 mice but not in the hE4 mice where significantly higher levels of apoE are measured ipsilateral to the lesion side (+35%; $p=0.0027$).

2.5.2 Hippocampal reactive sprouting is impaired in an APOE genotype-specific fashion

At DPL 30, when reactive sprouting is thought to be completed, increased AChE staining is observed in the outer portion of the molecular layer of the dentate gyrus of the hE3 mice (**Fig. 5 A, C**), consistent with the cholinergic terminal sprouting normally reported in response to ECL in rodents. However, no increased staining was observed in

the molecular layer of the hE4 mice hippocampus (**Fig. 5 B, D**). Commissural/Associational (C/A) fiber sprouting in the inner molecular layer (IML) was also used to confirm the presence of terminal proliferation in response to ECL. As opposed to the typical expansion of the IML observed during the reinnervation phase in hE3 mice, the hE4 mice strain completely failed to display widening of the innermost layer, suggesting impaired reactive sprouting and remodeling of the neuronal network. The degree of AChE staining and the width of the IML (in the ipsilateral DG) at DPL 30 are summarized in **Fig. 6**.

2.5.3 Astroglial response to entorhinal cortex lesion is determined by APOE genotype

We used GFAP ICC and protein levels as indicators of astroglial response during both the deafferentation and reinnervation phases in the hE3 and hE4 mice. **Fig. 7A** and **B** illustrate the increases in GFAP ratio (ipsilateral/contralateral) in the hE3 (40%; $p=0.0004$) and hE4 (55%; $p<0.0001$) mice at DPL 14 which remained elevated until DPL 30. However, the absolute increase of GFAP levels on the ipsilateral side was found to be significantly higher in hE4 compared to hE3 only at DPL 30 (**Fig. 7C**, $p= 0.03$).

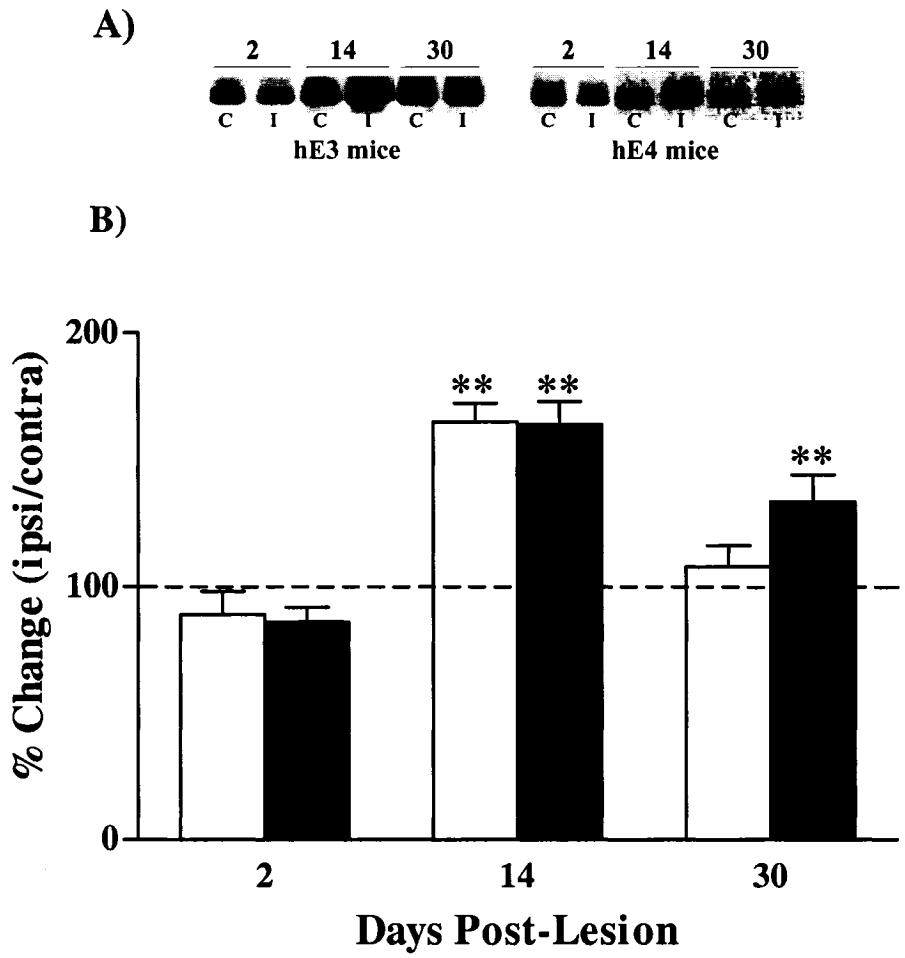

Figure 4. Hippocampal apoE expression following deafferentation. A) Representative Western blots of human apoE (hE3 and hE4 mice). C: contralateral side; I: ipsilateral side. B) Quantification of apoE by image analysis, white bars: hE3; black bars: hE4. ** $p<0.01$ ipsi vs. contralateral side which is considered 100% for each animal (n= 7 for both genotypes at each time point).

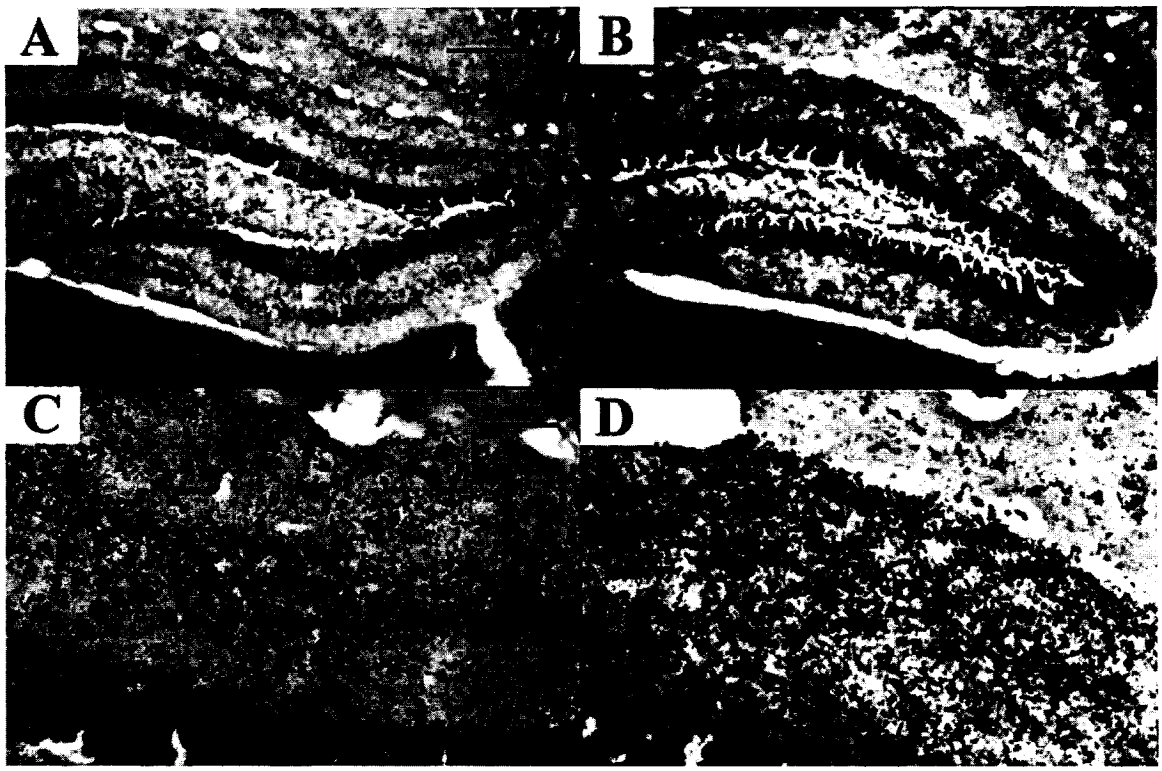

Figure 5. Acetylcholinesterase activity following ECL in hE3 and hE4 mice at DPL 30. Representative photomicrographs showing the hippocampal formation ipsilateral to the lesion. A,C: hE3; B,D: hE4. Scale bar: A,B: 200μm; C,D: 50μm.

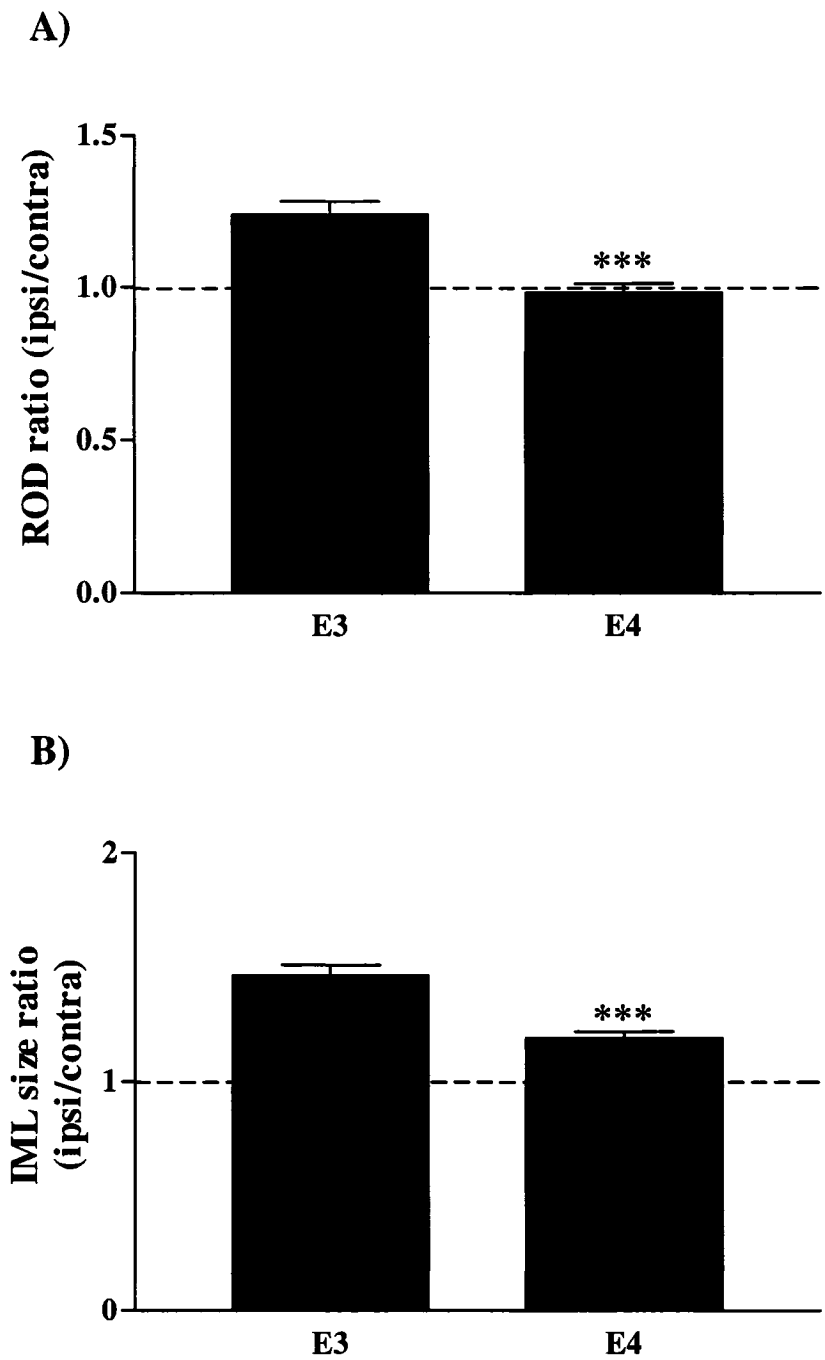

Figure 6. Acetylcholinesterase quantification in the Outer Molecular Layer and Inner Molecular Layer width at DPL 30. A) OML AChE relative optical density (ROD) and B) IML width were measured as described in the Methods section. Results are expressed as a ratio between the ipsi and contralateral sides. *** $p<0.0001$ hE4 vs. hE3 mice (n= 10 E3; 13 E4).

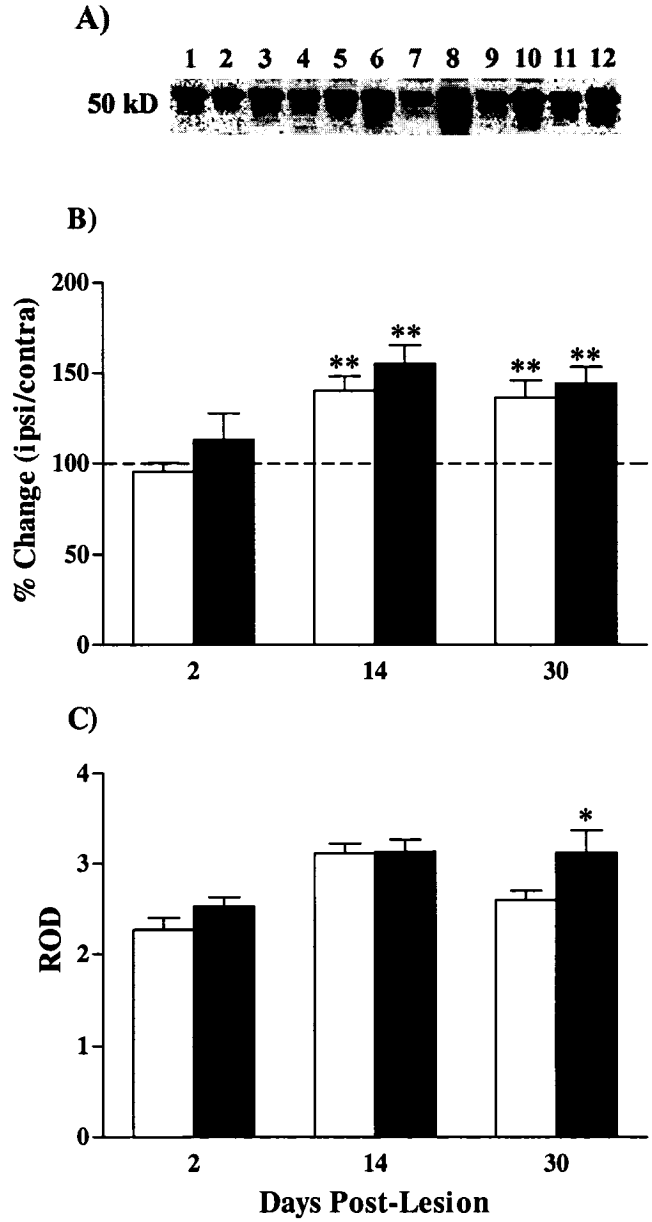

Figure 7. Hippocampal Glial Fibrillary Acidic Protein expression following ECL in hE3 and hE4 mice. A) Representative Western blot of GFAP expression. Band sequence is as follows: 1-2:E3 DPL2; 3-4:E4 DPL2; 5-6:E3 DPL14; 7-8:E4 DPL14; 9-10:E3 DPL30 11-12:E4 DPL30 (odd: contra, even: ipsi). B) GFAP expression ratio. C) GFAP relative optical density (ROD) on the side ipsilateral to the lesion. White bars: E3; black bars: E4. * $p<0.05$ E4 vs. E3. ** $p<0.01$ ipsi vs. contralateral side which is considered 100% for each animal (n= 7 for both genotypes at each time point).

Astrocytes distribution and localization in the outer molecular layer of the DG at DPL 30 reveal a marked difference between the two genotypes. A representative staining for GFAP of hE3 astrocytes shows a stratified organization typical of the repair process whereas hE4 astrocytes are very reactive, hypertrophied and show a completely disorganized profile (**Fig. 8**).

2.5.4 Kinetic of IL-1ra response to injury differs between APOE genotypes

We measured IL-1ra levels in hippocampus following ECL as an indicator of the inflammatory response and found time-dependent changes that are specific to the lesion side. **Fig. 9** illustrates differences in the time-course response of IL-1ra ipsilateral to the lesion. A peak response was observed at DPL 2 in hE3 ($p=0.0002$ vs. contralateral) that was delayed to 14 DPL in hE4 ($p=0.0019$). IL-1ra levels on the contralateral side were unchanged across time and genotypes (14.26 ± 2.71 pg/mg protein).

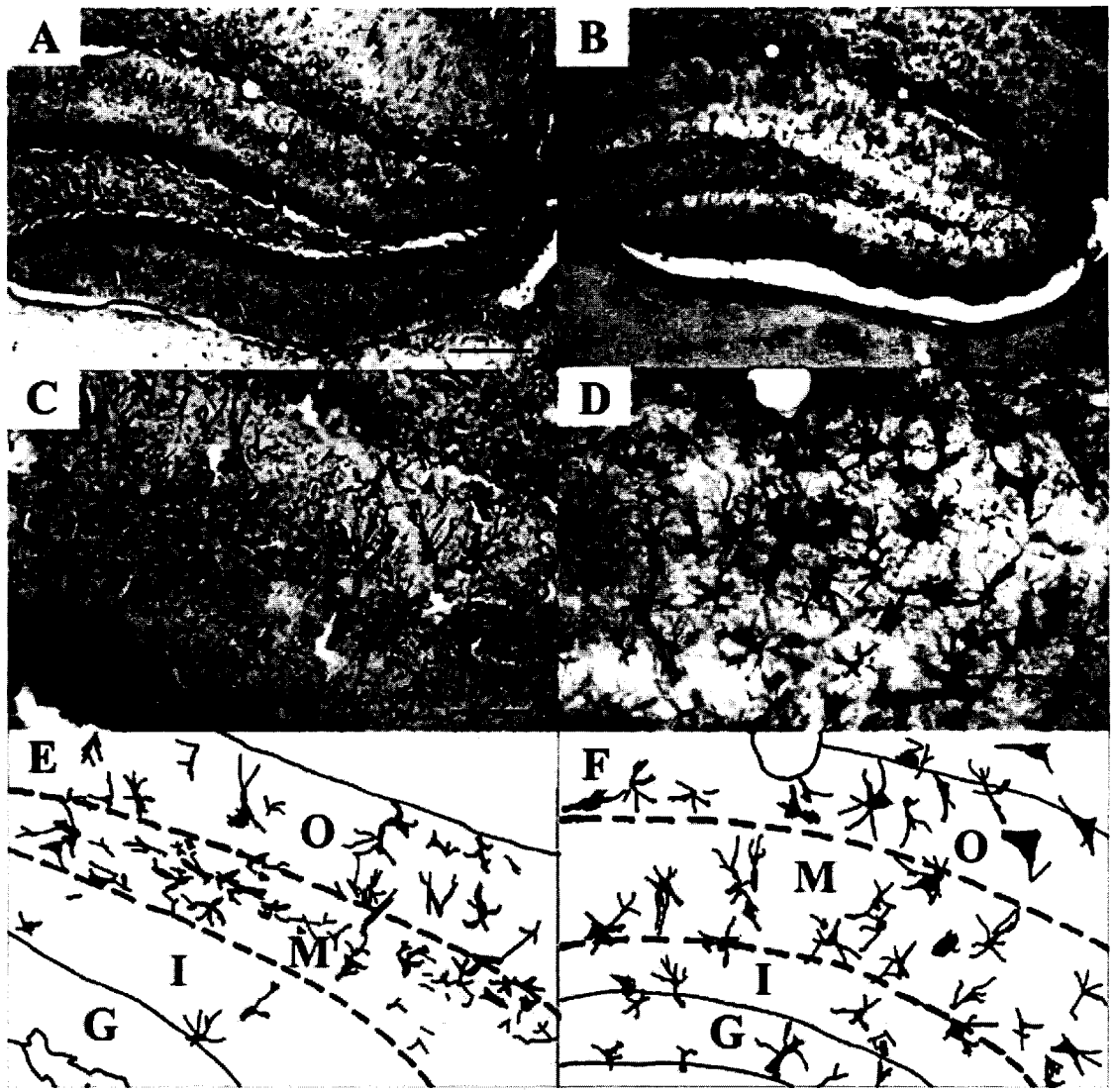

Figure 8. Astroglial organization following ECL in hE3 and hE4 mice at DPL 30. A-D: Representative photomicrographs of GFAP immunostaining showing the hippocampal formation ipsilateral to the lesion. E-F: Drawings of astrocyte organization (observed in C and D respectively) with layer identification. A,C,E: hE3; B,D,F: hE4. G: granule cell layer; I: inner molecular layer; M: median molecular layer; O: outer molecular layer. Scale bar: A,B: 200μm; C,D: 50μm.

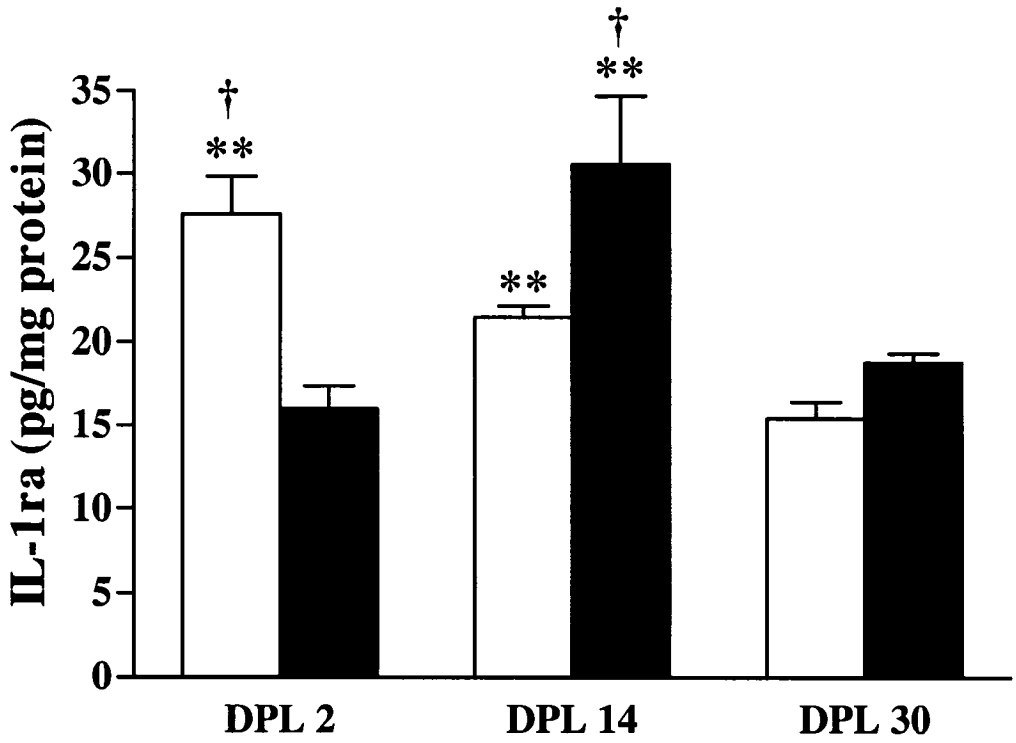

Figure 9. Hippocampal IL-1ra expression following ECL in hE3 and hE4 mice. ELISA was performed and results from hippocampi ipsilateral to the lesion side are reported. White bars: hE3; black bars: hE4. ** $p<0.01$ ipsi vs. contra; † $p<0.01$ E3 vs. E4 (n= 4 for both genotypes at each time point).

2.5.5 Genotype-specific tau phosphorylation and PP2A expression

Tau phosphorylation status was assessed using a phospho-specific monoclonal antibody recognizing dually phosphorylated tau on Ser202 and Thr205. As shown in **Fig. 10A**, ECL induced a marked phosphorylation of hippocampal tau in hE3 at DPL 2 when compared to hE4 (insert represents unlesioned animals of both genotypes). At DPL 14, E3 and E4 animals exhibited similar levels of phospho-Tau; corresponding to a decreased phosphorylation in hE3 and an increase in hE4. Phosphorylation state was back to baseline levels (see insert) at DPL 30 for both strains. **Fig. 10B** illustrates the absence of effect of the lesion on the expression of the catalytic subunit of PP2A. We did not perform ICC for tau because we have never observed neurofibrillary tangles (NFT) during the post-ECL time points.

2.5.6 Amyloid levels differs according to APOE genotype

Hippocampal Aβ42 levels were assessed by ELISA in both strains of mice following ECL and found relatively unchanged during the entire time course (data not shown). On the other hand, Aβ40 levels decrease by 54% (p=0.0184) on the ipsilateral side to the lesion in hE4 at DPL 2 while hE3 showed no difference (**Fig. 11**). At DPL 14, both strains had similar Aβ40 levels on both sides. Finally, there is an accumulation of hippocampal Aβ40 ipsilateral to the lesion in hE4 at DPL 30 (+ 44% , p=0.0176). Note that the tissue preparation used here does not distinguish between soluble and insoluble amyloid and that ICC was not performed because fibrillary Aβ was never detected at the post-ECL time points used here.

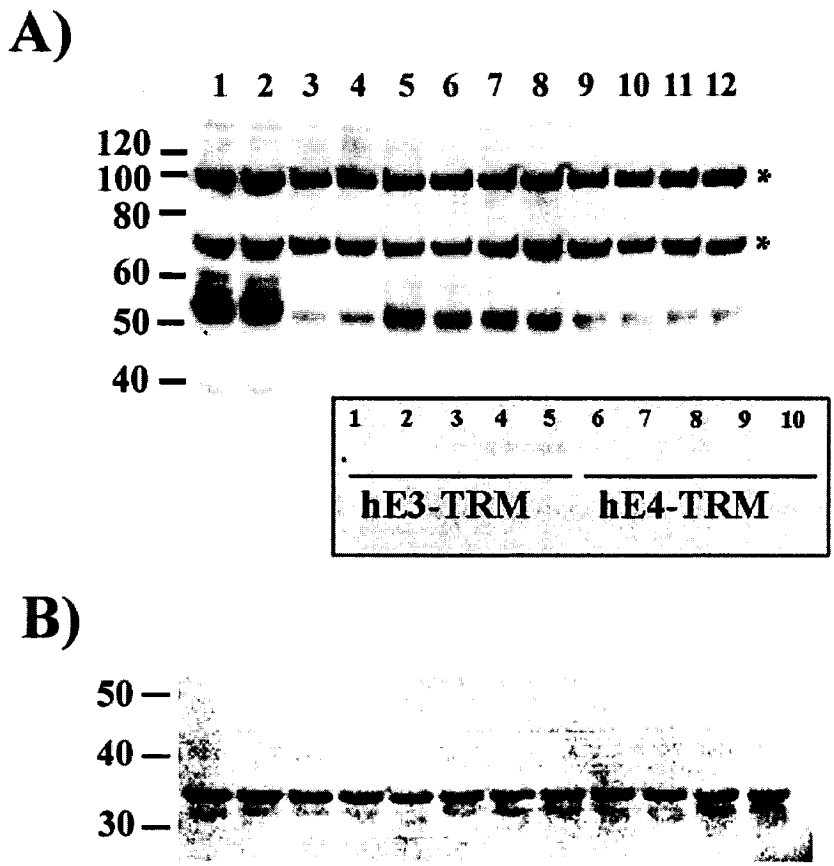

Figure 10. Hippocampal phospho-tau and PP2A expression following ECL in hE3 and hE4 mice. A) Western blot for the AT8 epitope of phospho-tau represented at ~52kD. The higher bands marked by * are the result of non-specific binding of the streptavidin-HRP detection reagent. *Insert: Basal phospho-tau expression in hippocampus of unlesioned mice.* B) Western blot for PP2A expression. Band sequence is as follows on A and B: 1-2:E3 DPL2; 3-4:E4 DPL2; 5-6:E3 DPL14; 7-8:E4 DPL14; 9-10:E3 DPL30 11-12:E4 DPL30 (odd: contra, even: ipsi).

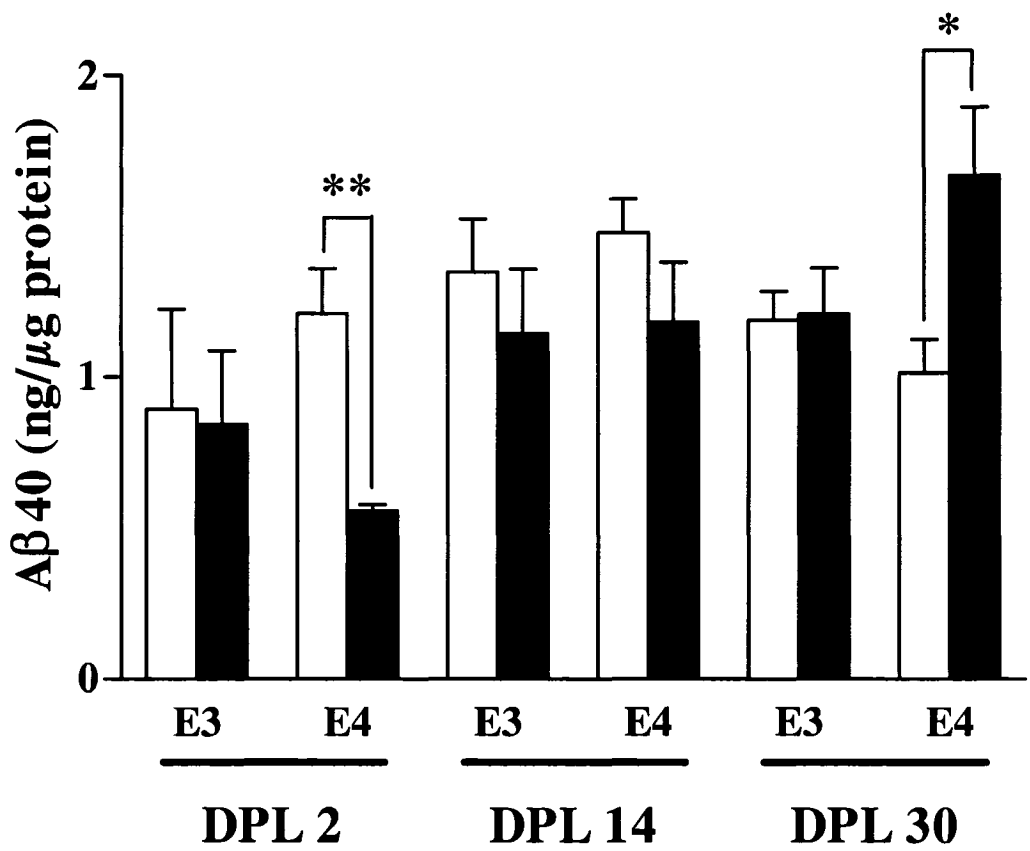

Figure 11. Hippocampal $A\beta_{1-40}$ expression following ECL in hE3 and hE4 mice. ELISA was performed as described in the Methods section. White bars: contralateral hippocampus; Black bars: ipsilateral hippocampus. * $p<0.05$ and ** $p<0.01$ ipsi vs. contra (n= 6 for both genotypes at each time point).

2.6 DISCUSSION

Modeling synaptic loss in animal models is a significant challenge for understanding AD pathology. The majority of AD cases is linked to the apoE polymorphism therefore, it is important to develop a model which addresses the pathogenesis of substantial synaptic loss in an APOE4 background for a better understanding of the disease. The ECL model is one of the best characterized model of deafferentation, designed to mimic synaptic loss in humans. We chose to perform ECL in the human TR mouse model to test the hypothesis that apoE4 associates with deficiencies in repair mechanisms and reinnervation of the dentate gyrus of the hippocampus. Due to the unique design of the TR mice, they provide an exquisite model for human apoE isoform comparison studies. Similar to non-demented humans, the hE3 and hE4 mice express similar levels of apoE in the same spatial pattern as humans and are able to respond appropriately to external stimuli (intervention) (Sullivan et al., 2004). Moreover, the fact that human apoE expression remains under the entire control of the host's regulatory sequences allows to completely eliminate the contribution of the apoE promoter polymorphisms reported to act both as a risk factor for AD (Lambert et al., 1998a; Lambert et al., 2002) and modulator of apoE expression in humans (Lambert et al., 1998b). Here we show that the hE4 mice respond to injury in an unfavorable manner for supporting synaptic remodeling compared to hE3 mice.

The major pathological changes associated with AD are first observed in the entorhinal cortex (Ball, 1978; Hyman et al., 1984; Roberts et al., 1993) and lead to widespread synaptic loss in the dentate gyrus of the hippocampus as evidenced by the severe memory loss in patients. The ECL model has been used extensively in rodents and is also characterized by a marked deafferentation of the dentate gyrus followed by reactive sprouting and synaptic replacement. We propose that neuronal repair is impaired in AD, and that apoE3 is more efficient in this repair process compared to apoE4. Each time point in this study was chosen for a reason, which is to represent the critical events following injury (i.e. the degenerative phase (0 to 6 days post-lesion), reactive sprouting and synapse replacement (6-30 days post-lesion) and completion of the reinnervation (30 days post-lesion and later) (Blain et al., 2004; Champagne et al., 2005).

Similar to other studies using ECL, we see a slight decrease in apoE levels at DPL 2, and a significant increase during the reactive phase (DPL 14) in both lines of mice. By DPL 30 the levels of apoE3 return to basal while apoE4 levels remain high. Considering the critical role of apoE during tissue remodeling in the brain, the maintained response in hE4 mice could be interpreted as incomplete synaptic recovery. Consistent with this interpretation is the absence of AChE induction in the hE4 mice. Upregulation of AChE (a marker of septal afferent sprouting) normally accompanies the plastic response to ECL (Fagan and Gage, 1994; Lynch et al., 1972; Scheff et al., 1977; West et al., 1982) as demonstrated in hE3 mice. Moreover, the marked expansion of the IML resulting from C/A fiber sprouting (Lynch et al., 1976), which normally follows ECL, is significantly smaller in hE4 mice compared to the hE3 mice. Together, these results suggest a delayed or compromised reactive sprouting response in hE4 mice which is similar to what is observed in human brains (Arendt et al., 1997) and in other apoE4 transgenic models (Buttini et al., 1999; White et al., 2001). However, the possibility that hE4 mice experience greater initial lesions cannot be ruled out.

Glial cells are important for reinnervation and respond by producing apoE (Diedrich et al., 1991; Poirier et al., 1991a), growth factors such as NGF (Carman-Krzan et al., 1991; Lindsay, 1979) and also become phagocytic for clearance of degeneration debris (Bechmann and Nitsch, 1997). Following deafferentation, astrocytes migrate in an outward fashion through the molecular layers of the hippocampus (Gall et al., 1979; Rose et al., 1976). This phenomenon is still not clear but is thought to represent a repair process associated to the removal of degeneration debris by phagocytic astrocytes. Our data show that astrocytes on the side ipsilateral to the lesion are more reactive in hE4 mice at DPL 30. Moreover, the hE4 mice do not display the outward astrocyte migration and stratified organization at a time when hE3 mice do. Indeed, in hE4 mice astrocytes are found throughout the molecular layers (IML, MML and OML) as well as in the granule cell layer of the DG whereas in hE3 they are mostly found in the two superior layers (MML and OML) of the DG. Interestingly, the increase in AChE activity exhibited by hE3 mice is located in the same area where effective migration of astrocytes occurs. Reinnervation thus appears to require outward migration of astrocytes and its absence or delay in the hE4 mice provides an explanation for the impaired reactive

sprouting also observed in these mice. ApoE knockout mice reacted to ECL in a similar fashion as the hE4 mice, exhibiting a delayed astrocyte migration (Champagne et al., 2005) as well as accumulation of degeneration products (Fagan et al., 1998). Our data combined with the apoE knockout studies suggest that apoE4 may impart a partial loss of function compared to apoE3 when expressed in the CNS.

IL-1 has been shown to activate astrocytes during the reinnervation process (Fagan and Gage, 1990). Since IL-1 protein levels are extremely difficult to detect at baseline, we measured its natural antagonist IL-1ra. Its upregulation always parallels that of IL-1 after brain damage (Wang et al., 1997) except for a slight delay in the time course of expression (Gabellec et al., 1999; Loddick et al., 1997). It also parallels microglial activation soon after the deafferentation phase (Gehrmann et al., 1991). A marked difference in the time course of IL-1ra expression was found following hippocampal deafferentation of the hippocampus in the two lines of mice. The hE3 mice showed an increase in IL-1ra expression soon after the deafferentation which was significantly delayed (DPL 14) in hE4 mice, consistent with a delay in the anti-inflammatory response. IL-1 exerts neurotrophic effects by stimulating the release of NGF from astrocytes (Carman-Krzan et al., 1991). This in turn stimulates neovascularization (Giulian et al., 1988b) as well as cholinergic sprouting following hippocampal deafferentation (Fagan and Gage, 1990). IL-1 levels are increased in Alzheimer's brain tissue and CSF (Cacabelos et al., 1991; Griffin et al., 1989) and IL-1ra levels are reduced in AD CSF (Tarkowski et al., 2001), consistent with an exacerbated inflammatory response. Moreover the APOE4 copy number is associated with the extent of microglial response (Egensperger et al., 1998; Lombardi et al., 1998).

Our results suggest that the reduced astrocytic response observed in hE4 mice as well as the compromised reinnervation could be the result of an exacerbation of the inflammatory response during the degenerative phase. Alternatively, the hE4 mice may exhibit an absence of proper feedback regulation of inflammatory signals. The expression of apoE4 may impair the regulation of IL-1ra and IL-1, resulting in a slower astrocyte organization and culminating in delayed repair processes.

The synaptic loss observed in the ECL model is one of the three pathological hallmarks of AD. Neurofibrillary tangles and amyloid deposition are the other two

hallmarks of AD which we tested for in the targeted replacement mice. The APOE4 gene is linked to neurofibrillary tangles and hyperphosphorylation of tau ((Genis et al., 1995; Genis et al., 2000). We observed a pronounced increase of tau phosphorylation (AT8 epitope) in the hippocampus at DPL 2 in hE3 but not in hE4 mice; coinciding with the active phase of deafferentation in this model. Tau hyperphosphorylation is mostly due to decreased phosphatase activity rather than increased kinase activity (Planel et al., 2001). Protein phosphatase (PP)2A is responsible for the dephosphorylation of the tau epitope investigated here (Sontag et al., 1996). However, the hE3 mice showed no reduction in PP2A catalytic subunit levels. These results suggest that apoE3 induces tau phosphorylation early on but does not address what happens at later stages in the injury. Since we only used AT8 for detection of hyperphosphorylated tau, we can not rule out that other sites on tau may be differentially phosphorylated according to apoE genotype (Kobayashi et al., 2003). Basal levels of AT8 signal were virtually undetectable in our model suggesting a shift in tau phosphorylation in response to ECL.

The decrease in Aβ 1-40 observed at DPL 2 in hE4 is consistent with the synaptic localization of APP and the loss of synapses occurring at this time point following ECL. Inexplicably, the same expected effect is not observed in hE3 mice. At DPL 30 we observe significantly more Aβ 1-40 in the hE4 mice hippocampus ipsilateral to the lesion compared to the contralateral side, a phenomenon not seen in hE3 mice. Interestingly, it also coincides with increased levels of apoE compared to hE3 mice. This is consistent with the hypothesis we and others proposed a few years ago claiming apoE4 is less efficient at scavenging soluble Aβ *in vivo* and *in vitro* (Beffert et al., 1999b; Holtzman et al., 1999; Yang et al., 1999). The high levels of apoE and Aβ measured in hE4 mice at DPL 30 may eventually enhance the deposition of Aβ (Schmechel et al., 1993) if the Aβ levels do not return to basal levels (Dolev and Michaelson, 2004; Fagan et al., 2002; Hartman et al., 2002; Nicoll et al., 1995). We plan to look at later time points to answer this question.

In summary, our combined apoE/ECL model elicits many features of AD which should aid our understanding of apoE isoform-specific effects in synaptic injury. This model will also be of great use in testing molecules that are currently under development for the treatment of AD.

2.7 ACKNOWLEDGEMENTS

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3.0 STUDY 2

A ROLE FOR LIPOPROTEIN LIPASE DURING SYNAPTIC REMODELING IN THE ADULT MOUSE BRAIN

Jean-François Blain, Eric Paradis, Sophie B. Gaudreault, Danielle Champagne, Denis Richard and Judes Poirier

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3.1 FOREWORD

In periphery, lipoprotein lipase (LPL) is involved in triglyceride hydrolysis and lipoprotein binding to the cell surface (Goldberg, 1996). Whether it is by itself or in conjunction with apoE, LPL was shown to promote the internalization of a subset of HDL particles (Schorsch et al., 1997; Panzenboeck et al., 1997; Rinninger et al., 1998b; Merkel et al., 2002a) that are similar to those found in the brain (LaDu et al., 1998; Fagan et al., 1999). *In vitro* LPL promotes neurite outgrowth of sympathetic neurons (Postuma et al., 1998) while in an animal model of peripheral injury, it is proposed to be involved in cholesterol recycling.

LPL is also present in the brain but its specific function has yet to be determined. Considering the role it has in periphery, its relationship with apoE-lipoproteins, and the fact that apoE is involved in supplying cholesterol for terminal sprouting, we hypothesized that LPL might also be involved in lipoprotein metabolism following injury. To address this question we monitored LPL expression as well as the one of some of its receptors following entorhinal cortex lesion, an animal model that mimics the neurodegeneration of the entorhino-hippocampal pathway in AD brain.

3.2 ABSTRACT

Lipoprotein lipase (LPL) is a member of a lipase family known to hydrolyze triglyceride molecules found in lipoprotein particles. This particular lipase also has a role in the binding of lipoprotein particles to different cell-surface receptors. LPL has been identified in the brain but has no specific function yet. This study aimed at elucidating the role of LPL in the brain in response to injury. Mice were subjected to hippocampal deafferentation using the entorhinal cortex lesion and mRNA and protein expression were assessed over a time-course of degeneration/reinnervation. Hippocampal LPL levels peaked at 2 days post-lesion (DPL) both at the mRNA and protein levels. No change was observed for receptors of the LDL-receptor family or RAP at DPL 2 in the hippocampus but the glia-specific syndecan-4 was found to be significantly upregulated at DPL 2. These results suggest that LPL is involved in the recycling of cholesterol and lipids released from degenerating terminals after a lesion through a syndecan-4-dependent pathway.

3.3 INTRODUCTION

Cholesterol was shown to play a significant role in the pathophysiology of Alzheimer's disease (AD). Alterations of cholesterol transport by apolipoprotein E and cholesterol production by the HMG-CoA reductase were shown to be compromised in the AD brain whereas polymorphic variants of these genes were shown to be associated with the common form of AD (Poirier et al., 1993a; Strittmatter et al., 1993a; Poirier, 2003). The recent discovery that cholesterol lowering agents selectively inhibiting the HMG-CoA reductase are protective against AD illustrates the growing importance of cholesterol homeostasis in the disease process (Jick et al., 2000; Wolozin et al., 2000; Poirier et al., 2002).

The majority of studies that have investigated the role of cholesterol in AD have focused on the relationship that exists between apoE, HMG-CoA reductase and β-amyloid metabolism. Several other proteins known to be involved in cholesterol homeostasis in the cardiovascular system, and also present in the CNS, are now being investigated in the context of AD pathophysiology.

Among these, lipoprotein lipase (LPL; EC 3.1.1.34) belongs to a lipase gene family which includes the hepatic lipase, pancreatic lipase and the newly discovered endothelial lipase. LPL is involved in the hydrolysis of triglycerides (Tg) found in Tg-rich lipoproteins in the blood, namely VLDL and chylomicrons. Moreover, after the initial interaction between LPL and lipoproteins on the vascular wall, the LPL remains associated with the remnant lipoproteins and subsequently acts as a ligand for lipoprotein receptors. LPL was also shown to selectively increase the uptake of HDL3 particles by adipocytes (Schorsch et al., 1997), peritoneal macrophages (Panzenboeck et al., 1997) and hepatocytes (Rinninger et al., 1998b). The dual action of LPL thus helps in providing triglycerides, free-fatty acids and cholesterol to the cells. Among lipoprotein receptors, members of the LDL receptor family such as the α2-macroglobulin/LRP (Beisiegel et al., 1991), the LDL receptor itself (Medh et al., 1996), the VLDLR (Takahashi et al., 1995) and the GP330/LRP2 (Kounnas et al., 1993) were shown to bind LPL. More recently it was reported that LPL could also interact with the apoER2, enhancing VLDL binding in an apoE independent fashion (Tacken et al., 2000).

However, its major binding sites are the heparan sulfate proteoglycans (HSPGs), which were reported to be critical for LPL binding and LPL-mediated binding of lipoproteins to the membrane (Eisenberg et al., 1992; Mulder et al., 1992; Mulder et al., 1993). Most notably, there is a family of proteoglycans called the syndecans which are transmembrane proteins that were shown to mediate binding and catabolism of LPL-lipoprotein complexes independently of other cell-surface receptors (Fuki et al., 1997). Four members are currently known in this family: syndecan-1, syndecan-2/fibroglycan, syndecan-3/N-syndecan and syndecan-4/ryudocan. They are all expressed in the brain, syndecan-1 being mostly restricted to the cerebellum, syndecan-2 and 3 being expressed on neurons of different regions of the brain including the dentate gyrus of the hippocampal formation and finally, syndecan-4 expressed on glial cells (Hsueh et al., 1999).

Little is known about LPL functions in the CNS. It has been reported to be present throughout the brain with its highest levels in the hippocampus (Ben Zeev et al., 1990; Nunez et al., 1995; Paradis et al., 2004a). It is suggested that LPL could serve as a transport for cholesterol and vitamin E to neurons thus helping to their survival and to the plasticity and regeneration of neuronal processes (Ben Zeev et al., 1990; Nunez et al., 1995; Huey et al., 2002; Paradis et al., 2003).

In the present study we show that in response to deafferentation, the mouse hippocampus exhibits a marked induction of LPL mRNA and protein levels but downregulation of apoE protein levels during the degeneration phase. The alterations coincide with increases in mRNA and protein levels for the glia-specific proteoglycan syndecan-4 subtype which is a well known LPL receptor. Altogether, these results indicate that brain lipoprotein lipase is involved in the recycling and/or scavenging of lipids and cholesterol released from the degenerating terminals through glial cell-surface syndecan-4 binding sites.

3.4 METHODS

3.4.1 Animals

Male C57Bl6/J mice aged between 3-4 months were purchased from Jackson Laboratories (Ann Harbor, MI, USA).

3.4.2 Entorhinal Cortex Lesions (ECL)

Unilateral electrolytic ECL were adapted to mice from a technique described earlier in rats (Lynch et al., 1972). Briefly, anesthesia was induced by intramuscular injection of 1 μl/g of body weight of a ketamine/xylazine/acepromazine mix. Skull was shaved and the animal is placed in a stereotaxic frame in a flat skull position. The lambda 0 was taken by aligning the electrode with the suture lines. The skull was drilled to allow the electrode to pass through the four different coordinates. The electrode is inserted at a 6° angle in the right side of the brain following these coordinates: RC (+0, +0, +0.5, +1.0); L (-3.0, -3.5, -4.0, -4.0); DV (-3.0, -4.0 at each point). A 1mA current was then applied for 10 sec at each point. Hole is patched with Bone Wax and skin sutured. Animals are given a subcutaneous injection of Ringer's solution to prevent dehydration and 1μl/g of body weight of butorphanol as anti-inflammatory. They are placed under a lamp for 24 h, nursed for 2-3 days and are finally sacrificed 2, 6, 14, 21 or 40 days following the lesion. Note that the contralateral side of the brain serves as an internal negative control.

3.4.3 Tissue homogenization

The brains were dissected and separated into left and right: frontal cortex, entorhinal cortex, striatum, temporal cortex, hippocampus and cerebellum. These different tissues were sonicated on ice in a phosphate buffered solution containing proteases inhibitors (AEBSF, leupeptin, bestatin, aprotinin, E-64, pepstatin A; Sigma-Aldrich Canada Ltd, Oakville, ON). Protein concentration was assessed using the BCA technique (Pierce Biotechnology Inc, Rockford, IL).

3.4.4 Membrane preparation

Aliquots of the whole tissue extracts were spun at 3000 rpm for 8 min at 4°C and the supernatant collected. This operation was repeated 3 more times and the final supernatants were centrifuged at 14000 rpm for 90 min at 4°C. The pellets were resuspended in PBS and protein concentration determined using the BCA technique.

3.4.5 PAGE and Immunoblot analysis

PAGE for LPL, apoE, synaptophysin, Glial Fibrillary Acidic Protein (GFAP), LDLR, LRP, RAP and syndecan-4 were performed using 10 to 25 μg of protein from total extracts in reducing conditions. 10 μg of proteins from membranes in non-reducing conditions were used for apoER2 detection. For syndecan-4, protein extracts were incubated for 3h at 37°C in the presence of 5mU/μl of Heparinase III (IBEX Pharmaceutical, Montreal, QC) prior to electrophoresis. Proteins were then transferred to a nitrocellulose membrane and blotted with either a polyclonal anti-mouse-LPL (generous gift from Dr I.J. Goldberg, Columbia University), a polyclonal anti-human apoE marked with HRP (IIC, Marrietta, CA), a polyclonal anti-mouse-LDLR, anti-mouse-LRP, anti-mouse-apoER2 or anti-mouse-RAP (generous gifts from Drs U. Beffert & J. Herz, U.T. Southwestern), a polyclonal anti-syndecan-4 (generous gift from Dr A. Bensadoun, Cornell University), a polyclonal anti-cow-GFAP (Dako Diagnostics Canada Inc, Mississauga, ON) or a monoclonal anti-synaptophysin (Boehringer Mannheim, Germany). Chemiluminescent detection is made using an ECL kit (Perkin-Elmer, Boston, MA) and exposing to Kodak XAR5 film. Density analysis was performed using a MCID image analyzer.

3.4.6 Real Time PCR

RNA extraction was performed using QIAGEN's RNeasy kit (QIAGEN Inc., Mississauga, ON). Primer pairs used for PCR amplification were as follows: mLPL-fwd 5'- CCC AAT GGA GGC ACT TTC C –3' with mLPL-rev 5'- ACG GAT GGC TTC TCC AAT GT –3'; mSynd4-fwd 5'- CAG GGC AGC AAC ATC TTT GA –3' with mSynd4-rev 5'- CAC GAT CAG AGC TGC CAA GAC –3'; mActin-fwd 5'- TGA CCG AGC GTG GCT ACA –3' with mActin-rev TCT CTT TGA TGT CAC GCA CGA T –3'. RT-PCR was performed using the SybrGreen method in a GeneAmp 5700 Sequence detection system from PE Applied Biosystems.

3.4.7 Brain perfusion and in situ hybridization

Animals were sacrificed by a lethal injection of anesthetic solution at different time points, followed by cardiac perfusion for 2 min with ice cold saline and 15 min with a solution of 4% paraformaldehyde (PFA, pH: 7.4). Brains were removed and placed in

4% PFA containing 10% sucrose overnight at 4°C before being frozen. Tissues were kept at –80°C until slicing. 30 μm sections were cut on a Microm D-6900 cryostat (Heidelberg, Germany) and immediately placed in an antifreeze solution (sodium phosphate buffer, pH 7.2-7.5; ethylene glycol 30%; glycerol 20%) and kept at –20°C until mounted on glass slides. *In situ* hybridization of LPL was described elsewhere (Paradis et al., 2004a). Briefly, sense and antisense RNA probes were obtained by *in vitro* transcription of LPL cDNA (primer pairs; mLPL-A 5'- TAG TTC CAG CAG CAA AGC AG –3' with mLPL-B 5'- TCT TCA AAG AAC TCG GAT GC –3') inserted in pGEM-T vector using SP6 and T7 polymerase (Promega Corp., Madison, USA). *In situ* hybridization studies were conducted to localize LPL mRNA on coronal tissue sections taken in the dorsal hippocampus region of lesioned mice.

3.4.8 Analysis of in-situ hybridization results

To quantify the expression of LPL mRNA, darkfield microphotographs of NTB2-emulsified brain sections were acquired with a DAGE-3CCD camera (DAGE-MTI, Michigan City, USA) mounted on an Olympus BX-60 microscope (Olympus America Inc., Melville, USA). Analysis of these images was done with ImagePro plus (MediaCybernetics, Silver Spring, USA). For this, the entire system was calibrated for each set of experiments. Slides were quickly reviewed to identify the most intense signal across all regions to be analyzed. The selected slides were then placed under the microscope, and live image acquisition was performed. An histogram reporting the number of saturated pixels was generated, and light intensity was adjusted to prevent the saturation of the signal. These settings were maintained for every slide, and the histogram was produced for every image acquired to confirm that the signal was not saturated under any circumstances. For all the optical density measurement and comparisons, brain sections located around rostrocaudal level bregma –1,94mm were selected. For every region analyzed, 3 readings were taken in the labeled area and 3 background readings were taken in the unlabeled, but immediately surrounding tissue. These readings were averaged and subtracted from each other to determine the signal intensity in the regions of interest. This procedure was performed 3 times on each hemisphere (ipsilateral and contralateral to the lesion), yielding a total of 3 measurements

for each region analyzed per slide. These 3 optical density measurements were then averaged and included as individual scores in the statistical analyses.

3.4.9 Combined immunohistochemistry and in situ hybridization

Immunohistochemistry was combined with *in situ* hybridization to determine the cell type that expresses LPL mRNA in the mouse brain after the lesion (Paradis et al., 2004a). Briefly, brain sections were first processed for immunohistochemical detection using a conventional avidin-biotin-immunoperoxidase method. Anti-Iba1 and anti-GFAP were used for identification of microglia and astrocytes respectively. Thereafter, tissues were rinsed in sterile KPBS, mounted onto poly-L-lysine-coated slides, desiccated overnight under vacuum, fixed in paraformaldehyde (4%) for 3 min, and digested for 30 min at 37°C with proteinase K (10mg ml in 100mM Tris HCL, pH 8.0, and 50mM EDTA). Pre-hybridization, hybridization, and post-hybridization steps were performed as described elsewhere (Paradis et al., 2004a). Slides were exposed for 7 days, developed in D19 developer for 3.5 min at 15°C, and fixed in rapid fixer for 5 min. Thereafter, tissues were rinsed in running distilled water for 1-2 h, rapidly dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

3.4.10 Statistical analysis

All the results except *in situ* hybridization were analyzed using a one-way ANOVA with Dunnett's post test in GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA). For in situ hybridization, statistical analysis was performed using the JMP 4.0 statistical software (SAS institute Canada, Ste-Foy, Canada). After the normal distribution of the data was confirmed with a Shapiro-Wilk test, the variance was analyzed with a Bartlett test. Data showing unequal variances were then analyzed with a Welch ANOVA test. Data with equal variances were analyzed with a standard ANOVA test. In every situation, the means were compared with a Tukey-Kramer HSD test.

3.5 RESULTS

3.5.1 ApoE and synaptophysin protein levels as markers of synaptic remodeling

The use of unilateral ECL provides an experimental advantage which is the simultaneous generation of a contralateral, unlesioned hemisphere, that serves as a negative control for each animal investigated in these experiments. We have used the unilateral ECL model to study the impact of deafferentation/reinnervation on the expression of specific proteins in the hippocampus of the adult mouse. We contrasted the expression of apoE (a cholesterol transporter) with that of synaptophysin, a marker of synaptic integrity and plasticity. Expression levels were assessed by Western analysis in hippocampal homogenates and results are expressed as ratios of ipsilateral to contralateral side to the lesion. We found a significant reduction (- 35%) in the levels of apoE at 2 days post-lesion (DPL; **Fig. 12A**) followed by a marked induction at DPL 14 (+ 93%), consistent with previous results obtained in the rat ECL model (Poirier et al., 1993b). ApoE was back to control levels by DPL 40. **Figure 12B** illustrates the pattern of expression of synaptophysin during the same time-course. The pattern of changes are similar although there is a slight delay in the synaptophysin induction when compared to apoE. The response is specific for the hippocampus as no change in apoE levels were observed in the ipsilateral frontal cortex at either time point following the lesion (**Fig. 12C**).

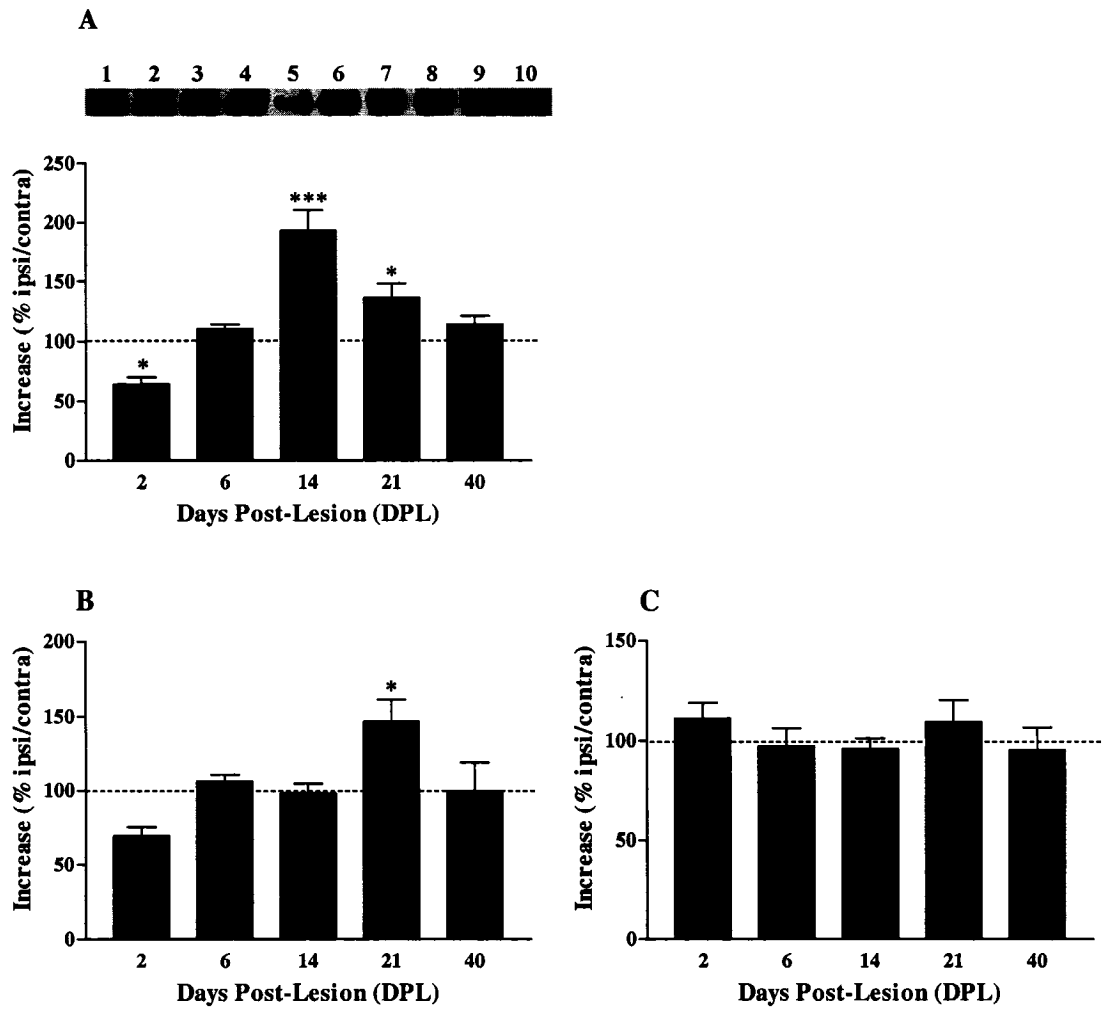

Figure 12. ApoE and synaptophysin are modulated in hippocampus following ECL. Protein levels were measured by Western blot in hippocampus and frontal cortex. *A*, Representative Western blot of ApoE levels in the hippocampus. Odd numbers represent the side contralateral to the lesion site and even numbers the side ipsilateral to the lesion. *B*, Synaptophysin levels in the hippocampus. *C*, ApoE levels in the frontal cortex. All values were normalized to Tubulin levels and are expressed as a ratio between the ipsilateral and contralateral side of the lesion. Results are mean ± SEM of n = 6-8 animals. * $p < 0.05$, *** $p < 0.001$.

3.5.2 LPL mRNA expression and localization following ECL

Real-time quantitative RT-PCR was used to assess the hippocampal LPL changes. **Figure 13** shows that LPL mRNA prevalence is increased in the hippocampus in response to the ECL at the beginning of the degeneration phase. Indeed, mRNA levels are significantly increased at DPL 2 ($p < 0.05$) and return to baseline levels at DPL 6, 14 and 21. *In situ* hybridization was performed to map the regional changes in LPL mRNA expression following ECL. As shown in **Figure 14**, LPL expression started to increase at DPL 2 in the alveus (**Fig. 14A-B**), then was also observable in the external capsule at DPL 6 (**Fig. 14C-D**). At DPL 14, a signal was still measurable in the alveus (**Fig. 14E**) but returned to baseline values by DPL 21 (**Fig. 14F**). **Figure 15A** illustrates the variation measured in these 2 regions where 10 to 18-fold increases were observed at the peak of expression, suggesting a permanent alteration of the neuronal circuitry in those areas. No significant difference in signal intensity was observed between time points in the lesioned side. However, the surface of the alveus/external capsule region revealed a significant increase at DPL 6 (1.57 ± 0.20 mm^2, $p<0.0002$) compared to DPL 2 (0.52 ± 0.11 mm^2) and DPL 14 (0.31 ± 0.06 mm^2). LPL mRNA prevalence remained unchanged in the CA3 region of the hippocampus (**Fig. 15B**), an internal control region where deafferentation is minimal.

Co-localization experiments using *in situ* hybridization and immunohistochemistry were performed to assess the cellular origin of these mRNA increases. LPL mRNA increases in the alveus and external capsule at DPL 6 co-localized with the microglial immunological marker Iba-1 (**Fig. 16A-B**) and astroglial marker GFAP (**Fig. 16C-D**).

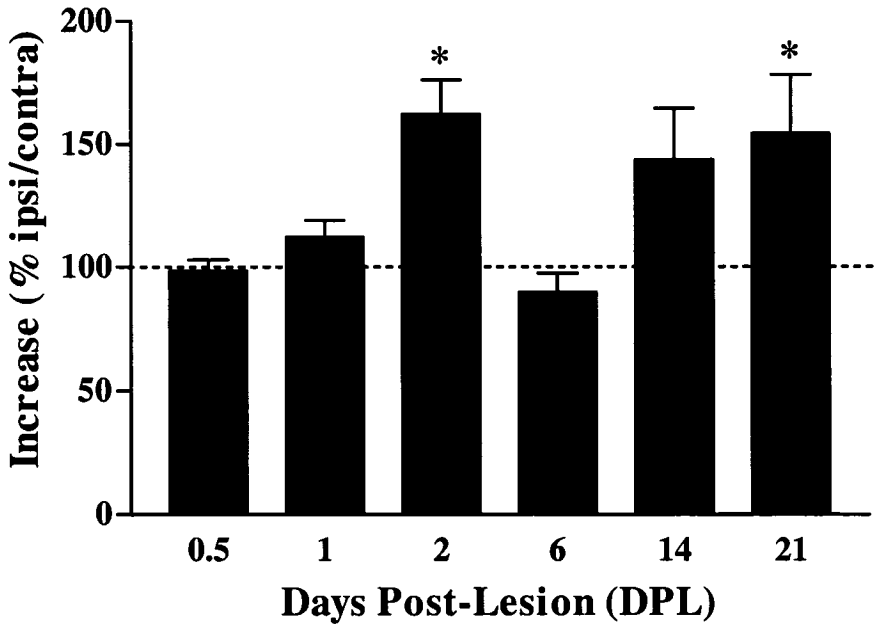

Figure 13. LPL mRNA expression is induced in hippocampus following ECL. LPL mRNA was measured by semi-quantitative RT-PCR as described in the Methods. LPL levels were normalized to Actin levels and expressed as a ratio between the ipsilateral and contralateral side of the lesion. Results are mean ± SEM of n = 5-6 animals. * $p < 0.05$.

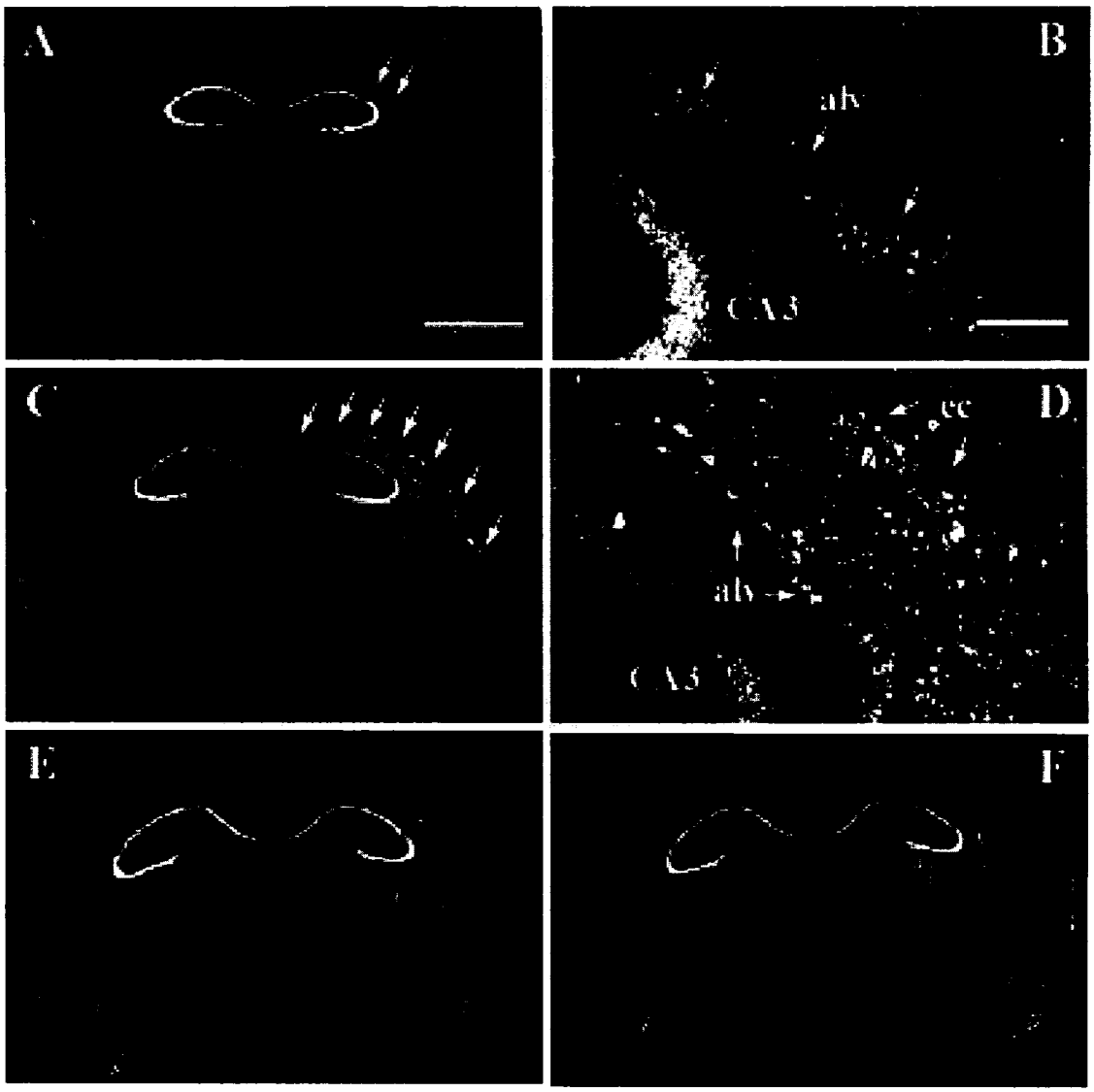

Figure 14. LPL mRNA expression is induced entorhinal fiber pathways following ECL. Darkfield photomicrographs of dipped NTB2 emulsion slides showing the unilateral hybridization signal for LPL mRNA (indicated by arrows) on coronal sections of mouse brain at DPL 2 (panel A-B), 6 (panel C-D), 14 (panel E) and 21 (panel F). On panel A, C, E and F the tissue sections were photographed with a 1,25X objective (scale bar 2 mm). On panel B and D, the tissue sections were photographed with a 10X objective (scale bar 250 μm). *alv*: alveus, *ec*: external capsule.

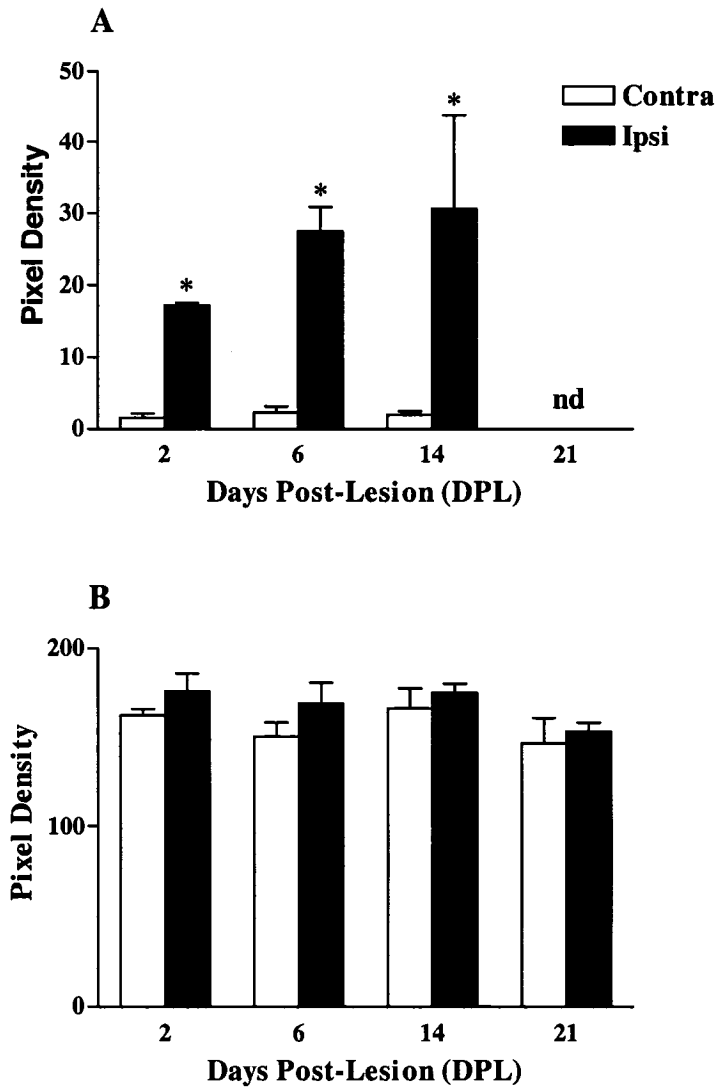

Figure 15. LPL mRNA expression is upregulated in entorhinal pathways during ECL. Quantification of LPL hybridization signals on the side ipsilateral to the lesion site compared to the side contralateral to the lesion at DPL 2 days (n=3), 6 days (n=3), 14 days (n=3) and 21 days (n=3). *A*, Alveus and external capsule. *B*, CA3. Data are means ± SEM of three independent experiments. Significance levels were determined by comparing LPL signals on the lesioned side of the brain with the control region on the same slide, at each time point; *, $p<0.006$.

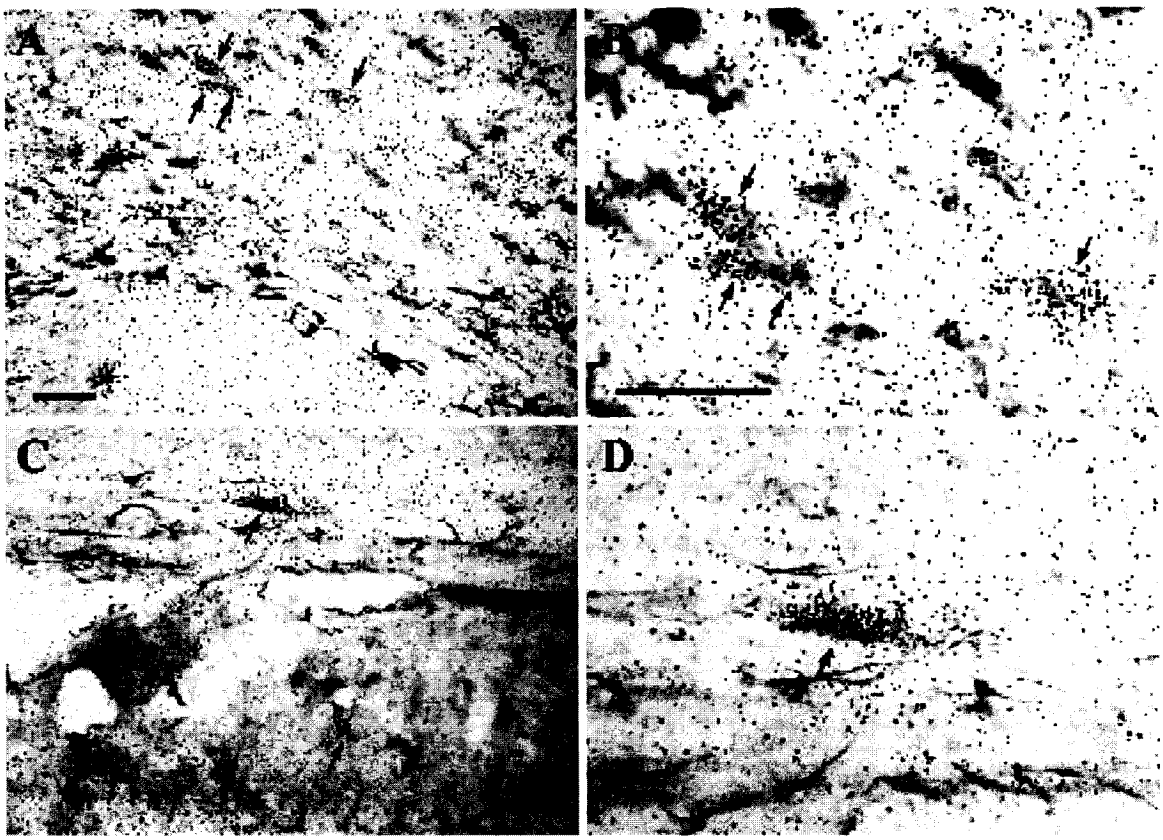

Figure 16. LPL is expressed by microglial and astroglial cells in the ECL. Bright-field photomicrographs of brain section (DPL 6) taken with a 40X (panel A and C) or 100X (panel B and D) objective showing the co-localization of LPL mRNA and cells immunoreactive to Iba-1 (panel A-B), or GFAP (panel C-D). Scale bars 100 μm.

3.5.3 LPL protein expression in hippocampus following ECL

Figure 17A shows the protein expression profile of LPL during deafferentation and reinnervation. Peak levels of expression for LPL were found at DPL 2 (+ 70%) where it coincided with the peak reduction for apoE protein levels (**Fig. 12**) in the same brain region. That particular period of time corresponds to the acute phase of deafferentation in the hippocampus in ECL animals. The profile of expression is specific for the hippocampus as no change was observed in the frontal cortex ipsilateral to the lesion (**Fig. 17B**).

3.5.4 Expression of receptors and binding sites in relation to LPL

Since LPL is recognized as a ligand by different cell-surface receptors, it prompted us to examine its possible involvement in the synaptic plasticity process. Accordingly, key members of the LDLR family known to bind LPL were examined in the deafferented hippocampus during the acute phases of deafferentation and reinnervation. **Figure 18A** shows that ECL caused a significant reduction (-25%) in LDLR expression at DPL 6 . However, LRP and its associated protein RAP (**Fig. 18B**) were not affected by the deafferentation process nor was apoER2 expression (**Fig. 18C**).

However, a careful analysis of the glial HSPG syndecan-4 expression revealed a different story. **Figure 19A** shows that syndecan-4 mRNA expression is upregulated 6 days following the lesion and that it stays significantly elevated until DPL 21. The time course of mRNA expression for syndecan-4 in the hippocampus parallels that of GFAP protein expression, an established marker of astroglial activation and proliferation (**Fig. 19C**). Interestingly, as it is the case for LPL protein levels, syndecan-4 protein levels are significantly increased at DPL 2 (**Fig. 19B**).

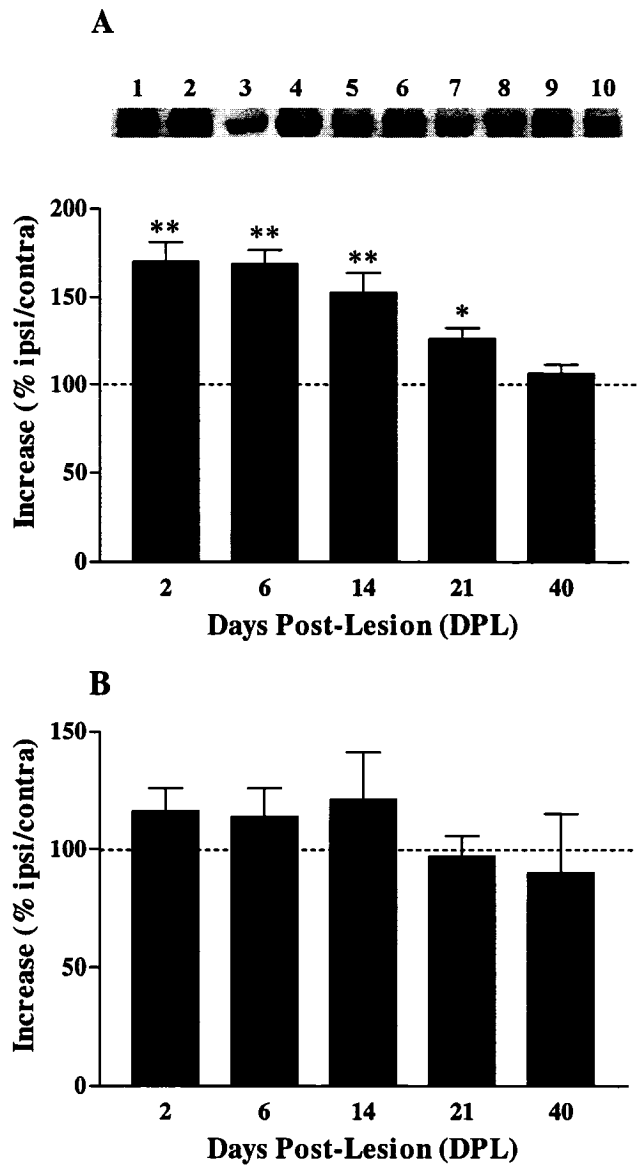

Figure 17. LPL expression is upregulated in hippocampus following ECL. LPL protein expression was measured by Western blot as described in the Methods. *A*, Representative Western blot of LPL levels in the hippocampus. Even numbers represent the side contralateral to the lesion site and odd numbers the side ipsilateral to the lesion. *B*, LPL levels in the frontal cortex. Protein levels were normalized to Tubulin levels and are expressed as a ratio between the ipsilateral and contralateral side of the lesion. Results are mean ± SEM of n = 7-14 animals. * $p < 0.05$, ** $p < 0.01$.

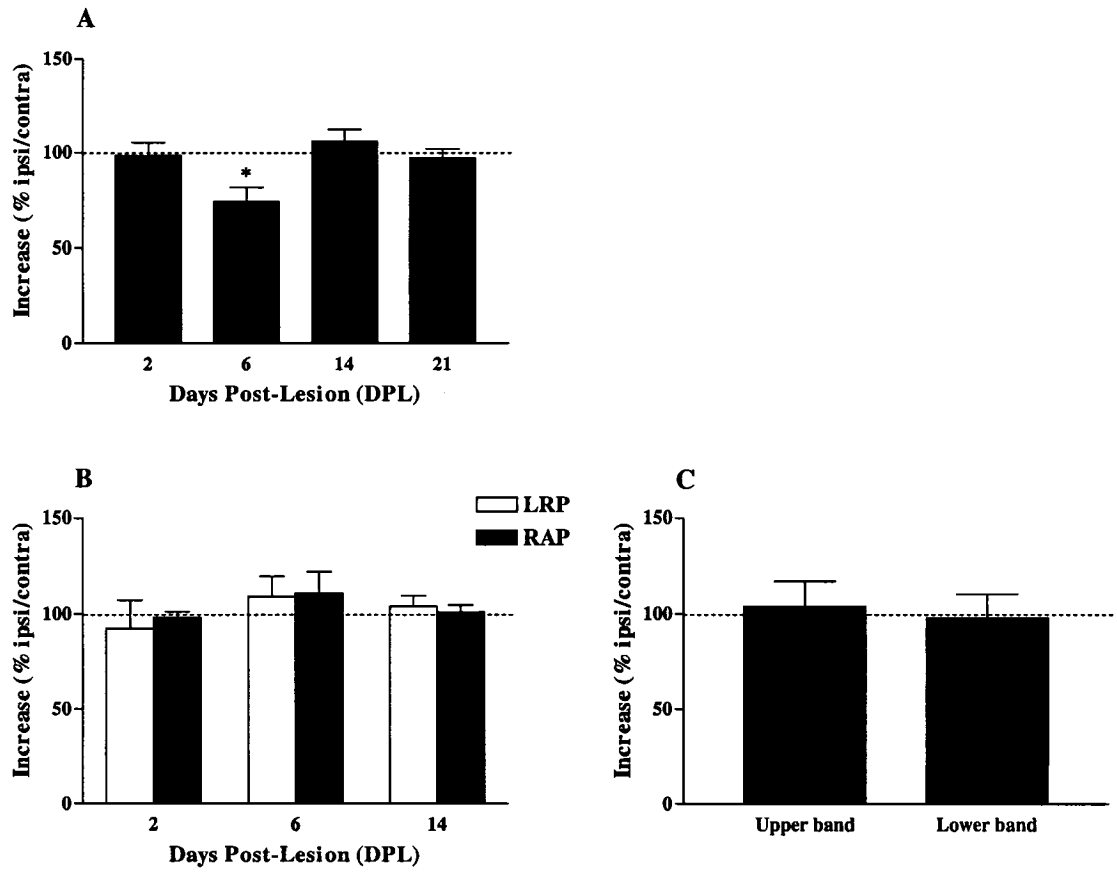

Figure 18. LDL receptor expression levels in hippocampus is downregulated in the degeneration phase of the ECL. Protein levels were measured by Western blot. *A*, LDLR. *B*, LRP and RAP. *C*, ApoER2. All values were normalized to Tubulin levels and are expressed as a ratio between the ipsilateral and contralateral side of the lesion. Results are mean ± SEM of n = 4-8 animals. * $p < 0.05$.

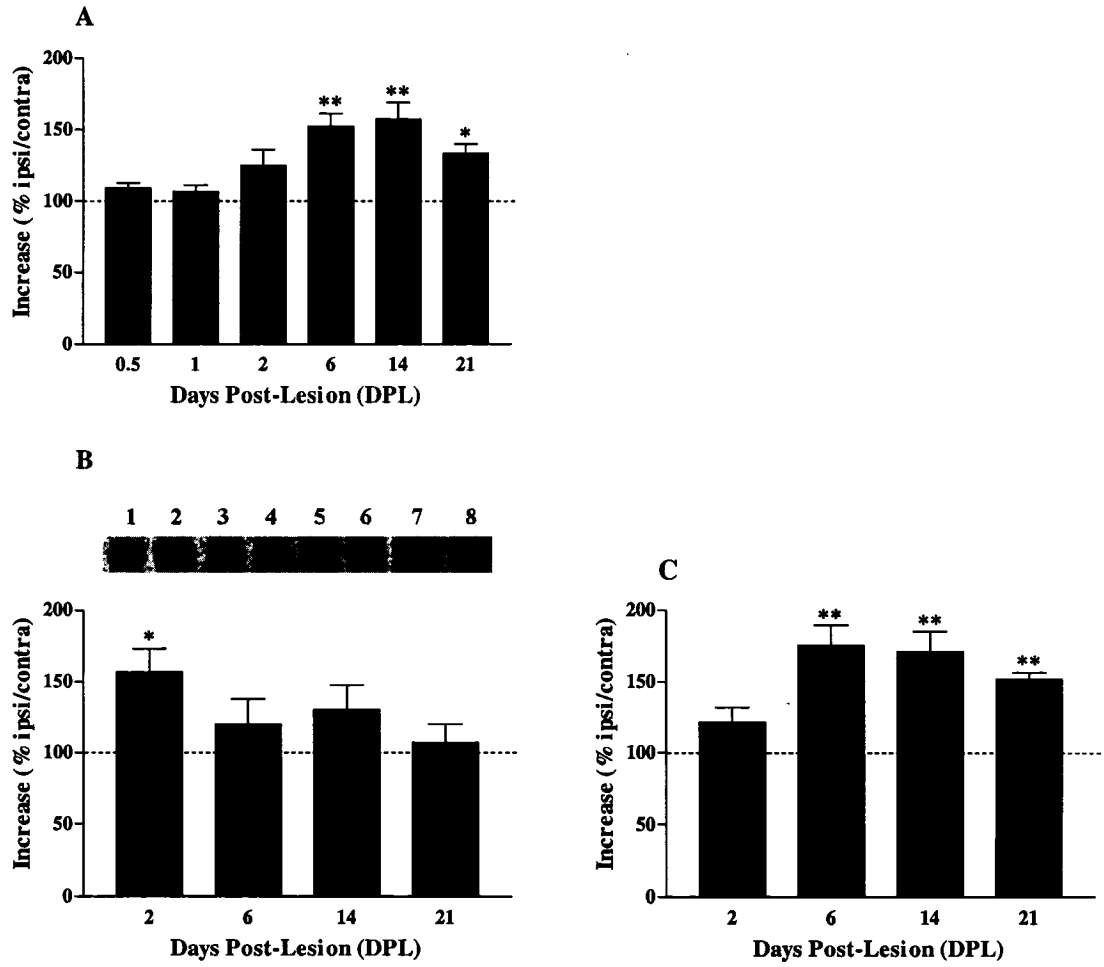

Figure 19. Syndecan-4 and glial proliferation are increased in hippocampus following ECL. Syndecan-4 mRNA and protein expression were measured by RT-PCR and Western blot respectively as described in the Methods. *A*, Syndecan-4 mRNA levels in hippocampus. Syndecan-4 levels were normalized to Actin levels and expressed as a ratio between the ipsilateral and contralateral side of the lesion. *B*, Representative Western blot of Syndecan-4 protein levels in hippocampus. *C*, Glial proliferation shown by GFAP protein levels in hippocampus. Protein levels were normalized to Tubulin levels and are expressed as a ratio between the ipsilateral and contralateral side of the lesion. Results are mean ± SEM of n = 5-10 animals. * $p < 0.05$, ** $p < 0.01$.

3.6 DISCUSSION

Lipoprotein lipase has an important role in cholesterol homeostasis as it was shown to selectively mediate the uptake of cholesterol esters from HDL3 particles in the periphery (Schorsch et al., 1997; Panzenboeck et al., 1997; Rinninger et al., 1998b). It is also HDL-like particles that are found in the brain (Fagan et al., 1999; LaDu et al., 2000b), however little is known about LPL function in the CNS. Our report suggests that LPL plays an active role in hippocampal remodeling and terminal reorganization in response to lesions of the entorhinal cortex area. This process involves the cell-surface, LPL-binding, proteoglycan syndecan-4.

Cholesterol has a relatively slow turnover rate in the brain compared to peripheral organs. Its concentration depends on several key regulatory events such as its synthesis, transport, binding, internalization, storage and degradation. Internalization of cholesterol via the cell-surface receptors leads to the suppression of HMG-CoA reductase expression and activity, the rate-limiting enzyme in cholesterol synthesis (Brown et al., 1986). In response to damage HMG-CoA reductase activity, is progressively suppressed in favor of lipoprotein internalization and degradation. Activity returns to control levels once regeneration is complete. This process is at the basis of the recycling of cholesterol released from the dying or degenerating neurons toward the surviving neurons undergoing synaptic replacement and dendritic remodeling using the apoE/LDL receptor pathway, in both the PNS and CNS (Goodrum, 1990; Poirier et al., 1993b; Goodrum et al., 1995). Cholesterol internalization via this pathway causes an increase in esterification (for storage purposes) and a downregulation of LDL receptor expression (Brown et al., 1986; Poirier et al., 1993b), consistent with a feedback inhibition (Poirier, 1994).

In the ECL mouse, hippocampal apoE levels increase after the lesion as it was previously reported in the PNS (Ignatius et al., 1986; Ignatius et al., 1987) and in the ECL rat (Poirier et al., 1991a; Poirier et al., 1991b). Its peak was observed 7 days prior to synaptophysin upregulation, a marker of synaptic integrity and plasticity. This observation is consistent with the fact that neurons undergoing dendritic remodeling and synaptic replacement send biological signals (such as IL-1, IL-6 or Aß (Das et al., 1994;

Bales et al., 2000; Petegnief et al., 2001)) to apoE-producing astrocytes to increase the lipoprotein production and secretion in the extracellular space.

Apart from its enzymatic role, LPL is also known to act as a bridge between lipoproteins and the cell surface, a role consistent with the notion that it is a key accessory protein to the apoE/LDL receptor pathway. To further examine this issue during both degenerative and reinnervation processes, the ECL model was used to map the time course and specificity of the LPL changes in the deafferented hippocampal area.

Quantitative real-time RT-PCR on isolated hippocampi reveals the LPL mRNA prevalence induction at DPL 2, during the most active portion of the deafferentation process. The absence of significant changes in hippocampus (CA3) LPL by *in situ* hybridization is not surprising considering that entorhinal cortex projects only modestly to the CA3 region. The upregulation seen by RT-PCR most certainly reflects the massive changes occurring in the dentate gyrus where *in situ* signal was too weak to be quantified. Moreover, real-time PCR amplification is a much more sensitive and selective technique as it relies on two different oligomers and is significantly less sensitive to mRNA degradation. LPL protein levels are also increased during the active degenerative phase, with a peak at DPL 2.

We also found that LPL mRNA levels are upregulated in the alvear pathway and external capsule during the degenerative phase. Glial cells (microglia and astrocytes) are responsible for the increased production of LPL in the deafferented zone. A similar process was described in the peripheral sciatic nerve crush injury model where local macrophages were shown to be responsible for increased LPL production and activity during the degeneration phase (Huey et al., 2002). The signal observed in the alveus and external capsule coincides with the path of entorhinal projections to the hippocampus (Groenewegen et al., 1982; Brothers et al., 1985; Deller et al., 1996; Dolorfo et al., 1998) and reflects the huge amount of lipid breakdown products generated by the catabolism of the myelin around the degenerating projections as described previously in the peripheral nerve crush injury model (Huey et al., 2002).

The results indicate that while the peak expression of the gene in the hippocampus is maximal at DPL 2, its impact on protein levels extends during the whole deafferentation period and the early phase of the reinnervation phase. The long lasting

effect on LPL protein levels could also be due, at least in part, to changes in translational regulation and reduced turn-over as shown before in other systems (Ong et al., 1989; Saffari et al., 1992; Ong et al., 1992; Klingenspor et al., 1996).

It was shown that LPL compensates for the defective function of apoE variants by binding to cell surface proteoglycans and lipoprotein receptors, thus mediating the entry of lipids into the cytoplasm (Mann et al., 1999).

To further characterize the possible interactions between the apoE pathway and LPL, we expanded the analysis to include the expression of three major binding sites for lipoproteins in the brain: the LDLR, LRP, apoER2 as well as the 39kD receptor-associated protein (RAP) which acts as a folding chaperone for lipoprotein receptors. ECL modulated the expression of LDLR for which we showed a significant decrease at DPL 6, resulting from cholesterol recycling from degenerating terminals. This is consistent with the knowledge that LDL receptor expression is downregulated following cholesterol internalization (Brown et al., 1975b). Levels of apoER2, LRP or RAP were found to be relatively unchanged when compared to control levels. The absence or modest modulation of these receptors in response to deafferentation and reinnervation lead us to investigate the possible involvement of cell-surface proteoglycans that are known to act as major LPL binding sites in certain situations *in vivo*.

The two major classes of cell-surface proteoglycans found in the brain are the integral membrane type syndecans and the GPI-linked glypicans. To date, no evidence has been obtained on the role played by glypicans in lipoprotein binding, possibly due to the placement of their heparan sulfate chains so close to the plasma membrane that large particles cannot approach for binding (Williams et al., 1997). In contrast, several lines of evidences suggest that syndecans can act as co-receptors for lipoprotein internalization through receptors of the LDL receptor family (Mulder et al., 1992; Mulder et al., 1993; Mann et al., 1999; Mahley et al., 1999). They can also serve as autonomous cell-surface receptors mediating the binding, association, internalization and degradation of LPL-enriched lipoprotein complexes (Fuki et al., 1997; Fuki et al., 2000a).

Syndecan-4 is the sole member of its subfamily to be specific for glial cells (Hsueh et al., 1999). Analyses of syndecan-4 protein levels in ECL mice revealed an increased expression coinciding with the peak expression for LPL. Syndecan-4 mRNA

induction peaked at DPL 6 and coincided with the increase in GFAP expression, consistent with its reported glial origin (Gall et al., 1979; Gehrmann et al., 1991b; Hailer et al., 1999). In contrast, protein levels were found to peak at DPL 2, nearly four days prior to astrocyte proliferation and mRNA induction. The increase in protein levels could be associated to early microglial proliferation (DPL 0 to 2) or post-transcriptional regulation as reported for another member of the syndecan subfamily, namely syndecan-1 (Yeaman et al., 1993; Sneed et al., 1994; Jiang et al., 1995). Another possibility could be an autocrine modulation by LPL to stimulate syndecan-4 expression in astrocytes. It was recently shown that overexpression of LPL or apoE in CHO cells significantly increased proteoglycan expression (Obunike et al., 2000). The exact mechanism regulating syndecan-4 expression yet remains to be identified.

In conclusion, we provide evidences supporting an involvement of LPL in the scavenging and recycling of cholesterol released from dying cells and degenerating terminals *in vivo*. This effect is most likely due to an HSPG-dependent mechanism requiring syndecan-4 expression. However, our results do not rule out the possibility that syndecan-4 acts as a co-receptor for one of the LDLR-family member. This potential new role for LPL in the normal and injured central nervous system will help us better understand how cholesterol homeostasis is regulated in response to damage and disease.

3.7 Acknowledgements

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4.0 STUDY 3

LIPOPROTEIN LIPASE POLYMORPHISMS ASSOCIATE WITH THE RISK AND SEVERITY OF ALZHEIMER'S DISEASE

Jean-François Blain, Nicole Aumont, Louise Théroux, Doris Dea and Judes Poirier

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4.1 FOREWORD

Polymorphisms in the lipoprotein lipase are reported to associate with differences in lipid levels in periphery (Chamberlain et al., 1989; Ahn et al., 1993; Chen et al., 1996; Anderson et al., 1999). Moreover they increase the risk of coronary artery disease (CAD) and associate with its severity (Wang et al., 1996; Anderson et al., 1999; Gambino et al., 1999). An association between AD risk and LPL polymorphisms in the coding region of the protein was reported (Baum et al., 1999) but was not reproduced by independent groups (Myllykangas et al., 2000; Retz et al., 2001; Martin-Rehrmann et al., 2002; Fidani et al., 2002). Moreover, a non-coding polymorphism was also reported to associate with AD (Scacchi et al., 2004).

Since CAD and AD share some pathological features (Sparks et al., 1990; Sparks, 1997) and that LPL is involved in lipoprotein metabolism in the injured brain (Study 2)(Blain et al., 2004), we wanted to know if the reported non-coding polymorphisms of LPL could also associate with AD risk and severity. To address these questions we genotyped LPL in two different cohorts of patients (clinically-diagnosed and pathologically-confirmed) and assessed biological markers in the brain of the pathologically-confirmed subjects.

4.2 ABSTRACT

Objective: To evaluate whether genetic variations in the lipoprotein lipase (LPL) gene, previously associated to lipid levels and coronary artery disease (CAD) risk and severity, were related to Alzheimer's disease (AD) risk and the presence of relevant biological markers. **Methods:** Clinically-diagnosed subjects (145 controls, 153 AD) and autopsy-confirmed patients (97 control, 153 AD) were genotyped for *Hind*III and *Pvu*II single nucleotide polymorphisms (SNPs) of LPL. Brain LPL mRNA levels, cholesterol levels, choline acetyltransferase activity, amyloid concentration, senile plaques and neurofibrillary tangles counts were measured and correlations with LPL SNPs were calculated. **Results:** SNPs did not associate with the disease risk in the clinically-diagnosed patients however, the H+ ($p = 0.0494$) and P+ ($p = 0.0297$) alleles associated with the risk in the autopsy-confirmed patients. Moreover, we report significant associations between the LPL genotype of the *Pvu*II polymorphism and mRNA expression ($p = 0.012$), brain cholesterol levels ($p < 0.01$), choline acetyltransferase activity ($p < 0.05$), neurofibrillary tangle ($p = 0.019$) and senile plaque ($p = 0.016$) number in autopsy-confirmed patients. **Conclusions:** Although genetic variation does not inherently increase the level of risk in clinical subjects, polymorphisms in the lipoprotein lipase locus correlate with that risk in autopsy-confirmed patients and exert a marked impact on the classical pathophysiological markers in the AD brains.

4.3 INTRODUCTION

There are more than 110 genetic loci have been associated with sporadic Alzheimer's disease (AD) (for list and review see (Finckh, 2003)). Interestingly, there is a large cluster of genetic markers involved in cholesterol homeostasis including among others: apolipoprotein (apo)E4, apoCI, ATP-Binding Cassette (ABC)A2, LRP, VLDL receptor and cholesterol 24-hydroxylase (also known as CYP46). Lipoprotein lipase (LPL; E.C. 3.1.1.34), also involved in cholesterol homeostasis, was shown to be involved in normal synaptic remodeling in the injured adult brain (Blain et al., 2004).

LPL is an enzyme with a dual role in lipid metabolism. It is involved in the hydrolysis of triglycerides on the surface of lipoproteins and acts as a ligand for the different lipoprotein receptors (Beisiegel et al., 1991; Kounnas et al., 1993; Takahashi et al., 1995; Medh et al., 1996; Tacken et al., 2000) and heparan sulfate proteoglycans (HSPGs) (for review see (Kolset et al., 1999)). It is distributed in numerous tissues including the brain, where it is mostly expressed in the hippocampus (Goldberg et al., 1989; Ben Zeev et al., 1990; Paradis et al., 2004a). In periphery, apoC-II acts as an essential cofactor for LPL activity (LaRosa et al., 1970; Havel et al., 1973), however it is not expressed in the brain (Zannis et al., 1985; Hoffer et al., 1993). Nonetheless, catalytically inactive LPL retains its bridging function and promotes the active uptake of lipoproteins (Merkel et al., 1998).

LPL expression is increased during the degenerative phase following peripheral nerve crush (Huey et al., 2002) and entorhinal cortex lesion (Blain et al., 2004) where it is suggested to play a role in lipid redistribution. It is also found associated with amyloid (Aβ) and apoE in senile plaques of AD brains (Rebeck et al., 1995). Single nucleotide polymorphisms (SNPs) in the coding region of LPL were shown to associate with the disease incidence in clinically-diagnosed AD subjects (Baum et al., 1999). However, these observations failed to replicate in three other independent studies (Retz et al., 2001; Martin-Rehrmann et al., 2002; Fidani et al., 2002). More recently, a *Hind*III SNP in intron 8 of the LPL gene was found to associate with the disease prevalence in a cohort of clinically-diagnosed subjects (Scacchi et al., 2004).

This *Hind*III SNP, as well as a *Pvu*II SNP found in intron 6 of the LPL gene, associate with levels of triglycerides, HDL-cholesterol, the incidence of diabetes as well as coronary artery disease (CAD) incidence and severity (Chamberlain et al., 1989; Ahn et al., 1993; Wang et al., 1996). Interestingly, AD and CAD share common pathological features such as senile plaques (SP) and neurofibrillary tangles (NFT) (Sparks et al., 1990; Sparks, 1997).

Based on these evidences we screened two different cohorts of AD patients (clinically-diagnosed and autopsy-confirmed) for associations between the intronic LPL polymorphisms, the AD risk and severity as measured by the neurofibrillary tangles and senile plaques number, choline acetyltransferase activity as well as brain cholesterol levels.

4.4 METHODS

4.4.1 Study populations

Diagnosis of the clinical cases was done at the McGill Centre for Studies in Aging, with approval by Institutional Review Board. Informed consent was obtained from all patients. They all had a clinical diagnosis of probable AD and most were in stage 3 or 4 of the Reisberg global deterioration scale. Controls consisted of healthy spouses of AD patients and elderly volunteers. Human frontal cortex tissue samples from 153 autopsy-confirmed AD patients and 95 age-matched healthy controls were from the Douglas Hospital Research Centre Brain Bank (**Table 1**). Neuropathological analyses were consistent with the criteria used in the classification of Khachaturian (Khachaturian, 1985).

Table 1. Population characteristics.

	Clinically-diagnosed cases		Autopsy-confirmed cases	
	Control subjects (n = 145)	**AD patients (n = 153)**	**Control subjects (n = 97)**	**AD patients (n = 153)**
Age, years	75.9 ±5.8	77.8 ±5.7	75.3 ±9.1	76.6 ± 8.1
Sex, n(%) Female	95 (0.655)	92 (0.601)	34 (0.350)	74 (0.483)
MMSE score	>28	18.67 ± 5.55	---	---
APOE*2 frequency	---	---	0.06	0.03
APOE*3 frequency	---	---	0.81	0.64
APOE*4 frequency	---	---	0.13	0.33

MMSE: Mini-mental state examination

4.4.2 Lipoprotein lipase genotyping

DNA was extracted from brain tissue using DNeasy tissue kit (QIAGEN Inc., Mississauga, ON). DNA was subjected to PCR as described before (Ahn et al., 1993) with minor modifications. Briefly, amplification of a 1239bp *Hind*III restriction fragment was carried out for 35 cycles (1:30min at 95°C, 2:30min at 50°C and 3:00min at 72°C) using the following primer pairs: Forward 5'- TTT AGG CCT GAA GTT TCC AC-3' and Reverse 5'- CTC CCT AGA ACA GAA GAT C -3'. Amplification conditions for the 854bp *Pvu*II fragment were the same except for the annealing temperature which was set at 62°C. Primer pairs were as follows: Forward 5'- TAG AGG TTG AGG CAC CTG TGC -3' and Reverse 5'- GTG GGT GAA TCA CCT GAG GTC -3'. PCR products were digested with *Hind*III or *Pvu*II overnight at 37°C and separated on gel. Presence of the restriction site (+ allele) resulted in the formation of 576 and 665bp fragments for *Hind*III and 268 and 586bp fragments for *Pvu*II.

4.4.3 Quantitative RT-PCR

RNA was extracted from frontal cortex samples using QIAGEN's RNeasy kit (QIAGEN Inc., Mississauga, ON). Primer pairs used for PCR amplification were as follows: LPL-fwd 5'- ATC CAG AAA CCA GTT GGG CA –3'; LPL-rev 5'- GCT GGT CCA CAT CTC CAA GTC –3'; Actin-fwd 5'- TCA CCC ACA CTG TGC CCA TCT ACG A –3'; Actin-rev CAG CGG AAC CGC TCA TTG CCA ATG G –3'. RT-PCR was performed using the SybrGreen method in a GeneAmp 5700 Sequence detection system from PE Applied Biosystems. Following comparison of the primer efficiencies, quantification could be made using the comparative C_t method that uses a mathematical model (Pfaffl, 2001).

4.4.4 Neuropathological analyses

Neurofibrillary tangles (NFTs) and senile plaques (SP) numbers were determined as previously described (Etienne et al., 1986) for the cortical and hippocampal areas and were consistent with the criteria used in the classification of Khachaturian (Khachaturian, 1985).

4.4.5 Choline Acetyltransferase activity

Brain tissue samples were homogenized and incubated for 15 min in buffer containing [^{14}C]-acetyl-CoA, as previously described in details elsewhere (Araujo et al., 1988).

4.4.6 Brain cholesterol determination

Frozen human brain sections weighing 30 to 60 mg were homogenized in 1ml PBS for 30 sec with Vibra-cell Processor then mixed. Extraction was done according to Folch *et al.* (Folch et al., 1957) using a water-methanol-chloroform (3:4:8 v/v/v) mix. After vortexing and centrifugation, the aqueous phase was removed and kept for DNA measurements (Labarca et al., 1980) and the organic phase washed with 0.2 volume of distilled water. After centrifugation, the organic phase was removed and evaporated under a nitrogen stream. Acetonitrile-isopropanol (50:50 v/v) was added to the dried extract and injected on HPLC.

HPLC analysis adapted from Vercaemst et al. (Vercaemst et al., 1989) was performed on a POLARIS system equipped with a Microsorb-MV 100-5μ C18 column with a Metaguard 4.6 mm Metasil 5μ ODS (Varian, Inc., Mississauga, ON, Canada). The wavelength detector was set at 210 nm. Cholesterol and heptadecanoate were eluted isocratically at a flow rate of 1.0 ml/min with acetonitrile-isopropanol (50:50 v/v). A linear relationship was obtained between the injected amount of cholesterol and the peak area. The standard solutions were prepared by dissolving each standard in mobile phase. The standard curve range was 10 to 40 μg of cholesterol and 30 μg of heptadecanoate was added as an internal control.

4.4.7 β-Amyloid ELISA

Total Aβ concentration was determined as previously described (Beffert et al., 1999c) using 100 μg of protein from brain samples homogenized in a buffer containing 5M guanidine. Coating antibodies used were R163 and R165 for $A\beta_{1-40}$ and $A\beta_{1-42}$ respectively (generous gift from Dr P.D. Mehta, New York Institute for Basic Research, Staten Island, New York) and detection antibody was a biotinylated-6E10 (Signet Laboratories Inc., Dedham, MA).

4.4.8 Statistical analyses

Allele frequencies and linkage disequilibrium were assessed using contingency table χ^2-test. Logistic regression analysis (Enter method) was used to examine the simultaneous effect of LPL genotype, apoE4, age and sex on the risk of AD. Cholesterol concentration was compared using one-way ANOVA. The effect of genotype on the other biological markers (mRNA, ChAT activity, SP and NFT) was tested by Pearson's correlation. All the analyses were performed using SPSS 11.5 and GraphPad 3.0.

4.5 RESULTS

4.5.1 Polymorphisms and AD risk

Genotype distribution was in Hardy-Weinberg equilibrium for all SNPs except for a deviation of the *Pvu*II SNP in the AD group (Pearson's $\chi^2 = 6.158$; df =1; p = 0.013). *Pvu*II and *Hind*III SNPs were in strong linkage disequilibrium ($p < 0.0001$) as shown in **Table 2.**

No association between disease frequency and allele or genotype for either SNPs could be found for the clinically-diagnosed cohort of AD patients (**Tables 3 and 4**). Two-sided Pearson's chi-square analysis revealed that the P+ allele was more frequent in the AD group of the autopsy-confirmed cohort ($\chi^2 = 4.724$; df =1; p = 0.0297) (**Table 3**). This was also true for the H+ allele ($\chi^2 = 3.862$; df =1; p = 0.0494) (**Table 4**). The age and sex adjusted odds ratio (OR) for the risk of AD in homozygous carriers of the P+ allele was 2.59 (95% C.I.: 1.217 – 5.519) and 4.8 (95% C.I.: 2.428 – 9.489) for the presence of at least one allele of apoE4 (**Table 5**). There is no synergistic effect on AD risk between the P+ allele and apoE4 as demonstrated by the lack of significant interaction between the two (Wald $\chi^2 = 2.103$; df =1; p = 0.147). No significant association was found for H+ homozygous patients (Wald $\chi^2 = 0.289$; df =1; p = 0.591) even though presence of apoE4 remained highly significant (OR = 2.985; 95% CI: 1.260 – 7.070, p = 0.013).

Table 2. Association between LPL PvuII and HindIII SNPs in autopsy-confirmed cases.

	H-H-	H+H-	H+H+
P-P-	11 (5.3)	24 (19.5)	26 (36.2)
P+P-	8 (8.8)	41 (32.3)	52 (59.9)
P+P+	2 (6.9)	12 (25.2)	65 (46.9)

Observed number of cases (expected number) calculated by the model for the null hypothesis that LPL *Pvu*II and *Hind*III genotypes are independent. $\chi^2 = 30.9$, $p < 0.0001$. The (+) sign represents the presence of the cutting site.

Table 3. LPL PvuII genotype and allele distribution in control subjects and AD patients.

LPL genotype	Clinically-diagnosed cases, n(%)		Autopsy-confirmed cases, n(%)	
	Control subjects (n = 145)	AD patients (n = 153)	Control subjects (n = 97)	AD patients (n = 153)
P-P-	34 (0.234)	30 (0.196)	29 (0.299)	36 (0.235)
P+P-	73 (0.503)	73 (0.477)	45 (0.464)	60 (0.392)
P+P+	38 (0.262)	50 (0.327)	23 (0.237)	57 (0.373)
Statistics	$\chi^2 = 1.67$; p = 0.43		$\chi^2 = 5.06$; p = 0.07	
Allele frequency				
P-	0.486	0.435	0.531	0.431
P+	0.514	0.565	0.469	0.569
Statistics	$\chi^2 = 1.594$; p = 0.21		$\chi^2 = 4.724$; p = 0.0297 OR= 1.49; 95%C.I. 1.039 – 2.142	

Table 4. LPL HindIII genotype and allele distribution in control subjects and AD patients.

LPL genotype	Clinically-diagnosed cases[a], n(%)		Autopsy-confirmed cases[a], n(%)	
	Control subjects (n = 144)	AD patients (n = 152)	Control subjects (n = 95)	AD patients (n = 146)
H-H-	14 (0.097)	7 (0.046)	12 (0.126)	9 (0.061)
H+H-	55 (0.382)	65 (0.428)	32 (0.337)	45 (0.308)
H+H+	75 (0.521)	80 (0.526)	51 (0.537)	92 (0.631)
Statistics	$\chi^2 = 3.11$; p = 0.21		$\chi^2 = 3.75$; p = 0.15	
Allele frequency				
H-	0.288	0.260	0.295	0.216
H+	0.712	0.740	0.705	0.784
Statistics	$\chi^2 = 0.597$; p = 0.44		$\chi^2 = 3.862$; p = 0.0494 OR= 1.59; 95%C.I. 0.9996 – 2.308	

[a]Some samples could not be amplified explaining the slightly lower number of cases.

Table 5. Association between APOE*4 allele and LPL PvuII genotype in autopsy-confirmed AD cases.

Independent variable	Odds ratio	95% Confidence Interval	P value
*LPL*P+P+* genotype	2.6	1.217 – 5.519	0.014
*APOE*4*	4.8	2.428 – 9.489	< 0.0001
Age	---	---	0.442
Sex	---	---	0.139

Logistic regression was performed using the Enter method.

4.5.2 PvuII SNP on LPL expression

Using real-time quantitative RT-PCR we measured a 1.42-fold increase of LPL mRNA in AD brain compared to control. Two-tailed Pearson correlation analysis revealed a positive correlation between the P+ allele and mRNA concentration in AD (Pearson correlation = -0.349; p = 0.012, n = 51; **Figure 20**) which is not observed in controls (Pearson correlation = -0.019; p = 0.920, n = 30; not shown).

4.5.3 PvuII SNP and biological markers

Severity of AD pathology can be assessed in autopsy-confirmed cases by the measurement of characteristic biological markers. Correlation with these biological markers was assessed for the different LPL genotypes at the *Pvu*II SNP. Analysis of variance revealed that frontal cortex cholesterol concentration was significantly lower in the presence of the P+ allele in AD brains (F = 5.688; p = 0.006, n = 55; **Figure 21**) but not controls (F = 0.167; p = 0.847; n = 32; not shown). There was also an inverse correlation between P+ allele and ChAT activity in the whole population (Pearson correlation = -0.342; p = 0.048, n = 34) that was increased in the AD patients but did not quite reach significance (Pearson correlation = -0.399; p = 0.101, n = 18; **Figure 22**). NFT number in the fusiform gyrus (Fu) and parietal cortex (PCx) of AD brains positively correlated with the presence of the P+ allele (Fu: Pearson correlation = 0.325; p = 0.019, n = 52; PCx: Pearson correlation = 0.274; p = 0.047, n = 53). This was also true for SP number in the Fu and a trend toward significance was observed in PCx (Fu: Pearson correlation = 0.333; p = 0.016, n = 52; PCx: Pearson correlation = 0.239; p = 0.086, n = 53; **Figure 23**). Interestingly, there was absolutely no correlation between the LPL genotype and $A\beta_{40}$ (Pearson correlation = 0.043; p = 0.848, n = 22) nor $A\beta_{42}$ (Pearson correlation = -0.085; p = 0.699, n = 23) levels. Due to the low occurrence of the H-H- genotype, correlation analyses could not be conducted for the *Hind*III SNP and these biological markers.

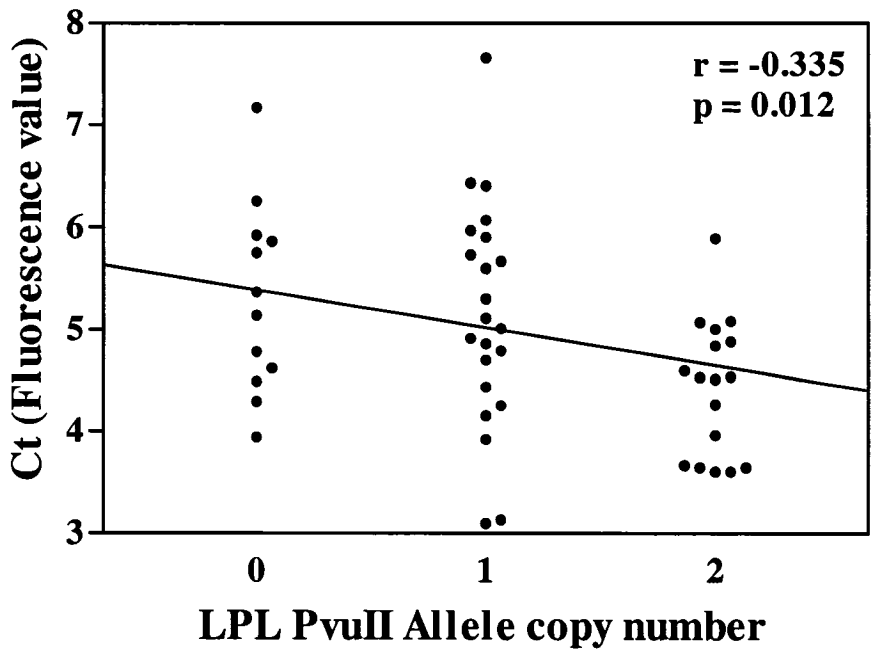

Figure 20. Gene-dose relationship between the PvuII genotype of LPL and mRNA concentration in the frontal cortex of AD patients. Measures of mRNA are expressed as *Ct* which represents a fluorescence emission value. Note that lower *Ct* values correspond to higher mRNA expression.

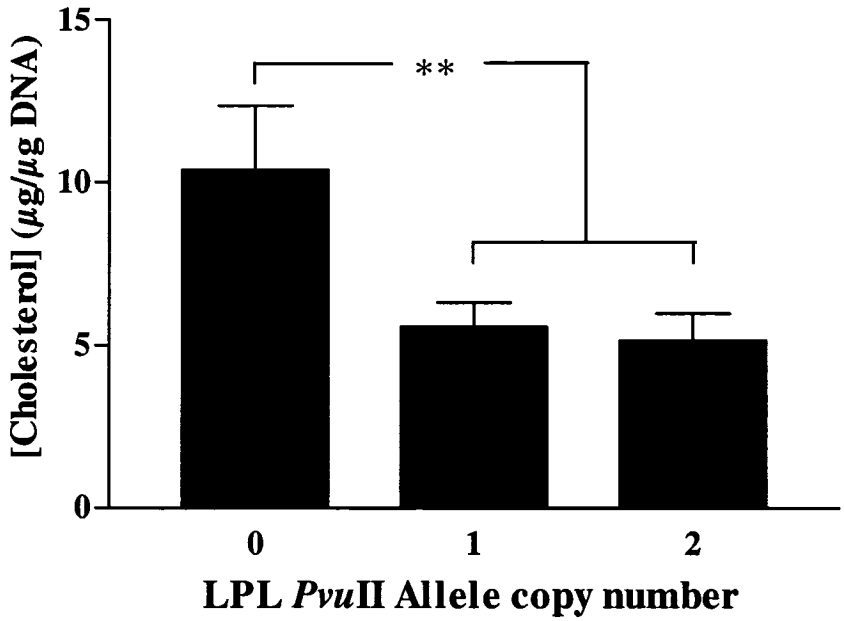

Figure 21. Frontal cortex cholesterol concentration in AD according to LPL PvuII genotype. Cholesterol was measured by HPLC. Each bar represents mean ± SEM of 14 (0), 22 (1) and 19 (2) patients. ANOVA: F = 5.688; ** p = 0.0058.

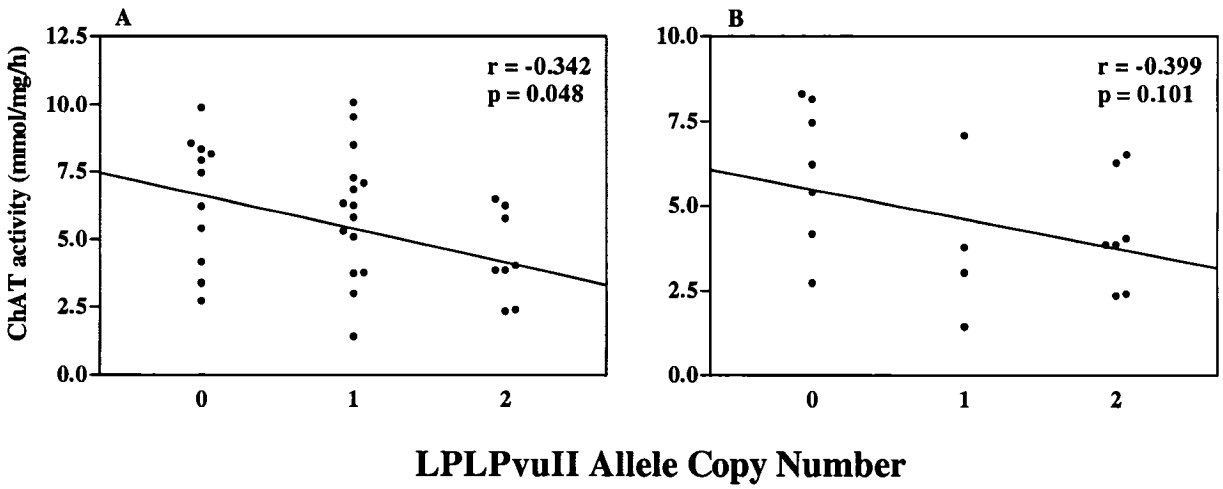

Figure 22. Gene-dose relationship between the PvuII genotype of LPL and hippocampal choline acetyltransferase activity. A) Whole population and B) AD patients only.

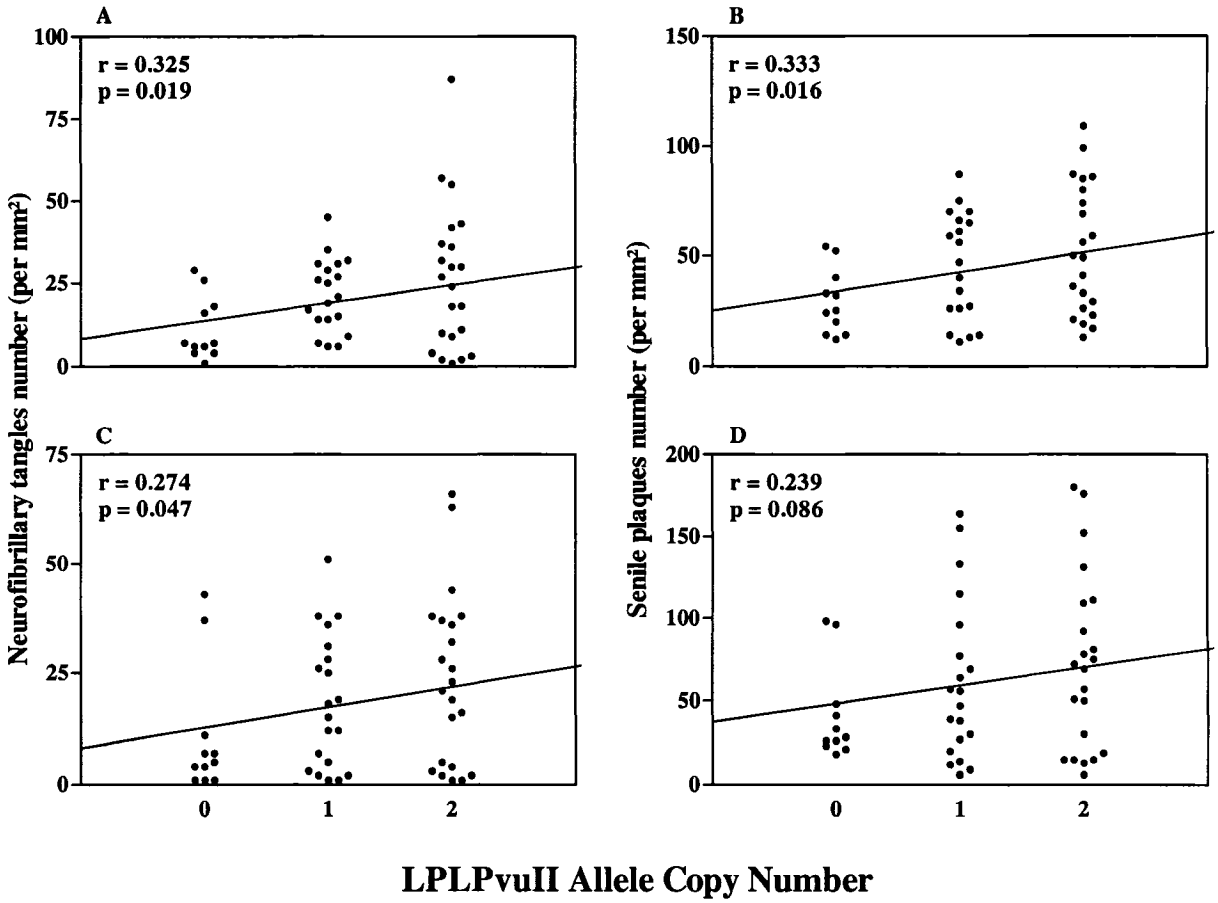

Figure 23. Gene-dose relationship between the PvuII genotype of LPL, NFTs and senile plaques. Neurofibrillary tangle number (A, C) and senile plaque number (B, D) in the fusiform gyrus (A, B) and the parietal cortex (C, D).

4.6 DISCUSSION

Lipoprotein lipase *Hind*III and *Pvu*II intronic polymorphisms were shown to associate with lipase deficiency (Gotoda et al., 1989), increased levels of blood triglycerides, lower HDL cholesterol as well as coronary artery disease risk and severity (Chamberlain et al., 1989; Mattu et al., 1994; Wang et al., 1996). Moreover, the H+ allele of the *Hind*III polymorphism associates with increased common AD risk in a clinically-diagnosed cohort of patients (Scacchi et al., 2004).

In this study, as well as reproducing the association of the *Hind*III SNP of LPL with the disease risk, we report for the first time an association between the *Pvu*II SNP of LPL, the disease risk and also biological markers as indicators of the disease severity.

It is striking that the polymorphisms analyzed only associate with the disease in the autopsy-confirmed cohort of patients. However, it is well accepted that about 10-15% of AD clinical diagnoses can be attributed to other types of dementia (e.g. Lewy bodies, vascular dementia...). Neuropathological confirmation of AD allows to reject these cases, creating pathophysiological homogeneity within the group. What is also of high interest is that the polymorphisms do not only associate with AD prevalence but also with biological markers that are strong indicators of AD's brain pathology. The low prevalence of the H-H- polymorphism does not allow for correlation analysis but we observed a trend for association between the H+ allele and all the AD markers analyzed (Blain & Poirier unpublished observations). On the other hand, there is a clear gene-dose relationship between the P+ allele (presence of the *Pvu*II cutting site) and the different biological markers of AD.

It is not clear how an intronic polymorphism can influence the biological activity of LPL leading to a modification the disease state. However, in this particular case, the *Pvu*II restriction site (5'-CAGCTG-3') is a consensus sequence for the E-box DNA motif. This particular motif is usually found in the promoter region and in enhancers. It binds transcription factors of the basic helix-loop-helix family (Blackwell et al., 1990) leading to induction or repression of genes. Interestingly, we report that AD brains express more LPL mRNA than control cases and that this increase is associated in an allele dose-dependent fashion to the presence of the *Pvu*II restriction site (P+ allele) of

LPL. The P+ allele keeps the E-box sequence intact suggesting that its presence could induce the expression of LPL. On the other hand, increased LPL mRNA expression was also observed following hippocampal deafferentation and found to be associated with the presence of proliferating glial cells (Blain et al., 2004). Thus we can not rule out the possibility that the gliosis observed in AD brain could also be responsible, at least in part, for the increased mRNA expression.

In periphery the P+ allele of LPL is associated with higher blood triglycerides and lower levels of HDL cholesterol (Chamberlain et al., 1989; Mattu et al., 1994; Wang et al., 1996). The brain contains virtually no triglycerides and produces lipoprotein particles of the HDL-like type (Fagan et al., 1999; LaDu et al., 2000b). However, in this study the P+ allele correlated with levels of tissue free cholesterol and accounted for 17.8% of the variance observed. Since lipoproteins transport esterified cholesterol, lower tissue cholesterol is more likely an indicator of degeneration rather than lipoprotein cholesterol. It is interesting to note that increased NFT number and decreased ChAT activity are also markers of degeneration and they both correlate in a gene-dose fashion with the P+ allele.

Decreased neuronal membrane cholesterol enhances Aβ production (Abad-Rodriguez et al., 2004), a characteristic feature of AD. We did not find any correlation between LPL polymorphisms and Aβ levels however, the correlation is strong with the number of senile plaques. It was already shown that LPL could be found associated with apoE and amyloid in senile plaques (Rebeck et al., 1995). LPL is a ligand for different receptors of the LDL receptor family thus it seems possible that it could also interact with Aβ to mediate its internalization alone or in concert with apoE.

The function of LPL in the brain is still not very clear. It is proposed to help bridging lipoproteins to cell-surface receptors, act as transport for cholesterol and vitamin E and support survival and plasticity of neuronal networks (Ben Zeev et al., 1990; Rinninger et al., 1998b; Blain et al., 2004; Paradis et al., 2004a). Our results suggest that carrying the P+ allele of LPL increases the risk and severity of AD. Although of weaker strength, this association is similar to what is observed for apoE4 and AD. In the AD brain there is an imbalance toward Aβ production that seems to saturate its potential clearance mechanisms. ApoE is clearly involved in removal or deposition of Aβ and our results combined with others (Rebeck et al., 1995) suggest a role for LPL as well. As the

P+ allele associates with increased LPL expression, the combined higher Aβ production could favor amyloid deposition and increased plaque number. More investigations will be necessary to elucidate the exact role of LPL in the pathogenesis of AD and its possible interaction with other key players regulating cholesterol homeostasis such as apoE and its receptors.

4.7 ACKNOWLEDGEMENTS

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5.0 GENERAL DISCUSSION

It is well accepted that Alzheimer's disease (AD) is a multi-factorial disease with genetic and environmental components. In the recent years, the body of evidence linking cholesterol homeostasis to AD grew significantly and highlighted its central role in the development of the disease. Whether it's the presence of the ε4 allele of apoE (Corder et al., 1993; Poirier et al., 1993a; Strittmatter et al., 1993a), blood cholesterol levels and Aβ deposition (Sparks et al., 1990; Sparks et al., 1994), increased levels of brain caveolin-1 (Gaudreault et al., 2004), cholesterol-dependence of Aβ production (Bodovitz et al., 1996; Simons et al., 1998) or the protective effect of cholesterol-lowering drugs (Jick et al., 2000; Wolozin et al., 2000; Poirier et al., 2002), a link between lipoprotein metabolism, cholesterol homeostasis and AD pathophysiology seems to get confirmed.

In the brain, the most cholesterol-rich organ of the body, cholesterol is thought to be located either in myelin or cell membranes. The flux of cholesterol across AD brain is higher than in healthy brain suggesting that a net movement of cholesterol between brain cells occurs *in vivo* (Lutjohann et al., 2000). In fact, in a neurodegenerative disorder like AD, the brain replaces the lost input connections by new ones made with surrounding healthy neurons that undergo a process called reactive synaptogenesis or sprouting (Lynch et al., 1972; Lynch et al., 1973; Geddes et al., 1985; Cotman et al., 1988). Using the ECL model in the rodent as a reactive synaptogenesis model, it has been shown that when sprouting occurs, a huge amount of apoE is secreted by astrocytes in order to deliver cholesterol as the building blocks for membrane synthesis (Poirier et al., 1993b), a phenomenon also observed following peripheral nerve injury (Ignatius et al., 1986; Snipes et al., 1986). Interestingly, reactive sprouting is impaired in AD patients carrying the ε4 allele of apoE (Arendt et al., 1997) and transgenic mice models (Buttini et al., 1999; White et al., 2001a).

The exact mechanism(s) underlying the impaired synaptic replacement in ε4 carriers is(are) not yet understood but hypotheses have been put forward based on the current available data. The first one pertains to the concentration of apoE found in the brain according to the specific phenotypes. Indeed, apoE concentration is found to decrease in an ε4 allele-dose fashion in periphery (Utermann et al., 1980; Boerwinkle et

al., 1988) and in the CNS (Bertrand et al., 1995; Beffert et al., 1999c). Lower apoE concentration in ε4 carriers could lead to inefficient cholesterol transport for membrane synthesis and repair, loss of plasticity and ultimately, loss of synaptic integrity (Poirier et al., 1995b). Consistent with these observations is the age-dependent neurodegeneration observed in the brain of APOEko mice (Masliah et al., 1995) and the associated memory deficit (Gordon et al., 1995; Masliah et al., 1997) rising from the procedural component of the memory task (Champagne et al., 2002).

The second hypothesis suggests that the impairments associated to apoE4 are rather a gain of negative function (Nathan et al., 1994; Teter et al., 2002b) or loss of protective function associated with apoE2 and E3 (Rebeck et al., 2002). Indeed, apoE4 reduces neurite outgrowth *in vitro* (Nathan et al., 1994), a phenomenon explained by a lower binding ability of apoE4 to microtubules, leading to their depolymerization and destabilization of the cytoskeleton (Nathan et al., 1995). Experiments using organotypic hippocampal slices from human apoE transgenic mice reveal that mossy fibers sprouting is isoform-specific (E3 > E4) (Teter et al., 1999a) and that increasing apoE expression (using animals expressing one or two *APOE* genes) leads to an increased sprouting with apoE3 and a further decrease with apoE4 (Teter et al., 2002a). Moreover, the presence of apoE4 further increases the inhibition of LTP caused by oligomeric $A\beta_{42}$ compared to apoE2 and apoE3 (Trommer et al., 2005).

Finally, apoE also influences Aβ catabolism. Indeed, in AD brain the number of ε4 alleles correlates with $A\beta_{40}$ accumulation (Beffert et al., 1999c) as well as senile plaque density (Schmechel et al., 1993; Beffert et al., 1996). Increased plaque density is also observed in non-demented individuals carrying the ε4 allele (Sparks et al., 1996). Interestingly, increasing apoE by high cholesterol diet in mice (measured in brain tissue) (Howland et al., 1998) or Probucol treatment in humans (measured in CSF) (Poirier, 2003), reduces brain Aβ levels, arguing for an effect related to apoE concentration differences between ε3 and ε4 carriers. On the other hand, *in vitro* studies report apoE isoform-specific effects on Aβ clearance (Yang et al., 1999; Beffert et al., 1999b) while transgenic mice show an age and genotype-dependent deposition of Aβ (Holtzman et al., 2000). Since apoE levels in these models are similar between genotypes, a direct effect

of the isoform may also modulate Aβ accumulation. This isoform-specific effect is consistent with what we report in 'Study 1' using the human apoE-TR mice.

Considering that detrimental effects are consistently associated with apoE4 and that they do not necessarily associate with differences in apoE concentration, it is tempting to hypothesize that the presence of arginine (Arg) at both site 112 and 158 may cause apoE4 to interact differently with other proteins (e.g. cell-surface receptors) thereby causing a 'gain of negative function'.

How can we reconcile this detrimental effect of apoE4 in humans and the fact that it is also the ancestral form expressed in all non-human primates and rodents used for research today? Structure analysis of apoE has revealed that while Arg112 and 158 are conserved between species, the presence of an Arg in position 61 in humans compared to a threonine (Thr) in rodents and non-human primates causes a shift in lipoprotein preference (Dong et al., 1994). Indeed, the presence of Arg112 causes Arg61 to interact with a glutamic acid in position 255 creating a conformation that is different from the one observed for apoE2, E3 and apoE from rodents and non-human primates (Dong et al., 1996a). This difference in conformation is proposed to mediate some of the effects associated with apoE (Mahley et al., 2000). Creating a human apoE-TR mouse with a mutated form of apoE4 expressing a non-charged residue (e.g. alanine) in place of Arg61 would help to clarify the effect of conformation on the role of apoE in neurobiology.

Other discrepancies regarding the 'gain of negative function' of apoE4 include its lack of association with AD in African populations (Osuntokun et al., 1995; Sayi et al., 1997; Corbo et al., 1999) and the possibility for ε4 carriers to reach old age (Schachter et al., 1994; Rebeck et al., 1994; Sobel et al., 1995; Slooter et al., 1998).

It is in fact interesting to note that while apoE4 prevalence is rather high in Nigerians (29.6%) (Sepehrnia et al., 1989) and even reaches 40.7% in Pygmies, AD prevalence is low in African populations compared to Western populations of the same ethnic background (Hendrie et al., 1995). Moreover, as opposed to Western populations, apoE4 is not a associated with risks of coronary artery disease (CAD) in African populations either (Sepehrnia et al., 1989). Corbo & Scacchi note that the ε3 allele is most frequent in "populations with a long-established agricultural economy" while the ε4 allele is higher in "populations where an economy of foraging still exists, or food supply

is scarce or sporadically available" (Corbo et al., 1999). This suggests that environmental factors such as lifestyle (e.g. diet) and longer life span of the Western populations might have rendered the ε4 allele a susceptibility factor for the development of complex diseases such as CAD and AD (Sepehrnia et al., 1989; Corbo et al., 1999).

As for the presence of the ε4 allele among the oldest, it is reported to decrease with age (Rebeck et al., 1994). The rare presence of the ε4 allele among healthy individuals 80 years and older (Corder et al., 1993; Poirier et al., 1993a) may be the result of a selection related to the higher CAD risk associated with apoE4 (Davignon et al., 1987). In any case, inheritance of apoE4 was rather shown to associate with a younger age of onset than whether one is predisposed to develop AD (Meyer et al., 1998; Breitner et al., 1999; Miech et al., 2002; Khachaturian et al., 2004). Remembering that apoE4 is a susceptibility factor rather than a cause of AD, survival of these ε4 carriers into old age in absence of dementia suggests that combination with environmental or other genetic factors is necessary for apoE4 to mediate its 'gain of negative function'.

5.1 FURTHER INSIGHT INTO APOE4 IMPAIRMENT OF REACTIVE SPROUTING

Transgenic mice models were developed to try and understand the isoform-specificity of apoE in neurobiology. In a first model, pronuclear injection of an 11.1 kb DNA restriction fragment (containing the whole apoE gene with regulatory sequences as well as the apoC-I and apoC-I' sequences) into APOEko single cell embryos leads to varying levels of transgene expression due to differences in chromosomal location and copy number (Xu et al., 1996). In a second model, the APOEko background is again used to express the different human apoE isoforms under the control of cell-specific promoters that target their expression in either astrocytes (GFAP-apoE) (Sun et al., 1998) or neurons (NSE-apoE) (Raber et al., 1998). Even though glial-specific expression of apoE may be physiologically relevant, neuronal-specific expression is not, as apoE is found in glial cell and its neuronal expression remains marginal and very controversial. While GFAP-apoE mice astrocytes can be used for *in vitro* experiments, a problem arises *in vivo* as these mice are bred on an APOEko background. Indeed, APOEko mice have very high plasma cholesterol levels (Zhang et al., 1992) which is a risk factor for AD (Jarvik et al., 1995; Notkola et al., 1998). In consequence, cell-specific expression of apoE in the brain

would complicate the interpretation of *in vivo* data. Moreover, apoE is also expressed in microglia and oligodendrocytes which do not express GFAP. Finally, a third model uses a gene-targeting method that takes advantage of homologous recombination to replace the mouse apoE by the human isoforms thus causing a 'knock-in' of the gene sequence (Sullivan et al., 1997; Knouff et al., 1999). The many advantages of this model over the others include: 1) the presence of the host's regulatory sequences that leave the biological feedback loop intact (Sullivan et al., 1997), 2) the temporal and spatial pattern of expression which are respected and 3) the isoform-specific reproduction of plasma cholesterol levels as observed in humans (Sullivan et al., 2004). Another attractive characteristic of this model is that, as opposed to what is observed in the human brain, apoE concentration in the brain of these mice is the same whatever the genotype (Sullivan et al., 2004). This makes it an excellent model to study isoform-specific effects of apoE without the concentration bias observed in human CNS and periphery.

In the second study of this work we characterized the time-course of hippocampal apoE expression following an entorhinal cortex lesion (ECL) in the mouse (Blain et al., 2004). We report that soon after deafferentation (days post-lesion (DPL) 2) apoE levels are significantly decreased on the lesioned side, indicative of cholesterol use for glial cells proliferation. Twelve days later (DPL 14) we observe a peak in apoE expression that coincides with the start of the reinnervation process, which is ultimately observable by DPL 21 when synaptophysin (a synaptic marker) reaches its peak of expression. By DPL 30-40, apoE expression is back to basal levels indicating that reinnervation is completed. With these data in hand, we undertook a series of experiments described in 'Study 1'. The experimental design took advantage of the availability of the human apoE-TR mouse model to study the effect of the different apoE isoforms following ECL at 3 different time-points representative of the degeneration phase (DPL 2), reinnervation (DPL 14) and post-reinnervation (DPL 30) periods, as determined in 'Study 2'.

In the absence of the genotype-related concentration bias reported in human brain (Bertrand et al., 1995; Beffert et al., 1999c), we observe an impairment in reactive synaptogenesis similar to the one reported in apoE4 AD subjects (Arendt et al., 1997) and in apoE4 transgenic mice (White et al., 2001a). While apoE levels are similar between genotypes during the degeneration and reinnervation phases, a sustained increase in brain

apoE concentration in hE4-TR mice is measured at a time-point where reinnervation is thought to be complete. This sustain of apoE over-expression combined to the sprouting impairment we report for hE4-TR is compatible with the proposed notion that apoE redistributes cholesterol and phospholipids to neurons undergoing reinnervation (Poirier et al., 1993b; Poirier et al., 1995b) a process that is obviously not completed in this strain of mice. However, as apoE4 is present in sufficient concentration to accommodate reinnervation (see hE3 vs. hE4 at DPL 14), it indicates that the impairment observed in hE4-TR mice is not only related to apoE's role in lipid transport.

Our results contrast with those of White *et al.* who have used transgenic mice expressing multiple copies of the human *APOE* gene (White et al., 2001a). While they conclude similarly on apoE4's adverse effect on reinnervation, the model they use has flaws that prevent from getting a clear answer on the mechanism by which it mediates its detrimental action. Indeed, the line of *APOE4* mice they use expresses more apoE than the *APOE3* line (White et al., 2001a) and this has been shown to have repercussions on neuronal sprouting (Teter et al., 2002a). Moreover, the time-course of apoE expression following ECL they report for wild type mice in another study is completely different than the one reported for the transgenic animals. Indeed, no increase in apoE levels is observed in order to support reinnervation in wild type mice (White et al., 2001b) while apoE increases transgenic mice (White et al., 2001a). As opposed to that, despite a small difference in magnitude, the time-course of apoE expression in our studies using wild type and apoE-TR mice is exactly the same. This suggests that random expression of human apoE in the mouse genome, the presence of human regulatory elements, as well as the variable apoE expression levels have a profound impact on the way the animals respond to brain injury.

Another group reports similar detrimental effects of apoE4 on neurodegeneration (Buttini et al., 1999; Buttini et al., 2000). Their results should however be taken with care because they do not specify the gender of the animals used. Indeed, using the same transgenic model, which specifically expresses apoE in neurons (NSE-apoE), apoE4-associated behavioral deficits have been reported only in females (Raber et al., 1998) whereas androgens seem to protect females against those deficits (Raber et al., 2002). This contrasts with what is reported for the model we use for our experiments. When

comparing similar behavioral tasks (e.g. Morris water maze), despite marginal gender effects the hE4-TR mice do not show the gender-specific memory deficits observed in NSE-apoE4 transgenic mice (Raber et al., 1998) when compared to hE3-TR mice (Grootendorst et al., 2005). These findings suggest that the non-physiological neuronal expression of apoE may be particularly susceptible to sex hormones control. This hypothesis is consistent with the reported regulation of neuronal-specific enolase (NSE) by gonadal steroids (Scouten et al., 1985). Interestingly, neuronal-specific expression of apoE under another type of promoter (Thy1-apoE) causes prominent axonopathy and a disruption of axonal transport (Tesseur et al., 2000b) as well as increased tau phosphorylation (Tesseur et al., 2000a), suggesting that neuronal apoE expression has adverse effects even in the absence of insult.

Considering apoE's isoform-specific Aβ clearance (Yang et al., 1999; Beffert et al., 1999b; Holtzman et al., 2000; Tokuda et al., 2000; Fryer et al., 2005), it is not surprising to measure an accumulation of $A\beta_{40}$ in hE4-TR mice hippocampus in the late phase of the reinnervation process. Crossing human apoE4-TR mice with mice over-expressing APP also results in an increased ratio of $A\beta_{40}/A\beta_{42}$ in the brain, indicative of $A\beta_{40}$ accumulation (Fryer et al., 2005). Recent evidence suggests that, as well as modulating clearance, apoE also has an isoform-specific role on Aβ nucleation and deposition, but not fibrillization (Dolev et al., 2004). Moreover, the reversal of Aβ accumulation in young GFAP-apoE mice over-expressing APP (Holtzman et al., 1999) is lost in an apoE isoform-specific fashion in old animals (Holtzman et al., 2000). Since no plaques can be observed during the time-course we used in our studies, further time points would have to be examined to see if accumulation of Aβ would result in deposition into plaques.

The synaptogenesis impairment in the hippocampus observed in hE4-TR mice is similar to that reported for APOEko mice (Champagne et al., 2005). In this study, the absence of apoE impacted on the astroglial response and compromised the reinnervation process that normally occurs in wild-type animals. Following ECL, glial cells become activated and migrate through the molecular layers of the hippocampus in order to clear degeneration debris (Rose et al., 1976; Gall et al., 1979; Bechmann et al., 1997). This glial response is mostly the result of an inflammatory reaction that is controlled by apoE

(Whitson et al., 1994; Miyata et al., 1996; Puttfarcken et al., 1997; Barger et al., 1997; Jordan et al., 1998; Hu et al., 1998b) or exacerbated in its absence (Laskowitz et al., 1997; Laskowitz et al., 1998; LaDu et al., 2001). Similar to the response previously reported for APOEko mice, the hE4-TR mice exhibit marked astrogliosis during the post-reinnervation phase and fail to show the typical layer arrangement seen in hE3-TR and wild-type animals.

Consistent with a postulated role for apoE during inflammation, we observe a delayed expression of the anti-inflammatory cytokine IL-1ra in hE4-TR mice. These findings suggest that the presence of apoE4 might lead to an exacerbated inflammatory response that is detrimental to reinnervation whereas, when under apoE3's control, inflammation is beneficial in supporting reinnervation.

Ophir *et al.* report that the absence of apoE, or the presence of apoE4 impairs astrocyte activation following lipopolysaccharide (LPS) challenge (Ophir et al., 2003). These results contrast with those from 'Study 1' as well as those from our lab using APOEko mice following ECL (Champagne et al., 2005). Indeed, Champagne *et al.* reported an increased astrogliosis in APOEko mice compared to wild type animals as soon as DPL 6 (Champagne et al., 2005), and the present study reports increased astroglial reactivity in hE4-TR mice (up to DPL 30) as well as an exacerbated inflammatory response. In their study, Ophir *et al.* report that apoE3 prevented LPS-induced microglial activation whereas apoE4, or the absence of apoE, did not. These findings are consistent with apoE's isoform-specific role in the modulation of microglial activation and pro-inflammatory cytokine release (Egensperger et al., 1998; Laskowitz et al., 2001; Chen et al., 2005). On the other hand, they report the exact opposite for astrocyte activation (apoE3 > apoE4 = APOEko) (Ophir et al., 2003). Considering that the first event in the inflammation cascade is microglial activation, which then leads to astrocyte activation, these results are hard to reconcile. Even though we do not report a direct measurement of microglial activation, we show that a delay of the anti-inflammatory response associated with microglia in hE4-TR mice is the cause of increased astrogliosis. One of the major differences between the two models is that treatment with LPS does not induce cell proliferation (Suzumura et al., 1991; Lee et al., 1994; Casal et al., 2001) whereas ECL does (Gall et al., 1979; Hailer et al., 1999).

Moreover, as opposed to their mouse model, ours recapitulates the spatial and temporal gene expression pattern of apoE as well as the cascade of cellular events that occurs following an insult to the brain.

Proposed apoE isoform-specific differences in the reactive synaptogenesis process are summarized in **Figure 24**. These differences suggest that albeit lower levels of apoE in ε4 carriers, the possession of this allele may also exacerbate the inflammatory reaction seen in AD brains. This hypothesis is consistent with *in vitro* studies reporting that apoE4 potentiates complement activation (McGeer et al., 1997), does not suppress brain inflammation following LPS treatment (Lynch et al., 2003), induces IL-1ra expression to a lower extent (Grocott et al., 2001) and induces PGE2 and IL-1β expression as well as NO release from microglia (Brown et al., 2002; Chen et al., 2005). In AD brains, microglial activation also correlates in a gene-dose fashion with the presence of the ε4 allele (Egensperger et al., 1998).

Two meta-analyses of epidemiological studies looking at the potential protective effect of non-steroidal anti-inflammatory drugs (NSAID) have been conducted (McGeer et al., 1996; Szekely et al., 2004). They both report an overall protective role for NSAIDs against the development of AD. The influence of apoE genotype on NSAIDs protective role is reported by three studies with two showing a more marked protective effect in ε4 carriers (Alafuzoff et al., 2000; Yip et al., 2005), while the third did not find differences between non-ε4 and ε4 carriers (Anthony et al., 2000). The stronger effect of NSAIDs in ε4 carriers is consistent with the exacerbated inflammatory response we report in hE4-TR mice as well as the one observed in AD brain. It is noteworthy that the HMGCR inhibitors statins, which were shown to have a protective role against AD (Jick et al., 2000; Wolozin et al., 2000), also have an anti-inflammatory potential which is independent of their cholesterol-lowering function (Stuve et al., 2003; Sun et al., 2003b; Cordle et al., 2005).

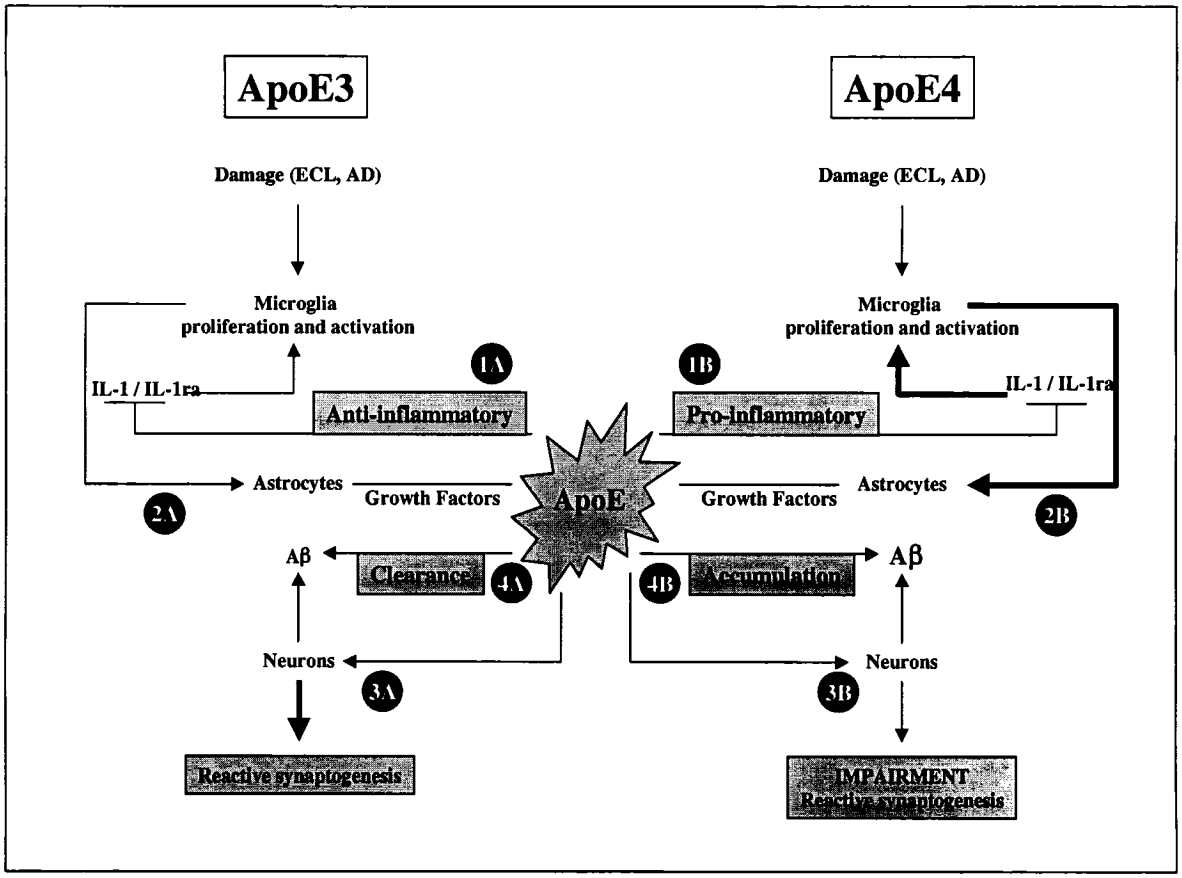

Figure 24. Isoform-specific effects of apoE in reactive synaptogenesis.

Microglial cytokine release (e.g. IL-1/IL-1ra) promotes astrocyte activation. ApoE released by activated astrocytes limits the inflammation in the case of apoE3 (1A) or exacerbates this state in the case of apoE4 (1B). Depending on the inflammatory state, astrocytes either start migrating (2A) or stay hyperactivated (2B). ApoE secretion is thus controlled to support reactive synaptogenesis (3A) and amyloid clearance (4A) or, in the case of apoE4, sustained in reaction to reactive sprouting impairment (3B) and amyloid accumulation (4B).

5.2 Is there more to it than apoE? A role for LPL.

Looking at the time-course of apoE expression in 'Study 2', it is interesting to note the significant decrease in apoE expression during the degeneration phase (Blain et al., 2004). Glial cells, which are the major apoE-producing cells in the brain, are known to undergo proliferation shortly after a lesion (Gall et al., 1979; Hailer et al., 1999). Since apoE expression is regulated by the cholesterol content of the cell (Mazzone et al., 1989; Zechner et al., 1991), the decrease we report is consistent with cholesterol utilization by proliferating glial cells, a phenomenon also reported for vascular smooth muscle cells (Majack et al., 1988).

With decreased apoE levels, internalization of cholesterol and phospholipids released from degenerating terminals would have to proceed through an alternative mechanism to the usual apoE/LDLR family pathway. It has been shown that activated glia could incorporate cell debris and lipid particles through phagocytosis (Cheng et al., 1994; Bechmann et al., 1997; Kluge et al., 1998). However, lipoprotein particles could also be internalized through an apoE-independent pathway involving other cell-surface molecules.

In periphery, LPL has been shown to modulate lipoprotein metabolism by two different mechanisms: 1) hydrolysis of Tg-rich lipoproteins and 2) binding and internalization of different lipoprotein classes through members of the LDLR family (Beisiegel et al., 1991; Chappell et al., 1992; Kounnas et al., 1993; Takahashi et al., 1995; Medh et al., 1996; Tacken et al., 2000) as well as the cell-surface proteoglycans (Saxena et al., 1991; Eisenberg et al., 1992; Mulder et al., 1992; Mulder et al., 1993). Both of these roles are not dependent on each other (Merkel et al., 1998) but the lipolytic role of LPL is dependent on the presence of the co-factor apoC-II (LaRosa et al., 1970; Havel et al., 1973; Ekman et al., 1975). Considering the virtual absence of triglycerides in the adult brain (LaDu et al., 1998) and that apoC-II is not synthesized in the brain of most mammals (Zannis et al., 1985; Datta et al., 1987; Lenich et al., 1988; Hoffer et al., 1993), it seems that a potential role for LPL in the brain would have to be related to its lipoprotein-binding capacity.

Consistent with this hypothesis, we observe an induction of LPL expression coinciding with that of the cell-surface, glia-specific proteoglycan syndecan-4 in the degeneration phase (DPL 2) (Blain et al., 2004). LPL is known to naturally bind to proteoglycans of the syndecan family (Fuki et al., 1997; Fuki et al., 2000a) and to promote internalization of subtypes of HDL particles similar to those found in the brain (Schorsch et al., 1997; Panzenboeck et al., 1997; Rinninger et al., 1998b). Our results thus suggest that the LPL-syndecan-4 pathway may be directly involved in cholesterol recycling. Alternatively, since LRP is also expressed on reactive astrocytes (Tooyama et al., 1993), syndecan-4 may act as its co-receptor to promote LPL-mediated recycling. Using the sciatic nerve crush model, Huey *et al.* proposed a similar role for LPL post-injury (Huey et al., 2002). However, as opposed to them, we do not observe any change in hippocampal LPL activity even with an external source of apoC-II (data not shown). In fact, the very low activity levels we measure (in presence of apoC-II) are consistent with those reported for the adult brain (Nunez et al., 1995).

The involvement of LPL during the degenerative phase following damage is also consistent with its increased expression in the AD brain undergoing sustained degeneration (see 'Study 3').

While LPL and apoE can increase binding of lipoproteins to cell surface in an additive fashion (Mann et al., 1999), apoE's presence is not necessary for the binding to occur (Tacken et al., 2000). LPL can even compensate for defective apoE variants causing type III HLP by favoring the interaction of lipoproteins with cell-surface receptors and proteoglycans (Mann et al., 1999). These observations suggest that the decreased apoE concentration reported following degeneration is not a limiting factor for cholesterol recycling through the LPL-Syndecan-4 pathway to occur (**Figure 25**).

A recycling mechanism utilizing internalization through syndecan-4 (or LRP-syndecan-4), and repackaging of cholesterol into newly synthesized lipoproteins would seem most plausible. However, the cell might see it as an unnecessary energy expense. Alternatively, lipoproteins could be docked at the cell surface, trapped by the interactions between LPL and syndecan-4. Synthesis of apoE and incorporation into these docked particles would then induce their release for redistribution to neurons. Depending on the lipidation state of the docked lipoproteins, ABCA1 might also come into play. Indeed, it

was recently reported that ABCA1 expression is necessary for normal apoE secretion by astrocytes (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). Thus, ABCA1 might be involved in the transfer of cholesterol to the docked lipoprotein particles. It is noteworthy that a link between ABCA1 and AD risk also exists (Wollmer et al., 2003; Katzov et al., 2004).

5.3 LPL IN AD: IS THERE MORE TO IT THAN LIPOPROTEIN METABOLISM?

Binding of LPL to heparan sulfate proteoglycans (HSPG) is mediated through its heparin-binding domains (Saxena et al., 1990; Saxena et al., 1991; Hata et al., 1993). It is noteworthy that proteins known to play a significant role in AD pathogenesis, namely apoE and Aβ, also have the ability to bind heparin and HSPG (Weisgraber et al., 1986; Mulder et al., 1992; Snow et al., 1994; Cotman et al., 2000). Moreover, HSPG, apoE and LPL are all found in senile plaques associated with Aβ (Rebeck et al., 1995; van Horssen et al., 2002).

As stated earlier, in 'Study 3' we report an association between AD and increased frontal cortex LPL mRNA levels. This association is genotype-specific for a polymorphism in LPL that also associates with the highest levels of LPL expression. Overall, the highest LPL levels always associate with the worst case scenario whether we look at AD risk, brain cholesterol concentration, choline acetyltransferase activity, neurofibrillary tangles or senile plaques number. Interestingly, LPL levels do not associate with Aβ concentration nor apoE genotype, even though they correlate with increased plaque number. It is thus tempting to suggest that LPL could promote Aβ deposition into senile plaques.

A common characteristic of LPL, apoE and Aβ is that they were all shown to bind to LRP that mediate their degradation (Beisiegel et al., 1989; Beisiegel et al., 1991; Chappell et al., 1992; Rebeck et al., 1995; Kounnas et al., 1995b). Indeed, *in vitro* studies report that LRP has a neuroprotective role by mediating Aβ clearance in conjunction with either apoE or α_2-macroglobulin (Narita et al., 1997; Du et al., 1998; Qiu et al., 1999; Kang et al., 2000; Van Uden et al., 2000). Interestingly, increased Aβ deposition is also observed in RAP-deficient mice which have a 75-80% decreased expression of LRP (Van Uden et al., 2002).

Our data strongly suggest that LPL is involved in cholesterol recycling through syndecan-4 (Blain et al., 2004), but do not rule out the possibility that this proteoglycan acts as a co-receptor for LRP. Moreover, LPL increases the binding of apoE-lipoproteins to LRP (Beisiegel et al., 1991). Considering this and the reported apoE-Aβ-LRP interactions, it seems plausible that, as well as modulating lipoprotein

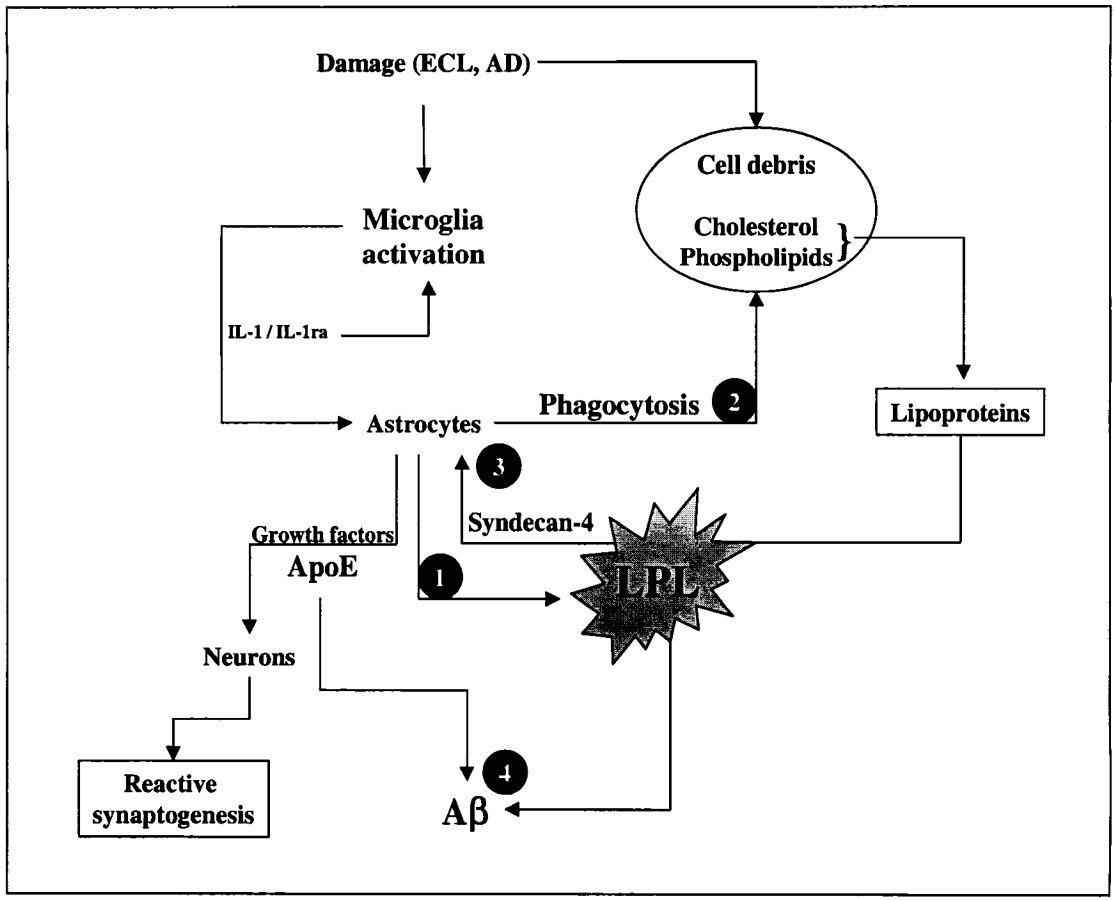

Figure 25. LPL functions in the brain in response to damage.

In response to degeneration, astrocytes increase their expression of LPL and syndecan-4 (1). As well as using phagocytosis for the recycling of terminal degeneration debris (2), we propose that LPL is involved in docking/internalization of the lipoproteins through a syndecan-4-mediated pathway (3). In sustained degeneration situations like AD, LPL together with apoE are also involved in clearance/accumulation of amyloid (4).

metabolism in the brain, LPL may also be involved in targeting Aβ for degradation through LRP. Its accumulation in senile plaques may be the result of a sequestration by the receptor. It is not yet known if LPL can bind to Aβ by itself. Since the genetic association between LPL and senile plaque number is independent of apoE genotype, it would be interesting to investigate if a direct LPL-Aβ interaction is possible without the participation of apoE.

5.3 ApoE and LPL: Potential Therapeutic Targets?

Understanding the role of the different players in AD pathophysiology will help for the development of new therapeutic strategies. While the association between apoE4 and AD has been known for more than a decade, the exact mechanism by which it confers such a risk is still not clear. Since it is the major risk associated with AD, it would seem to be a therapeutic target of choice. However, not knowing exactly how it mediates its adverse effects causes a barrier in the development of such apoE-based therapies. ApoE's primary role in the brain is to transport cholesterol for the support of synaptic plasticity (Poirier, 1994; Poirier et al., 1995b). However, it has also been shown to have a role in Aβ metabolism, neuroinflammation, oxidative stress and NFT formation. The leading hypothesis suggests that lower brain expression of apoE in ε4 carriers would impair the maintenance of synaptic plasticity (Bertrand et al., 1995; Beffert et al., 1999c). Based on these data, a proof-of-principle study using the non-statin cholesterol-lowering drug Probucol, which had been shown to up-regulate apoE expression (Aburatani et al., 1988), report an overall stabilization of symptoms in mild to moderate AD patients over the course of a 6 months treatment (Poirier et al., 2002). Interestingly, ADAS-Cog variation correlated with apoE expression levels and ε4 carriers did not respond as well to the treatment as non-ε4 carriers (Poirier et al., 2002). Having eliminated the concentration bias observed in humans, we report that apoE4 exacerbates neuroinflammation and causes Aβ to accumulate following ECL. Considering this, one might want to be careful in increasing apoE expression in ε4 carriers, as benefits related to synaptic plasticity maintenance may be overridden by the apoE4-related adverse effects. Accordingly, a negative effect of increased *APOE4* gene expression has been observed *in vitro* (Teter et al., 2002a; Teter et al., 2002b).

On the other hand, the choice of LPL as a therapeutic target is not as obvious as for apoE. Indeed, we report LPL's involvement both in cholesterol recycling (beneficial) and in amyloid deposition (detrimental). As opposed to apoE, LPL doesn't seem to play a major role in reinnervation but rather in degeneration, a result corroborated by the increased expression we report for AD brains. Based on the results we obtained from AD brains, it seems that the adverse effect of increased LPL levels (associated with the P+P+ genotype) dominates as it correlates with increased plaque numbers, increased NFTs and a lower ChAT activity. However, our results do not provide a direct proof that LPL is involved in Aβ deposition since protein-protein interaction between the two has not been reported yet. It is thus conceivable that LPL is a passive player in plaque formation in that it is present on lipoprotein particles and gets deposited together with apoE. On the other hand, LPL and Aβ both being heparin binding proteins, it is highly plausible that they interact together. If this interaction proved to occur, it will be imperative to see how it affects Aβ biology because it could either lead to internalization and degradation or deposition. Before rushing to any conclusion, more experiments need to be performed as targeting LPL expression, in order to modulate Aβ deposition, might also affect neuronal survival since it was shown to protect against oxidative insults (Paradis et al., 2003).

6.0 CONCLUDING REMARKS

With the three studies presented here, we advanced the knowledge of lipoprotein metabolism in the context of brain injury and Alzheimer's disease. While the prevailing hypothesis pertaining to the lower concentration of apoE associated with the ε4 allele is not refuted by our study, we provide new evidence showing that apoE4 may exacerbate the inflammatory reaction in the brain following a damage, that ultimately leads to an impairment of the reactive sprouting. Moreover, we report a new potential role for lipoprotein lipase and one of its receptor, syndecan-4, as cholesterol recycling molecules in neurodegenerative states. Finally, we observe a correlation between polymorphisms of the LPL and its expression levels as well as with AD risk and severity as measured by its classical biological markers. As a future direction of these study it would be interesting to see how LPL interacts with Aβ and the role of apoE in modulating this interaction.

This could have strong impacts on the way Aβ deposition is regulated and open new avenues for therapy.

Taken together these results highlight the importance of lipoprotein metabolism in 1) the maintenance of synaptic integrity in the brain 2) its underlying repair mechanisms and 3) its impact on amyloid metabolism.

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