A PUTATIVE ROLE FOR PCSK9 IN SYNAPTIC REMODELLING AND PLASTICITY IN RESPONSE TO BRAIN INJURY: IMPLICATIONS FOR ALZHEIMER'S DISEASE

By

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Acknowledgement

As a child, every dream you have seems realistic and achievable. The years go by and new ones appear but the most important dreams are the ones you stick to and eventually accomplish. In order to realise your dreams, guidance people will pass through your life, some will only cross your path but each of them, in their own way, will support you in the achievement of your goal. They will stand by your side, support you during the difficult moments when you think your dream becomes unrealistic and you feel like giving it up. They stand by you and make you realise that you have what you need to achieve your dream. This book is actually the accomplishment of a very long and demanding dream that has implicated the time, comprehension, patience, support and love of several members of my entourage. Therefore, every one of you mentioned below owns a little piece of that doctoral thesis…

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Abstract

Since the association of the ε4 allele of the apolipoprotein E (apoE) with Alzheimer's disease (AD) risk, growing evidences support a role for cholesterol metabolism in the pathophysiology of this disease. Many genes involved in lipid metabolism have now been studied and associate with the risk of AD. PCSK9 is a proprotein convertase recently identified as the third gene linked to familial hypercholesterolemia. It is a key regulator of plasma cholesterol concentrations by enhancing the degradation of cell surface low-density-lipoprotein receptor (LDLR). The present project derives from the global hypothesis that in the brain, PCSK9 may play a role in cholesterol homeostasis by regulating the expression of the LDLR proteins under normal and especially, neurodegenerative conditions.

A first study was conducted to evaluate potential variations in PCSK9 expression in the brain of autopsy-confirmed AD compared to age-matched control subjects. A genetic association study was also performed to determine the effect of five common PCSK9 polymorphisms on AD risk and modulation of gene expression. Using the entorhinal cortex lesion (ECL) model in a second study, a role for PCSK9 in reactive synaptogenesis was evaluated in response to brain damage in this *in vivo* paradigm in mice. A third study investigated in an *in vitro* model of reactive neuronal plasticity, the effect of PCSK9 on synaptogenesis and remodelling processes in response to neuronal injury.

The results show a cortical and hippocampal upregulation of PCSK9 expression in the brain of end-stages AD patients which do not result from the five studied genetic variants. No correlations were observed for PCSK9 with markers of AD pathology; suggesting an involvement of PCSK9 in response to neurodegeneration. Consistent with this idea, PCSK9 levels were increased during the active phase of neuronal membrane remodelling following ECL. *In vitro*, overexpression of PCSK9 in normal or neuronal-like cells undergoing post-injury reactive plasticity caused increased synaptic density which supports a role for PCSK9 in synaptogenesis and plasticity. While PCSK9 was found to negatively affect the LDLR levels in AD brains and both the LDLR and apoER2

during reactive plasticity *in vitro*, levels of the LDLR in ECL mice was not affected by PCSK9 but instead, together with apoE, levels were upregulated in the early phase of synaptic remodelling.

Together, these findings indicate that PCSK9 plays an important role in compensatory neuronal repair associated with age, brain injury or chronic degeneration as found in AD. Its expression in the brain possibly regulates cholesterol homeostasis and/or signalling pathways mediated by the apoE/LDLR pathway or other members of the LDLR family during axonal and synaptic remodelling. These findings are consistent with the relationship that exists between lipid homeostatic processes and AD pathology and indicate that PCSK9 may be a new player in the regulation of these processes that worths further investigation in a context of neurodegenerative disorders.

Résumé

Depuis la découverte que l'allèle ε4 de l'apolipoprotein E (apoE) est associé avec un risque plus élevé de développer la maladie d'Alzheimer (MA), un nombre grandissant d'études démontre que le cholestérol joue un rôle important dans les mécanismes pathophysiologiques reliés à cette maladie. Plusieurs gènes impliqués dans le métabolisme du cholestérol ont fait l'objet d'études et démontré une association génétique avec la MA. PCSK9 est une proprotein convertase qui récemment, fut identifiée en tant que troisième gène impliqué dans l'hypercholestérolémie familiale. Cette convertase est un régulateur principal des niveaux de cholestérol plasmatique par sa capacité à promouvoir la dégradation d'un récepteur de surface, le récepteur des lipoprotéines à faible densité (LDLR). Les travaux de ce projet sont basés sur l'hypothèse initiale que dans le cerveau, PCSK9 pourrait également jouer un rôle dans l'homéostasie du cholestérol en contrôlant l'expression protéique des récepteurs de LDL sous des conditions normales et plus particulièrement lors de conditions neurodégénératives.

Le premier volet expérimental avait pour objectif de comparer les niveaux d'expression de PCSK9 dans des cerveaux de patients décédés et identifiés à la MA ou considérés comme contrôles sains. De plus, une étude d'association génétique a été effectué afin de déterminer l'effet de polymorphismes de PCSK9 sur le risque de la MA ainsi que sur le contrôle de l'expression de PCSK9. Dans la deuxième étude, l'utilisation d'un modèle murin de lésion du cortex entorhinal a permis d'évaluer le rôle potentiel de PCSK9 dans des processus de synaptogénèse induits suite à un dommage neuronal. La troisième étude avait pour objectif de déterminer *in vitro*, les effets de PCSK9, lorsque surexprimé, sur le remodelage et plasticité neuronale en réponse à une lésion dans un modèle cellulaire.

Les résultats de ce projet ont démontré entre autre, une augmentation des niveaux d'expression de PCSK9 dans les régions du cortex frontal ainsi que l'hippocampe de cerveaux obtenus de patients Alzheimer en fin de maladie. Cette augmentation n'est pas causée par l'un des cinq variants génétiques de PCSK9 étudiés et aucune corrélation a été observé entre cette convertase et les marqueurs pathologiques caractérisant la MA; ce qui

suggère l'implication de PCSK9 en réponse à la neurodégénération. Conformément à cette hypothèse, les niveaux de PCSK9 sont également augmentés lors de la phase active de remodelage membranaire suite au dommage neuronal induit par le modèle de lésion du cortex entorhinal. *In vitro*, la surexpression de PCSK9 dans des cellules de type neuronal a causé une augmentation de la densité synaptique tant sous des conditions normales que lorsque les cellules sont activement en mode de réparation suivant la lésion, supportant ainsi un rôle pour PCSK9 dans les mécanismes de synaptogénèse et de plasticité synaptique. Alors que les niveaux de récepteurs aux LDL (LDLR) dans les cerveaux Alzheimer en plus des récepteurs de type apoER2 lors de processus de plasticité *in vitro* sont affectés de façon négative par PCSK9, les niveaux de LDLR suite à la lésion du cortex entorhinal ne semblent pas affectés par PCSK9 mais plutôt, parallèlement à apoE et PCSK9, sont augmentés dans la phase active de remodelage synaptique.

Globalement, ces résultats indiquent que PCSK9 joue un rôle important dans les mécanismes compensatoires de réparation neuronal associés au vieillissement, à un dommage cérébrale ou à des conditions chroniques de dégénération tel qu'observées lors de la MA. Son expression dans le cerveau réflète possiblement une régulation de l'homéostasie du cholestérol ou des voies de signalisation par le contrôle de récepteurs de surface aux LDL lors de remodelage membranaires et synaptiques en réponse à un dommage neuronal. Ces résultats sont en accord avec le lien existant entre les processus d'homeostasies lipidiques et les mécanismes pathologiques associés à la MA et indiquent que PCSK9 pourrait être un nouveau joueur participant à ce phénomène. Ainsi, PCSK9 mérite de plus amples investigations dans un contexte de maladies neurodégénératives.

Contribution to original knowledge

***Study 1*:** The results of this study demonstrated the first evidence of an increased cortical and hippocampal expression of PCSK9 in AD brain. Moreover, the results provide evidence of a specific association of PCSK9 rs2483205-C, rs483462-A and rs662145-T alleles with the risk of AD in the male sub-population. These findings represent the first report investigating PCSK9 functions in the human brain, genetic associations between PCSK9 polymorphisms with risk of AD and with landmark biological markers of the disease.

***Study 2*:** This study reports a novel role for PCSK9 in the adult brain in response to injury. Results demonstrated a hippocampal upregulation of PCSK9 during the active phase of reinnervation in an *in vivo* mouse model of neuronal compensatory response to damage. Moreover, the data showed that PCSK9 upregulation temporally coincides with peak increases in apoE and LDLR levels during the early phase of synaptic repair in the deafferented hippocampus; suggesting a putative role for PCSK9 in the regulation of cholesterol homeostatic processes.

***Study 3*:** The results of this study report the first evidence of an increased synaptic density when PCSK9, a potent regulator of plasma cholesterol, is overexpressed in neuronal differentiated PC12 cells. Furthermore, PCSK9 overexpression correlates with the reduction of both LDLR and apoE type 2 receptor protein levels during synaptic recovery in response to mechanical-injury of differentiated PC12 cells. This novel report documents for the first time the effects of PCSK9 overexpression on synaptogenesis and synaptic remodelling *in vitro* using a cellular model of reactive compensatory plasticity.

Contribution of authors

***Study 1*:** Stéphanie Bélanger Jasmin, Louise Théroux, Doris Dea and Judes Poirier. **Increased expression of proprotein convertase PCSK9 in late-onset Alzheimer's disease brain.** Submitted for publication to *Neurobiology of Aging*, 2010.

Dr. Judes Poirier supervised the research project. Louise Théroux prepared brain tissues and provided assistance with the genotyping. Doris Dea provided assistance and participated to the mRNA purification and RT-PCR. The first author elaborated the hypothesis of this work, carried out the work, analysed the data and wrote the manuscript.

***Study 2*:** Stéphanie Bélanger Jasmin, Vanessa Pearson, Louise Théroux and Judes Poirier. **PCSK9 is involved in post-injury synaptic remodelling.** Submitted for publication to *European Journal of Neuroscience*, 2010.

Dr. Judes Poirier supervised the research project. Vanessa Pearson participated in the animal project by helping with the *in vivo* lesions of the mouse entorhinal cortex, mRNA extraction and sample homogenates. Louise Théroux provided assistance with the ELISA and immunohistochemistry technique and participated in HMGCoAR activity measurement. The first author elaborated the hypothesis, carried out the work, analysed the data and wrote the manuscript.

***Study 3*:** Stéphanie Bélanger Jasmin, Louise Théroux, Doris D and Judes Poirier. The proprotein convertase **PCSK9 promotes synaptogenesis and synaptic remodelling in differentiated PC12 cells.** Submitted for publication to *Neuroscience Letters*, 2010.

Dr. Judes Poirier supervised the research project. Louise Theroux participated in the cell culture project by helping with the mechanical injury to neuronal differentiated PC12 cells. Doris Dea provided assistance and participated to the mRNA extraction and RT-PCR. The first author elaborated the hypothesis, carried out the work, analysed the data and wrote the manuscript.

Abbreviations

Aβ	amyloid beta
ABCA1	ATP binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
ACAT	acyl-coenzyme-a cholesterol acyltransferase
ACIs	acetylcholinesterase inhibitors
AD	Alzheimer's disease
ADH	autosomal dominant hypercholesterolemia
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor
apoB	apolipoprotein B
apoC	apolipoprotein C
apoD	apolipoprotein D
APOE/apoE	apolipoprotein E
apoJ	apolipoprotein J
apoER2	apoE type 2 receptor
APP	amyloid precursor protein
BACE1	β-site APP-cleaving enzyme 1
BBB	blood-brain-barrier
BDNF	brain-derived neurotrophic factors
CE	cholesterol ester
CLU	clusterin
CNS	central nervous system
CR1	complement receptor 1
CSF	cerebrospinal fluid
CT	carboxy terminal
CYP46	cholesterol 24-hydroxylase
DG	dentate gyrus
DPL	days post-lesion
EC	entorhinal cortex

ECL	unilateral entorhinal cortex lesion
EGF	epidermal growth factor
EOAD	early onset Alzheimer's disease
FAD	familial Alzheimer's disease
FC	free cholesterol
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FTD	frontotemporal dementia
GC	granule cell
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GWAS	genowe-wide association study
HDL	high-density-lipoprotein
HMGCoAR	3-hydroxy-3methylglutaryl Coenzyme A reductase
HPLC	high-performance liquid chromatography
HPTR	hypoxanthine phosphoribosyltransferase
IGF	insulin-like growth factor
IMT	intima media thickness
LDL	low-density-lipoprotein
LDLR	low-density-lipoprotein receptor
LOAD	late onset Alzheimer's disease
LPL	lipoprotein lipase
LRP	low-density-lipoprotein receptor related protein
LTP	long term potentiation
MA	maladie d'Alzheimer
MAPT	microtubule-associated protein tau
MCI	mild cognitive impairment
ML	molecular layer
min	minutes
NCAM	neural cell adhesion molecule
NPC	Niemann-Pick type C disease

NeuN	neuronal nuclei
NGF	nerve-growth factor
NMDA	N-methyl-D-aspartate glutamate receptor
NFT	neurofibrillary tangles
OML	outer molecular layer
PBS	phosphate buffered saline
PCs	proprotein convertases
PCSK9	proprotein convertase subtilisin/kexin type 9
PHF	paired helical filaments
PICALM	phosphatidylinositol-binding clathrin assembly protein
PNS	peripheral nervous system
Pro	prosegment
PSEN1	presenilin 1
PSEN2	presenilin 2
RT-PCR	real-time polymerase chain reaction
sec	secondes
SEM	standard error mean
SNP	single nucleotide polymorphism
SP	signal peptide
SP	senile plaques
SREBP	sterol regulatory element binding protein
TBS	tris buffered saline
TGF-β	transforming growth factor
VEGF	vascular endothelial growth factor
VLDL	very-low-density lipoprotein
VLDLR	very-low-density-lipoprotein receptor
WT	wild-type

Chapter 1: Review of Literature

During the last century, the scientific and medical progress has allowed the treatment of youth and middle-age diseases which has in turn considerably increased life expectancy. The appearance of late life illnesses has significantly increased in high-income countries such that more than 10% of people over the age of 65 are dealing with cognitive impairments and dementia. This number rises exponentially with age and most experts accept that over the age of 70, 20% of the population is affected by dementia whereas over the age of 85 the incidence raises to 50% in this population. Alzheimer's disease (AD) is the most common cause of dementia as it represents the two third of all demented cases. Behind heart and cerebrovascular diseases and cancers, AD is the 7th leading cause of death but the fifth among those 65 and older. According to the World Alzheimer Report, more than 35 million individuals worldwide are dealing with this disease and by 2030, this number is expected to nearly double to 65.7 million people (Alzheimer's Disease International, 2010). In Canada only, the incidence by 2038 will correspond to one new case every 2 minutes.

Usually, people affected with AD have other medical conditions and compared to other older people, often takes longer time to die which increases the costs for the many years of needed care. The global cost for dementia is 315 billion annually which well overwhelms the amount governments spend on AD research. Not only AD represents one of the greatest medical and social challenges, it is an emerging economic problem for our societies. By managing many diseases that make the population to live longer, we basically give more time to dement thereby reorient people towards AD. In hope of a better situation, much more efforts are needed to find what causes this disease or where it comes from so that earlier diagnostic can be made and effective prevention treatments developed.

1.1 Alzheimer's disease

In 1906, the first described case of what became known as Alzheimer's disease was publicly reported by the German psychiatrist and neuropathologist Alois Alzheimer after the death of his fifty-one-year-old woman patient named Auguste Deter. No longer after, he identified in the brain of this demented woman the presence of amyloid plaques and neurofibrillary tangles that now represent the most important histopathological hallmarks of AD. Today, this disease is characterised by common clinical symptoms that vary in every individuals over the course of the disease. Although the earliest clinical observations are often mistakenly believed to be age-related concerns or resulting from anxiety and stress, the most recognised symptom is the inability to acquire new memories such as recalling recent facts.

The progression of the disease has been characterised by 7 different stages that correspond to the widely used concept of mild, moderate, moderately severe and severe AD (Reisberg and Ferris, 1985). During the early stages, individuals feel that they have memory loss which is generally related to short-term memory deficits. For instance, the patient has difficulties to remember names of newly introduced person, to plan and organise tasks such as planning a dinner or paying bills, to make decisions, and they often loose or misplace valuable objects. As the disease advances, the progressive cognitive impairment is associated with confusion, irritability, mood swing, personality changes including suspiciousness and delusion, disorientation in time and place and loss of judgement and emotions. During the moderate to severe stage, memory problems worsen and long-term memory deficits become apparent (Forstl and Kurz, 1999). The persons may fail to recognise close relatives or the name of their spouse. In very late stages, the patient is completely dependent upon caregivers as he gradually looses its ability to carry out daily living activities (e.g. dressing, eating, washing) and losses the control of its body functions (inability to walk, hold its head up and swallow, becomes incontinent) to eventually die in most cases of bronchitis/pneumonia (Forstl and Kurz, 1999; Molsa et al., 1986). The duration of the disease varies from 3 to 20 years but the patient usually dies between 4 to 7 years after the diagnosis (Molsa et al., 1986).

However, detection of AD in its early clinical course has been proven to be an elusive objective. Clinical variability may depend on the patient's intelligence, language and memory skills, social and cultural background, medication and psychological history just to name those few. On the other hand, a distinction between dementia related to AD or other illnesses such as vascular dementia, frontotemporal dementia, Parkinson's disease or many others is critical to make an appropriate diagnosis. The use of a variety of cognitive tests, functional imaging methods and a specific stage system outlining key symptoms have largely improved the accuracy of the diagnosis but the sensitivity and specificity of the diagnosis still remain affected by alternative clinical causes. A definitive diagnosis is therefore only possible at autopsy by neuropathological examinations.

1.1.1 Neuropathological hallmarks of Alzheimer's disease

AD is a progressive and irreversible neurodegenerative disorder with distinctive clinical and pathological features. Occurring years before clinical symptoms appear, the pathological changes in AD brains consist of the prominent loss of nerve cells, appearance of plaques and tangles and inflammation. The distribution and the spread of these abnormalities, except for senile plaques, follow a stereotypical pattern in which the medial temporal (entorhinal cortex and hippocampus), limbic and association cortices and the basal forebrain are firstly affected whereas primary sensorimotor and visual cortices are affected only in later stages of the disease (Braak and Braak, 1991). Although structural imaging methods have been useful to monitor brain changes and atrophy that closely correlate with AD progression and cognitive deficits (Whitwell, 2010), the presence of both neurofibrillary tangles and amyloid plaques remain the most critical features of AD and are still considered essential biomarkers to define an AD diagnosis.

1.1.1.1 Synaptic loss

One of the earliest events in AD is the progressive degradation of synapses followed by neuronal loss in specific regions, particularly in the neocortex, entorhinal area, hippocampus, amygdala, nucleus basalis, anterior thalamus and several brainstem

nuclei (i.e., the locus coruleus and raphe nucleus) (Arnold et al., 1991; Busch et al., 1997; DeKosky and Scheff, 1990; Heinonen et al., 1995; Iraizoz et al., 1991; Masliah et al., 1994; Terry et al., 1991). However, the first and most heavily damaged area in AD is the entorhinal cortex (EC) (Crews and Masliah, 2010; Masliah et al., 1994; Samuel et al., 1994). Importantly, the latter is the major source of cortical afferents to the hippocampus which is a fundamental structure responsible for acquisition of new episodic and spatial memories and for long-term memory consolidation (Gabrieli et al., 1997; Maguire, 2001; SCOVILLE and MILNER, 1957; Witter, 1993). The EC consists of a central element for cortical and hippocampal interconnectivity as it receives cortical inputs, send them to the hippocampus but also forwards hippocampal outputs back to cortical regions (Witter, 1993; Witter, 2007).

It is not surprising that extensive degeneration of entorhinal neurites which leads to deafferentation of the hippocampus promotes and explains the clinical appearance of early memory impairments in AD patients (Gabrieli et al., 1997; Maguire, 2001; SCOVILLE and MILNER, 1957). Quantification of chemical markers directed against synaptic proteins, such as synaptophysin, performed on different cortical areas indicated that the hippocampus of AD subjects presents 44% to 55% loss of synapses whereas 15%-35% fewer number of synapses were observed in frontal cortices (Davies et al., 1987; Masliah et al., 1993; Samuel et al., 1994; Scheff et al., 2007). In agreement, the loss of synapses in the hippocampus has been demonstrated to closely correlate with hippocampal atrophy in AD subjects (Davies et al., 1987; Jack, Jr. et al., 2004; Masliah et al., 1994) as well as structural imaging techniques reported that cognitive deficits in AD follow progressive brain atrophic changes (Jack, Jr. et al., 2004; Morra et al., 2009; Ridha et al., 2008).

This gradual area-specific neuronal degeneration observed in AD leads to major deficits in cholinergic and glutamatergic functions as well as reductions in neurotransmission of noradrenaline, serotonine and dopamine (Adolfsson et al., 1979; Francis et al., 1985). Indeed, a reduction in the activity of cholinergic neurons is a well-known characteristic of AD (Davies and Maloney, 1976; Perry et al., 1978b). These

neurons in the basal forebrain provide the primary source of cholinergic innervations to the temporal cortex, hippocampus and amygdala; the main regions affected in AD (Ladner and Lee, 1998; Perry et al., 1978a; Perry et al., 1978b). Cholinergic dysfunctions due to reduced acetylcholine synthesis, choline uptake and/or loss of cholinergic neurons have been shown to contribute to the cognitive deficits observed in AD patients (Bartus et al., 1982; Ladner and Lee, 1998). As a consequence, strategies aiming to restore acetylcholine concentrations and cholinergic functions in the brain were developed as therapeutics for AD. Despite their modest effectiveness to attenuate symptomatic progression of the disease, acetylcholinesterase inhibitors (ACIs) are still widely used and the most prescribed therapeutic drugs for treatment of early and moderate stages of AD (Munoz-Torrero, 2008; Raschetti et al., 2007). Moreover, Poirier and colleagues have since demonstrated that response to ACIs treatment in AD patients is dependent on apolipoprotein E (APOE) genotype (Poirier and Sevigny, 1998).

There are several indications that the glutamatergic system, an important process in learning and memory, also participates in the pathomechanisms of AD. Evidences have indicated that both hyper and hypoactivation of the N-methyl-D-aspartate (NMDA) glutamate receptors result in neuronal dysfunctions and death (Ikonomidou and Turski, 1996). Memantine is the only glutamate receptor antagonist approved by the Food and Drug Administration (FDA) as a therapeutic treatment for moderate to severe AD (Reisberg et al., 2003; Winblad and Poritis, 1999). Similarly to ACIs, memantine treatment was shown to have small benefits on cognition (McShane et al., 2006; Van Der et al., 2006; van Dyck et al., 2007) although promising effects were reported when both memantine and donepezil (an ACI commercially named Aricept®) are used which may represent a better treatment for AD (Tariot et al., 2004). On the other hand, compounds that positively regulate the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors such as ampakine (drug in development) also demonstrated memory improvement in healthy elderly subjects (Ingvar et al., 1997; Lynch et al., 1997; Wezenberg et al., 2007).

1.1.1.2 Senile plaques

In his pioneering article (Alzheimer et al., 1995), Alois Alzheimer reported the presence of proteinaceous aggregates in AD brain that he called senile plaques (SP). Being more widely spread than neurofibrillary tangles (NFT) and synaptic/neuronal loss, the plaques are present in primary and association cortices as well as limbic areas and basal ganglia (Braak and Braak, 1991). It is only in the early 80s that the core filamentous material of these SP were defined as aggregates of amyloid beta (Aβ) peptides surrounded by abnormal or dystrophic neurites (Masters et al., 1985; TERRY et al., 1964). The formation of Aβ occurs through the sequential proteolytic cleavage of the amyloid precursor protein (APP) by three proteinases; α-, β- and γ-secretases (Hardy and Selkoe, 2002). This transmembrane protein coded on the chromosome 21 is metabolized by two competing pathways. The predominant non-amyloidogenic α-secretase/(ADAM10) pathway generates a soluble APP ectodomain and an 83-residue carboxy terminal (CT) fragment. Because this initial processing occurs within the β-secretase cleavage site, it prevents the formation of Aβ peptides. The C83 fragment is then digested by the γ-secretase to liberate an extracellular p3 peptide and an intracellular domain (Fig. 1). On the other hand, the amyloidogenic pathway is initiated by β- (BACE) and γ-secretases as their successive processing generates Aβ peptides of 37 to 42 amino acids in length (Fig. 1). Despite its association with AD pathology, the Aβ42 is produced to lesser extent than the primarily generated Aβ40 specie in an approximative ratio of 1 for 99 (Lublin and Gandy, 2010).

However, when produced, these Aβ42 monomers spontaneously aggregate into progressively larger soluble oligomers that eventually form the insoluble amyloid fibrils of the SP (Fig. 1). Because it was recently demonstrated that many healthy adults also accumulate numerous insoluble Aβ42 deposits in their brain with few signs of dementia (Aizenstein et al., 2008; Bourgeat et al., 2010; Price and Morris, 1999), the role of the amyloid plaques was brought into question. This “amyloid cascade hypothesis” was actually revised and now proposes that small soluble Aβ oligomers are most likely the important neurotoxic forms in AD (Hardy and Selkoe, 2002; Lambert et al., 1998). Indeed, they have been found to initiate mechanisms leading to tau hyperphosphorylation,

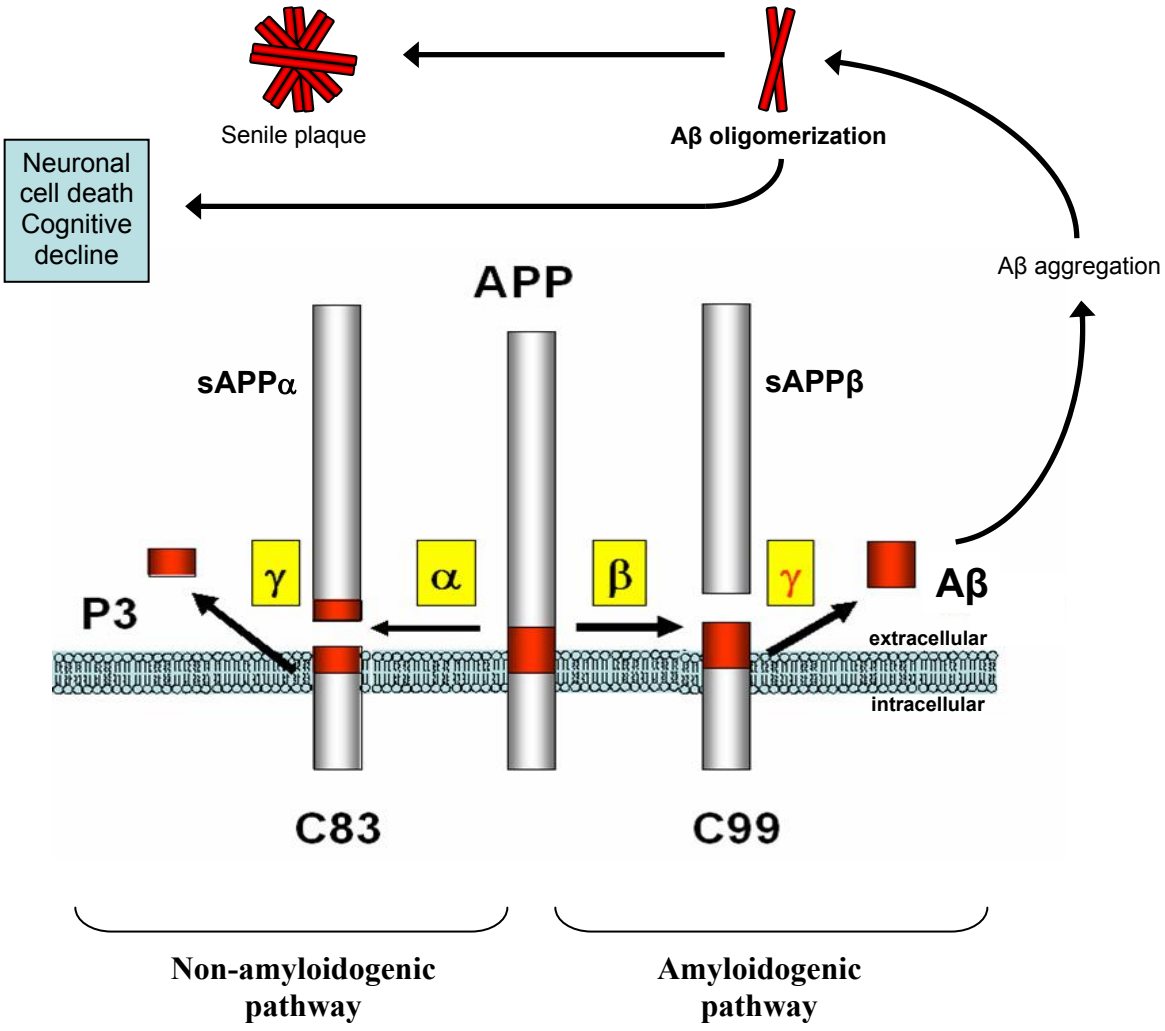

Figure 1: Processing of the Amyloid precursor protein.

The transmembrane APP proteolytic cleavage occurs via two competing pathways. The common non-amyloidogenic pathway consists of a primary cleavage of APP by the α-secretase generating a large N-terminal ectodomain (sAPPα) and a C-terminal fragment (C83). The C83 fragment is then digested by the γ-secretase which produces the extracellular p3 fragment. The amyloidogenic pathway is initiated by the β-secretase cleavage and generates sAPPβ and the C99 fragment. The C99 fragment is then a substrate for the γ-secretase cleavage liberating the Aβ peptide. Monomers of Aβ42 progressively aggregate into larger oligomers to eventually grow into fibrils and generate the senile plaques. Soluble Aβ oligomers are the most neurotoxic forms of Aβ. (Adapted from a picture posted on http://www1.tu-darmstadt.de/fb/ch/Fachgebiete/OC/AKSchmidt/Research.htm).

neuronal oxidative stress, impairment of synaptic plasticity, activation of microglia, synapse degeneration and neuronal death (De Felice et al., 2007; De Felice et al., 2008; Klyubin et al., 2008; Walsh et al., 2002). Furthermore, the levels of soluble Aβ42 oligomers in the brain but not total insoluble Aβ42 burden were shown to correlate with the severity of cognitive deficits in AD (Lue et al., 1999). Moreover, Jack and colleagues found, as others have, that the Aβ42 load in the brain of AD patients does not correlate with the rate of whole brain atrophy or clinical decline (Engler et al., 2006; Jack, Jr. et al., 2009; Josephs et al., 2008). Paradoxically, emerging evidences are now pointing towards neuroprotective and neurotrophic effects of Aβ42 monomers (Giuffrida et al., 2009; Kimura et al., 2006; Whitson et al., 1989).

The issue of whether the amyloid pathology is the triggered factor in the development of AD or a downstream element to neurodegeneration remains unresolved. However, several therapeutic interventions targeting Aβ production, aggregation into oligomers or plaques, and Aβ clearance are currently under investigation despite previous inconclusive attempts. Clinical trials with agents that inhibit BACE or γ-secretase activities are underway. While γ-secretase inhibitors have raised concern since they are involved in the Notch signalling pathway, agents in phase 3 trials have so far demonstrated relative tolerability (Neugroschl and Sano, 2010). Another hard blow for the amyloid hypothesis came from the negative trial of an anti-aggregation agent (Alzhemed from Neurochem). This phase 3 trial failed to show significant effect on endpoint cognition in an 18-months trial of 1052 patients (www.alzforum.org) (Alzheimer Research Forum, 2010).

Despite the latter disappointment, another agent who targets Aβ oligomers to prevent synaptic damage is currently in phase 2 trial whereas various immune agents are in clinical trials to determine whether immunotherapy slows cognitive decline of AD patients (Neugroschl and Sano, 2010). It is worth nothing that in 2002, the first phase 2 active immunity trial (AN1792) was halted because of meningoencephalitis in 60% of the patients who received the vaccine (Gilman et al., 2005). Long-term follow-up of the immunized patients showed no cognitive benefits despite reduction of plaques (Holmes et

al., 2008). The use of preformed antibodies to Aβ (passive immunization) instead of antigens that stimulate the patient's immune response (active immunization) is also being studied. Currently, phase 3 trials of monoclonal antibodies against Aβ (Elan and Wyeth's bapineuzumab), enrolling over 1000 patients, are underway. While results of the phase 2 trial on 240 patients with mild to moderate AD did not show clinical improvements, benefits on cognition were observed in non-APOE ε4 carriers (Salloway et al., 2009). However, results of the phase 3 studies will be required to determine whether such treatments are efficient and clinically improve cognition in AD patients.

1.1.1.3 Neurofibrillary tangles

The distribution of NFT parallels the regional pattern of synaptic loss observed in AD brains. These abnormal inclusions within neurons occur in several areas such as the EC, hippocampus, amygdala and deeper layers of the neocortex (Morrison and Hof, 1997). The main constituent of the tangles is the microtubule-associated protein tau (MAPT). The latter is a soluble, phosphorylated protein that stabilizes and promotes the polymerization of microtubules in neurons. Microtubules are cellular structures involved in axonal transport. When abnormally hyperphosphorylated, tau detaches from microtubules and aggregates into insoluble paired helical filaments (PHF) that merge together into NFT (Lee et al., 1991). In neurodegenerative tauopathies, tau proteins are mislocalized from the axons and become misrouted to cell bodies and dendrites (Shahani and Brandt, 2002). Impairment of axonal transport due to destabilisation of microtubules remains an intriguing possibility that leads to neurodegeneration in AD.

Unlike Aβ, the degree of NFT was reported to correlate with the rate of AD brain atrophy which led authors to suggest that tangles pathology is more directly related to neurodegeneration than amyloid pathology in AD (Josephs et al., 2008; Silbert et al., 2003). In addition, its extent and distribution was shown to positively correlates with AD severity and duration (Arriagada et al., 1992; Bierer et al., 1995). Of interest, one inhibitor of Aβ oligomerisation and *tau* aggregation is underway and had passed the phase 2 trial (e.g., methylene blue) (Wischik et al., 1996). This agent may represent a

potential therapeutic avenue to slow down cognitive decline in AD as recently observed, although blindness of this clinical trial remains a significant issue since this agent is known to cause sclera and urine to turn blue (Gura, 2008).

The deposition of hyperphosphorylated tau in insoluble filaments in the brain, known collectively as tauopathies, is also a pathological marker of other neurodegenerative disorders. Tauopathies associate with frontotemporal dementia (FTD), Niemann Pick type C (NPC) disease, progressive supranuclear palsy and corticobasal degeneration. Mutations in the MAPT gene on chromosome 17 were found in familial cases of FTD characterized by tau-positive inclusions and prominent degeneration of the limbic system and striatum (Haugarvoll et al., 2007).

Although synaptic loss, amyloid plaques and tangles are the cardinal neuropathological features of AD, astrocytes response, inflammation and oxidative damage have also been closely associated with AD. As to whether a particular pathology comes first and the others secondary or whether they are effects or causes of AD is a huge question of debate where everyone praises for their own theory. That being said, one century had passed since the discovery of AD and the cause(s) that drives this molecular cascade of pathological events is still elusive.

1.1.2 Causes of Alzheimer's disease

Epidemiological studies suggest that AD is a multifactorial disorder in which a variety of factors converges and leads to this clinicopathological syndrome rather than being a single aetiology disease. Indeed, a number of factors have been established to contribute to the development of AD; age, gender, head trauma, genetics and vascular diseases. Age is certainly the most important risk factor as ageing is accompanied with gradual accumulation of damage (Zhang et al., 1990). It was postulated that cell loss and impaired compensatory responses associated with neuronal loss, are observed in the ageing brain and exacerbated in AD (Coleman and Flood, 1987; Flood and Coleman, 1986). Besides, less than 1% of people of 60-64 years old are affected with AD whereas

more than 40% of people older than 85 years have the disease (Breteler et al., 1992). Gender was also reported as a risk factor for AD where women are more at risk than men. Explanations for a higher incidence of AD in women are not clear although unrecognized environmental factors, hormonal effects or unknown predisposing genes on the X chromosome are all presumptive possibilities. In addition, head trauma was considered to contribute to the development of AD as it may cause brain structural damage combined with Aβ and NFT accumulation (Roberts et al., 1991). However, genetic and vascular diseases contributions to AD are definitively the risk factors that received the most attention in the last decades and need to be discussed in more details (sections 1.2 and 1.3.5).

1.2 Genetics of Alzheimer's disease

Studies in twins have demonstrated a higher heritability for AD (58-79%) in identical (monozygotic) compared to dizygotic twins suggesting that this disease has a pre-eminent genetic component with the balance of variation explained by environmental or other nongenetic risk factors (Gatz et al., 2006). Identification of specific mutations and polymorphisms in genes that are associated with AD led to the distinction between early-onset and late-onset AD. The arbitrary cut-off to distinguish between both forms of AD is set to 60-65 years.

1.2.1 Early-onset or Familial Alzheimer's disease

Early-onset Alzheimer's disease (EOAD) represents ~5% of all AD cases worldwide (<65 years old) and often have a positive family history. Of those, familial cases are inherited in an autosomal dominant manner (familial Alzheimer's disease, or FAD) and have been linked to mutations in the genes for APP on chromosome 21, presenilin 1 (PSEN1) on chromosome 14 and presenilin 2 (PSEN2) on chromosome 1. Mutations in these genes affect the metabolism of Aβ as both PSEN1 and 2 are main components of the γ-secretase complex (Steiner et al., 2002; Wolfe and Handler, 1998). Of interest, patients with trisomy 21 (Down syndrome) are born with three copies of the

chromosome 21 and many develop in their 40s the classical neuropathological hallmarks of AD (Wisniewski et al., 1985), and by 65, nearly all Down symptoms exhibit the characteristic signs of AD. That led to the initial search for genetic linkage of chromosome 21 in families with autosomal dominant inheritance of AD. It is worth nothing that a significant sub-set of familial cases with age-of-onset >65 have been linked to the so-called APOE gene (Strittmatter et al., 1993).

1.2.2 Late-onset or sporadic Alzheimer's disease

Late-onset or sporadic Alzheimer's disease (LOAD) accounts for ~90-95% of all AD cases and occurs after 65 years of age. Several genes have been associated to LOAD but only few of them have shown reproducibility and strong genetic linkage within the general population. Until now, only the apolipoprotein E (APOE) gene has unequivocally been linked to AD whereas genes such as APOJ (also known as CLU for clusterin), APOC1, PICALM and CR1 identified by case control or genome-wide association studies (GWAS) have shown convincing evidence of association risk with AD although to much lesser extent (Harold et al., 2009; Lambert et al., 2009; Petit-Turcotte et al., 2001; Poduslo et al., 1998; Poirier et al., 1993b). A few weeks ago, the largest GWAS performed so far (>25 000 cases) led to the identification of two additional genes called BIN1 and EXOC3L2 (Seshadri et al., 2010).

1.2.2.1 Apolipoprotein E

In humans, the APOE gene is encoded on chromosome 19 and could be found as 3 different isoforms, namely, APOE2, APOE3 and APOE4. These isoforms differ from each other by amino acids substitution at residues 112 and 158 on a 299 amino acids protein. The APOE allele ε3 is the most common one found in a typical Caucasian population (~78%) whereas the APOE ε4 which is the allele that shows strong association with AD, is present in ~14% of people but in >50% of AD patients (Corder et al., 1993; Farrer et al., 1997; Saunders et al., 1993). The inheritance of one or two ε4 alleles is associated with higher risk of AD and younger age of onset in a dose-related manner

(Corder et al., 1993). Conversely, the APOE ε2 allele appears to be protective and delay age of onset (Corder et al., 1994). But how each of these alleles confers susceptibility to or protection from AD is unclear.

Apolipoproteins are lipid-binding plasma proteins involved in cholesterol transport. They especially bind phospholipids, triglycerides, cholesterol ester and free cholesterol, forming lipoprotein particles. They are the protein moiety of lipoproteins and they facilitate their transport and delivery among peripheral organs (Danik et al., 1999). While the liver constitutes the major site of apolipoproteins expression, the brain is the second greatest site in which apoE is the most prevalent and important lipid transporter of the central nervous system (CNS). ApoA, apoD, apoC1 and apoJ are also present in the brain but to much lower extent (Petit-Turcotte et al., 2001; Pitas et al., 1987b). Within the CNS, apoE is predominantly synthesized and secreted by astrocytes (>95%) and microglia, and acts as the principal ligand for members of the cell surface low-density-lipoprotein receptor (LDLR) (DeMattos et al., 2001; Pitas et al., 1987a).

ApoE has the metabolic function of coordinating the mobilization and redistribution of cholesterol to neurons for repair, growth and maintenance of myelin and neuronal membranes integrity during development, in adulthood or after neuronal cell injury or neurodegenerative conditions (Beffert et al., 1998b; Poirier, 2003). The formation and distribution of apoE/high-density-lipoprotein (HDL)-like particles is crucial for synaptic plasticity and neurites remodelling as indicated by their quick upregulation after injury (Laskowitz et al., 1998; Poirier, 1994). On the other hand, deletion of apoE in mice results in a marked impairment of synaptic plasticity, compromised dendritic elongation, loss of cholinergic activity in the hippocampal area but most importantly, an irreversible loss of cognitive performance as early as 3 months after birth (Krugers et al., 1997; Krzywkowski et al., 1999; Masliah et al., 1995; Masliah et al., 1997; Poirier, 1994). These findings have clearly highlighted the importance of apoE and lipid trafficking for synaptic remodelling and plasticity, neuronal survival and memory. However, the mechanism by which apoE predisposes individuals to AD is not

fully identified but dysfunction of cholesterol transport may in part contribute to neurodegeneration.

1.2.2.2 Apolipoprotein E and Alzheimer's disease

Several possibilities have been suggested to explain the effect of apoE in the brain of AD patients. These include apoE isoform-driven impact on apoE concentration and lipid transport, Aβ metabolism and tau hyperphosphorylation.

ApoE levels in humans were reported to be inversely correlated to the isoform-associated risk levels of AD. Those having the APOE ε2 genotype express the highest plasma and brain levels of apoE whereas the lowest levels of expression is observed in APOE ε4 carriers (majority of AD cases) (Bertrand et al., 1995; Poirier, 2005). Because concentration of apoE is central for synaptic integrity and remodelling, it is believed that low levels of apoE compromise these processes in an ageing brain and more so in brains suffering from damage or degenerative conditions (Poirier, 1994). That agrees with the observations that APOE ε4 carriers exhibit poor clinical recovery after brain injuries and impaired synaptic remodelling and defective compensatory plasticity in vulnerable brain areas in AD (Arendt et al., 1997; Beffert et al., 1998b; Friedman et al., 1999; Lichtman et al., 2000). ApoE3-containing lipoproteins were shown to promote while apoE4-lipoproteins decreased neurites outgrowth and reactive sprouting (Blain et al., 2006b; Nathan et al., 1994; Nathan et al., 2002). It is a possibility that low levels of apoE result in abnormal transport and uptake of HDL-like particles by neurons undergoing synaptic repair which therefore impair the brain to compensate for neuronal damage and influence the appearance of neuropathology and AD clinical symptoms overtime.

Accordingly, individuals carrying the APOE ε4 allele show greater amyloid load, plaque size and density, and NFT compared to non-ε4 carriers (Beffert et al., 1999; Schmechel et al., 1993; Tiraboschi et al., 2004). Moreover, Aβ deposition has been shown to be increased following head injury as well as in middle-aged and non-demented elderly subjects carrying the APOE ε4 allele (Arai et al., 1999; Nicoll et al., 1995). These

observations raised the hypothesis that apoE may modulate Aβ metabolism either via stimulation of amyloidogenic APP processing and/or reduction of Aβ clearance. In support of this idea, many studies have now suggested a role for apoE as an Aβ scavenging molecule that regulates extracellular Aβ concentration through apoE-LDLR internalization (Beffert et al., 1998a; Beffert and Poirier, 1996). Of interest, apoE immunoreactivity in both SP and NFT of AD brain tissues was reported years ago by Namba and colleagues (Namba et al., 1991). Although some inconsistencies exist, Ladu et al. (1994) were the first to report that apoE binding affinity to Aβ was stronger for apoE2 and apoE3 than apoE4 isoforms (LaDu et al., 1994). Furthermore, a negative correlation was observed between Aβ and apoE levels which is consistent with the increased Aβ and reduced apoE concentrations observed in AD subjects in a genotype-dependent manner: apoE4 > apoE3 > apoE2 (Beffert et al., 1999). In addition, apoE was shown to facilitate brain to blood removal of Aβ peptides by transport across the BBB, a clearing process that also follows an apoE2 > apoE3 > apoE4 gradient (Deane et al., 2008). These data strongly suggest that having low levels of apoE and/or a lower Aβ binding affinity, as in APOE4 carriers, is associated with reduced extracellular Aβ clearance via the apoE/LDLR uptake pathway and increased extracellular accumulation of soluble Aβ peptides leading to amyloid deposition and aggregation in AD brains. Another proposed mechanism is that apoE modulates Aβ formation via a direct binding to APP thus, lowering APP accessibility to γ-secretase processing (Irizarry et al., 2004).

In addition, distinct mechanisms have been proposed to link apoE with tau hyperphosphorylation. One has retained particular attention and lies on apoE effects, with apoE4 having the greatest effect, on GSK3 activity, the main kinase that phosphorylates tau (Cedazo-Minguez et al., 2003; Hoe et al., 2006; Hong et al., 1997; Ohkubo et al., 2003). A more compelling hypothesis linking apoE, GSK3, and tau phosphorylation would not necessarily require a direct apoE interaction with tau; rather, it is based on binding of apoE to members of the cell surface LDLRs, more specifically LDL receptor-related protein (LRP) which in turn affects GSK3 activity (De Ferrari and Moon, 2006; Herz and Bock, 2002). Moreover, neuronal proteolytic cleavage of apoE4 that generates

C-terminal truncated fragments is also believed to associate with increased tau hyperphosphorylation (Brecht et al., 2004).

All together, these findings indicate that apoE4 directly modulates Aβ metabolism and probably tau hyperphosphorylation in AD. Carrying the ε4 allele also represents a major risk factor for other types of vascular diseases such as atherosclerosis, coronary artery disease, hypertension, diabetes, obesity, stroke and several neurological disorders including cerebral amyloid angiopathy, tauopathies, dementia with Lewy bodies, Parkinson's disease and multiple sclerosis (Bu, 2009; Davignon et al., 1988; Kessler et al., 1997; Wellington, 2004) emphasizing the relationship that exists between the presence of the apoE4 allele and inadequate cholesterol homeostasis.

1.3 Cholesterol

Cholesterol is an essential component of mammalian cell membranes without which the cell cannot function. It is also the precursor of steroid hormones, bile acids and oxysterols which by themselves are important regulatory molecules in many metabolic pathways. Structurally, cholesterol determines the fluidity and confers stability to cellular membranes and in conjunction with other lipids, is essential for membrane extension and synapse formation. It has a crucial role in development and maintenance of neuronal plasticity and functions (Pfrieger, 2003a). Moreover, as cholesterol is the major component of myelin sheaths, it is fundamental for neural communication enabling a rapid and efficient electrical transmission.

1.3.1 Cholesterol and membrane physiology

Mammalian cell membranes consist of two leaflets composed of distinct lipid constituents and a variety of membrane proteins which are involved in various cellular processes but also serve as an attachment point for the intracellular cytosqueleton. By their composition, plasma membranes are selectively permeable as they control the movement of substances in and out the cells. The most popular accepted picture of

biological membranes is the fluid-mosaic model proposed by Singer and Nicholson in 1972 (Singer and Nicolson, 1972). This model assumes that lipids are the structural builders forming a fluid bilayer in which intrinsic and associated proteins span the membranes and perform particular functions such as cell adhesion, ion channel conductance and cell signalling. This bilayer membrane is believed to be a self-assembly of lipids in which their polar hydrophilic heads face the aqueous environment of the extracellular fluid and cytoplasm whereas the hydrophobic tails reorient towards the center to avoid aqueous solvent. In a mammalian cell, approximately 60% of total cellular cholesterol resides in plasma membranes (Liscum and Munn, 1999).

Although the lipid bilayer membranes have long been seen as a sea of randomly distributed lipids and proteins, recent research provides evidences for the existence of ordered membrane microdomains called lipid-rafts (Pike, 2004; Simons and Ikonen, 1997). Cholesterol and sphingolipids are assumed to spontaneously associate with each other to form these dynamic rafts of heterogeneous composition which implies sub-compartmentalization of the lipid bilayer. Because the lipid core of membranes is hydrophobic and prevents the free passage of substances across plasma membranes, these lipid-rafts may provide membranes with more stable and ordered platforms that control the association and binding of molecules, their membrane trafficking and signal transduction (Rajendran and Simons, 2005). Caveolae are typical examples of structurally defined domains. In many cases, they are joined together and form a flask-shaped membrane invagination that penetrates several micrometers into a cell while maintaining open contact with the surface (Minshall et al., 2003; Sharma et al., 2010).

From a fundamental point of view, the lipid composition of the bilayer membranes and microdomains coupled to protein organization and functions is a defining step in membrane evolution and properties. Sterols can influence membrane permeability and fluidity (Bjorkhem and Meaney, 2004). Minor changes in sterol structure or composition lead to pronounced effect on membrane physiology and activity leading to birth defects, functional disturbances of the peripheral nervous system (PNS) and CNS

and mental retardation as observed in patients with the Smith-Lemli-Opitz syndrome, due to a defect in the 7-dehydrocholesterol-7-reductase (Jira et al., 2003).

In addition, the lipid availability in cells such as neurons in the CNS, mediates their ability to form synapses and response to environmental variations by adapting synaptic wiring in a neuronal network. Essentially, lipids are the limiting-step in membranes elongation required for the generation of new neurons or for synaptic plasticity in which membrane terminals constantly retract and re-rextend to build new synaptic contacts.

1.3.2 Cholesterol metabolism

Contrary to the periphery where cholesterol can be synthesized in the body or obtained from the diet, cholesterol in the CNS essentially originates from local *de novo* synthesis since plasma lipoproteins do not cross the blood-brain-barrier (BBB) (Bjorkhem and Meaney, 2004; Dietschy and Turley, 2004). Therefore, to maintain a brain cholesterol concentration relatively constant, a tight regulation of its synthesis, transport, binding, internalization, storage and degradation is imposed locally.

Most of mammalian cells meet their cholesterol requirements by two separated but interrelated processes. One process is the endogenous synthesis of cholesterol which is a lengthy and energy consuming process that involves more than 20 enzyme-catalysed reactions. This *de novo* synthesis pathway is primarily regulated by the activity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) and the latter is an important target for pharmaceutical interventions, such as statins (Fig. 2). The second process involves the internalization of apoE-lipoprotein particles from the local environment through a receptor-mediated endocytosis mechanism. Members of the LDLR family, expressed by neurons and glia cells, bind apoE with high affinity and deliver cholesterol into the cells via endosomes which fuses to lysosomes causing release of free cholesterol (Fig. 3). Although brain cells have the capacity to synthesis their own cholesterol, mature neurons are believed to reduce their cholesterol production and

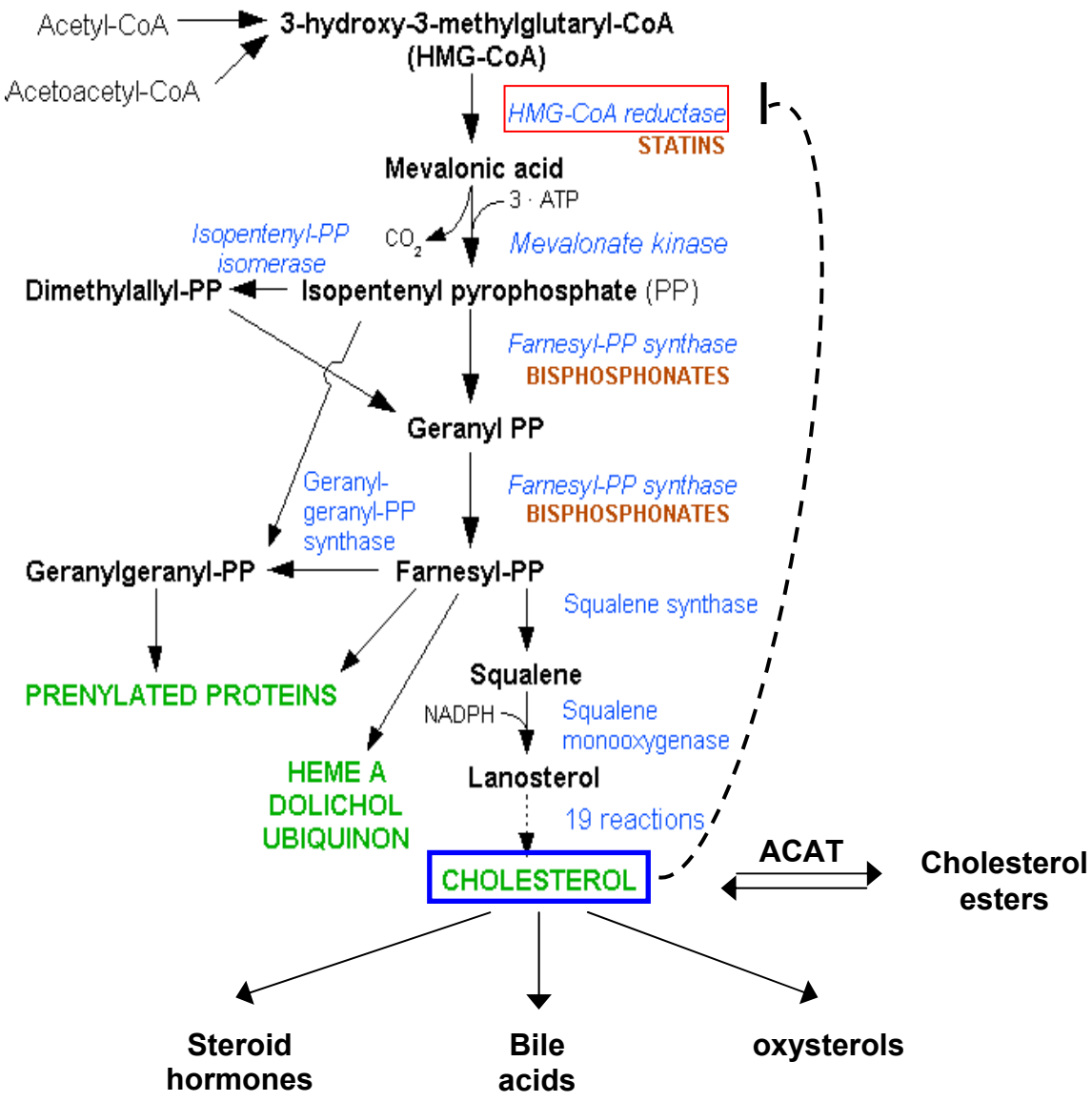

Figure 2: Cholesterol synthesis via the HMGCoAR pathway.

The synthesis of cholesterol is mainly regulated by the activity of the HMGCoAR, the enzyme that catalyzes the formation of Mevalonic acid from 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA). Cholesterol is the precursor of steroid hormones, biles acids and oxysterols but can also be esterified by the acyl-coenzyme-A cholesterol acyltransferase (ACAT) and serve as a cholesterol pool for eventual needs. Its synthesis can be inhibited by cholesterol-lowering drugs such as statins that target the activity of the HMGCoAR. (Adapted from a picture posted on http://www.chemistrydaily.com/chemistry/HMG-CoA_reductase_pathway).

mainly rely on cholesterol synthesized and secreted by astrocytes (Mauch et al., 2001; Pfrieger, 2003a). Evidences indicated that astrocytes produce 2 to 3 fold more cholesterol than neurons (Bjorkhem and Meaney, 2004) and in culture, the rate of their cholesterol synthesis is inversely proportional to the cholesterol content in the growth environment.

In agreement, scientists demonstrated that a cholesterol shuffle from astrocytes to neurons is needed to enhance axonal extension and formation of numerous and efficient synapses (Mauch et al., 2001; Pfrieger, 2003c). Inhibition of cholesterol synthesis in astrocytes reduces the secretion of both apoE and cholesterol and suppresses the growth stimulatory effect of the astrocytes-derived apoE-cholesterol lipoparticles. These experimental evidences strengthen the importance of cholesterol transport and internalization for neuronal functions.

Although a perfect balance between cholesterol influx and endogenous synthesis assures the maintenance of cholesterol homeostasis in neural cells, any excess of lipids must be removed from the brain to guarantee this homeostasis. It is possible that one or more members of the ABC transporter superfamily or apoE receptors (members of the LDLR family) may also mediate brain cholesterol efflux through the BBB as they are expressed on brain capillary endothelial cells (Gosselet et al., 2009; Ohtsuki et al., 2004). However, the most prevailing way those lipids in excess are excreted from the brain to the circulation is through their conversion to more lipophilic oxysterol 24S-hydroxylcholesterol that can freely cross the BBB (Fig. 3) (Bogdanovic et al., 2001). The conversion of free cholesterol to 24-hydroxycholesterol is mediated via the enzyme cholesterol 24-hydroxylase (CYP46) (Combarros et al., 2004). Interestingly, neurodegeneration together with brain atrophy are associated with reduced concentrations of brain oxysterols. Patients with AD have thus been found to have marked increased levels of 24S-hydroxycholesterol in the cerebrospinal fluid (CSF) during the early stages of the disease, potentially reflecting higher rate of cholesterol turnover in the brain due to intensive neurodegeneration (Papassotiropoulos et al., 2002).

1.3.3 Synaptic plasticity

While neurons of the CNS have a very limited capacity to regenerate following damage, they have been shown to have a strong ability to reorganize their neuronal network. Contrary to the PNS in which damaged axons re-grow and re-establish the original connections (= regeneration), the brain instead initiates a compensatory response via the proliferation of axons and terminals from undamaged neurons in order to form new branches and rebuild new synaptic connections to replace the lost ones. As mentioned before, this process that here refers to synaptic remodelling and synaptogenesis, necessitates a greater need for cholesterol which means that an active lipid transport is needed to support neuronal functions and repair.

In response to brain injury or neurodegeneration as in AD, a large amount of lipids is released from degenerating membranes and myelin. Therefore, an efficient recycling and redistribution of brain cholesterol is stimulated to meet the neuronal lipid requirements. Ultrastructural studies have shown that astrocytes and microglia progressively engulf and degrade those degenerating terminals from dead or dying neurons to rapidly clear the area for synaptic replacement (Fig. 3). Once internalized, these terminals represent a high pool of free cholesterol (FC) that activates the synthesis of apolipoproteins, mostly apoE and to lesser degree, apoJ (Fig. 3) (Poirier et al., 1993a). Within astrocytes, the ATP-binding cassette transporters A1 (ABCA1) coordinate the mobilization of cholesterol from the cytoplasm to the cell surface where it mediates lipidation of apoE lipoproteins to form apoE-HDL-like particles through the action of the lipoprotein lipase (LPL) (Fig. 3) (Abildayeva et al., 2006; Blain et al., 2004; Hirsch-Reinshagen and Wellington, 2007). In agreement, levels of apoE were found markedly decreased in the brain of ABCA1 deficient mice (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004) whereas both apoE and ABCA1 levels are upregulated in the reactive phase of neuronal remodelling in the deafferented mouse hippocampus (Chapter 3 and Appendix-1) (Blain et al., 2004).

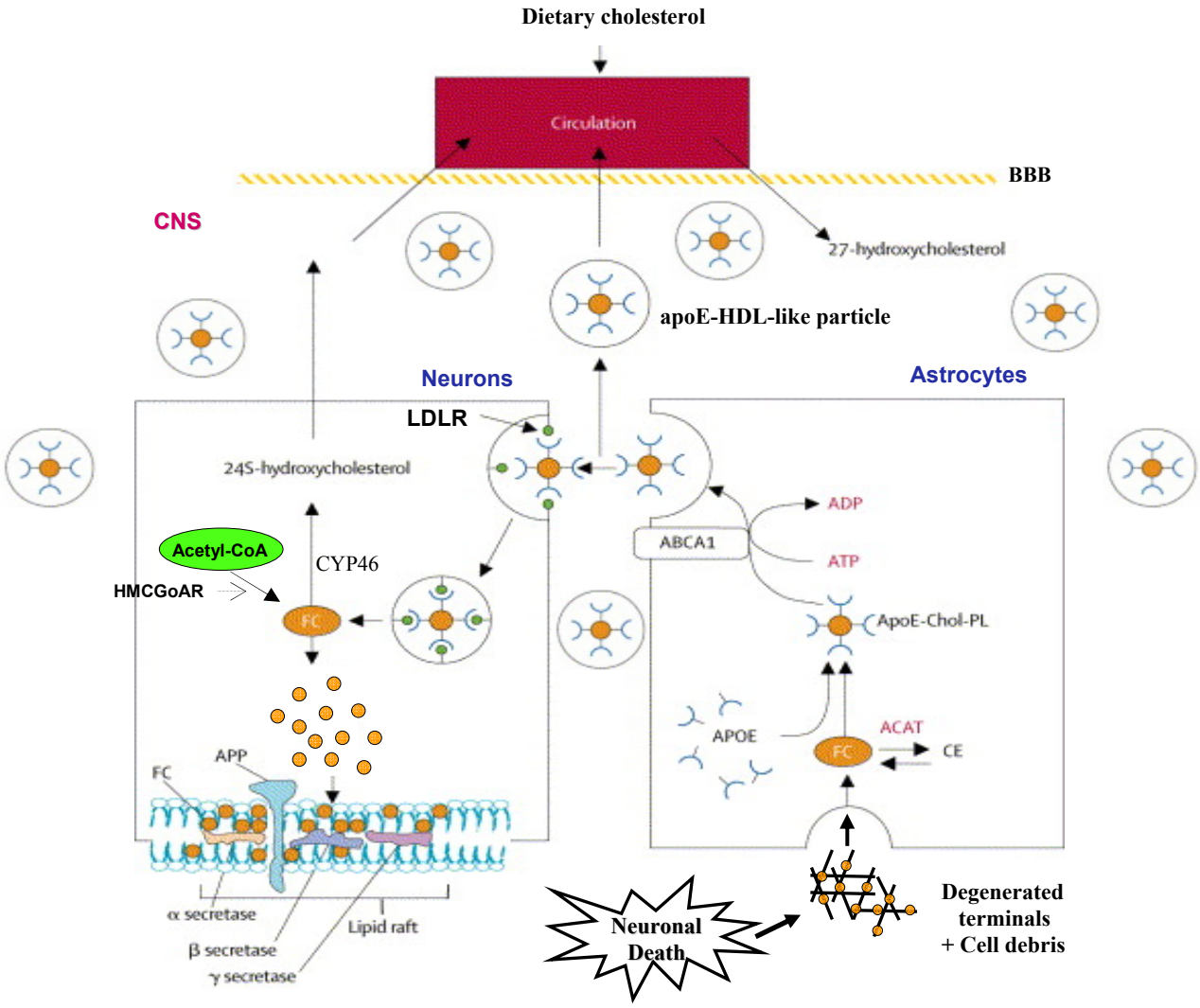

Figure 3: Cholesterol transport and distribution in the CNS.

Astrocytes are the major source of cholesterol for neurons. Endogenous synthesis of cholesterol is driven by the HMGCoAR pathway and is negatively regulated by high levels of intracellular lipids. Within astrocytes, ABCA1 facilitates apoE lipidation and secretion in the extracellular space. These astrocytes-derived apoE-HDL-like particles are recognized and internalized by LDLRs into neurons and provide a pool of free cholesterol (FC) that can either be stored as cholesterol esters (CE) formed by acyl-coenzyme-A cholesterol acyltransferase (ACAT), or be transported to terminals for membrane extension and synaptogenesis. In the injured brain, membranes of degenerating terminals represent high levels of cholesterol which are presumably cleared from the injured site by astrocytes. This excess of sterols is recycled and re-used for synaptic remodelling or excreted in part, to the circulation via its CYP46-mediated conversion into 24-hydroxycholesterol. Evidences are suggesting that the activity of secretases thus Aβ production, is influenced by intracellular and membrane microdomains lipid concentrations. (Modified from *Lancet Neurol 2005; 4:841-52*).

Once in the extracellular space, the resulting apoE-HDL-like complexes are recognized and internalized via the LDLR expressed at the cell surface of surviving adjacent neurons (Fig. 3) (Beffert et al., 1998b). The neuronal binding capacity of apoE-HDL-like particles increases dramatically after injury when cholesterol mobilization becomes critical for membrane-dependent synaptic remodelling processes and neuronal repair. Marked increase of LDLR in deafferentated granule cells in the hippocampus were observed in the early and middle phases of the reinnervation process (Poirier et al., 1993a). Other members of the LDLR family such as LRP1, very-low-density-lipoprotein receptor (VLDLR) and apoE type 2 receptor (apoER2) were also shown to indirectly mediate terminal proliferation and axonal extension primarily via signalling pathways rather than lipoproteins internalization (Beffert et al., 2004; Beffert et al., 2006).

These endocytosed apoE-cholesterol-containing complexes are hydrolysed and the intracellular release of FC into neurons can be utilised in two different ways. First, it may be esterified to form cholesterol esters (CE) through the activity of ACAT and stored as a reserve pool for future needs (Poirier, 2003) or it may be transported to dendrites or terminals of reinnervating neurons and serve as an immediate source of cholesterol for plasma membrane synthesis, neurites elongation and synaptic remodelling (Fig. 3) (Pfrieger, 2003a; Poirier et al., 1995).

As the intracellular concentration of cholesterol increases in astrocytes and neurons, cholesterol synthesis is progressively repressed at the level of HMGCoAR (Poirier et al., 1993a). Expression of both HMGCoAR and LDLR is regulated by cholesterol levels as they contain a sterol regulatory element in their promoters (Osborne et al., 1988). Sterol regulatory element binding proteins (SREBP) are transcriptional factors that mediate transcriptional activation of genes involved in cholesterol metabolism when cellular sterol levels are low (Espenshade and Hughes, 2007; Osborne, 2001). In opposite, elevated intracellular concentrations of cholesterol maintain inactive pool of SREBP which progressively prevent transcription of lipid-related genes and cholesterol synthesis thus mediating a negative feedback to HMGCoAR. A depressed cholesterol synthesis and increased apoE expression favour cholesterol re-utilization from

degenerating terminals through the apoE transport/LDLR-uptake pathway (Poirier et al., 1993a; Posse de Chaves et al., 2000).

This recycling and redistribution of cholesterol to neurons is a critical step during compensatory synaptogenesis. The absence of either apoE or LDLR in mice was shown to compromise synaptic integrity and plasticity and to cause cognitive deficits with age (Champagne et al., 2002; Mulder et al., 2004; Mulder et al., 2007). Consistently, the lack of one ABCA1 allele in aged APP23 mice was shown to be sufficient to lower apoE levels in their brain and show learning and memory deficits that may result from a disregulated cholesterol trafficking (Lefterov et al., 2009).

1.3.3.1 Synaptic plasticity in an animal model

As mentioned in a previous section (section 1.1.1.1), the entorhinal cortex (EC) located in the basal temporal lobe, plays a key role in relaying cortical-hippocampal informations. Entorhinal fibers represent the main inputs to the hippocampus and they predominantly terminate in the outer two-thirds of the molecular layer of the dentate gyrus (DG). This EC-hippocampal circuitry is of special interest because of its important role in higher cognitive functions such as learning and memory and its vulnerability to degeneration in AD.

The entorhinal cortex lesion (ECL) constitutes a classical *in vivo* model to study lesion-induced sprouting and reorganization of neural circuits in the CNS. Briefly, lesioning the EC causes the degeneration of entorhinal fibers that innervate the hippocampus resulting in the loss of up to 90% of all synapses in the outer molecular layer (OML) of the DG (Fig. 4) (Phinney et al., 2004). Deafferentation of the DG leads to the collateral sprouting of surviving axons from adjacent layers to form new synaptic contacts. These new afferents originate from cholinergic septal neurons (Lynch et al., 1972), glutamatergic commissural/associational pyramidal cells of the CA3/hilus area (Lynch et al., 1976) and to lesser extent, from neurons of the contralateral EC that usually

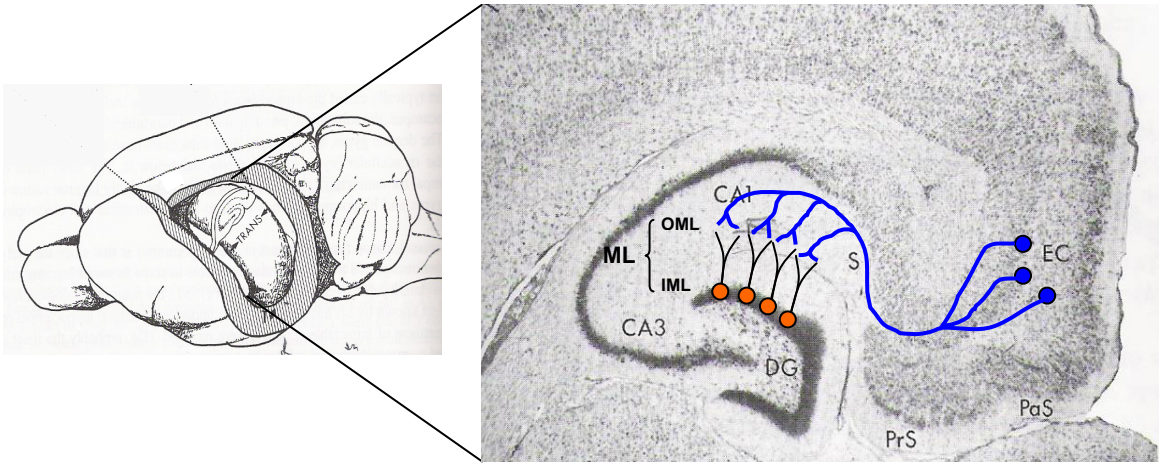

Figure 4: The entorhinal-hippocampal interconnectivity.

The main perforant path to the hippocampus originates from entorhinal fibers that send sensory information to the dentate gyrus (DG). Axons from the entorhinal cortex (EC) innervate distal and proximal granule cell dendrites located in the outer and middle third of the molecular layer (ML) of the DG. Once activated, granule cell axons (mossy fibers) project to CA3 pyramidal cells but also synapse on the polymorphic layer (*hilus*) which provides associational connections to other levels of the DG. In turn, the CA3 cells project to CA1 pyramidal cells that activate the subiculum (S) and forward information back to deep layers of the EC and then, to cortical areas. Transformations that occur through this entorhinal-hippocampal connectivity are presumably essential for storing information as long-term memories. The outer and inner molecular layer (OML and IML), the presubiculum (PrS) and parasubiculum (PaS) are also illustrated. (Adapted from *Neuroscience 1989; 31 no.3: 571-91*).

innervate the inner third of the ML (Matthews et al., 1976; Steward et al., 1973; Steward et al., 1988). Within few weeks, synaptic remodelling is completed and 60-80% of the lost entorhinal inputs have been replaced by new ones.

During the early phase of reactive synaptogenesis, this sequence of compensatory changes was found to correlate with the induction of apoE and increased LDL binding sites in the deafferented ML of EC-lesioned rats (Poirier et al., 1993a). Levels of synaptic proteins like synaptophysin and growth-associated phosphoprotein GAP-43 or cytokines, neurotrophic factors and cell adhesion molecules that stimulate axonal growth, are also increased during this particular phase of synaptic remodelling (Deller et al., 2006; Masliah et al., 1991). In opposite, cholesterol synthesis in the granule cell neurons was found to be reduced by more than 60% (Poirier et al., 1991a; Poirier et al., 1993a). Together, these findings indicate once again that the cholesterol transport/internalization system via the apoE/LDLR pathway is essential for neurites extension and synaptic reorganization in response to damage or neurodegeneration. Interestingly, these histochemical changes observed following ECL in rodents are believed to be similar to those observed in the adult CNS affected by damage or disease-related degeneration (Cotman and Anderson, 1988; Masliah et al., 1991). Therefore, this laminar model of reactive synaptogenesis constitutes an excellent model to study the different molecular mechanisms involved in the degeneration and regeneration phases occurring in the early and pre-clinical stages of AD.

1.3.4 Cholesterol and Alzheimer's disease

The first relationship between cholesterol metabolism and AD came from the discovery that inheritance of the APOE ε4 allele is the strongest known risk factor for the development of LOAD (Poirier et al., 1993b; Strittmatter et al., 1993). As mentioned previously, apoE is the main lipid transporter in the CNS and it has the metabolic function of regulating intercellular cholesterol transport via the apoE/LDLR uptake pathway (Poirier, 2005). Evidences are now supporting the functional importance of apoE and cholesterol delivery not only for axonal extension and reactive synaptogenesis following

brain injury but also in physiological ongoing synaptic plasticity and for the general maintenance of neuronal integrity. Therefore, a defect in cholesterol transport and delivery systems is an appealing hypothesis as it ties together the APOE genetic risk, the amyloid production and tangles formation.

1.3.4.1 Cholesterol, amyloid metabolism and tau phosphorylation

The first study reporting a link between cholesterol and amyloid metabolism in the brain was originally published by Sparks and colleagues (Sparks et al., 1994). Rabbits or transgenic mice fed with a cholesterol-enriched diet (inducing hypercholesterolemia) showed increased amounts of amyloid plaques in their brain (Hooijmans et al., 2007; Refolo et al., 2000; Shie et al., 2002; Sparks et al., 1994). Conversely, treatment with cholesterol synthesis inhibitors reduces this Aβ accumulation (Fassbender et al., 2001; Refolo et al., 2001) demonstrating that the extent of AD pathology can be modulated *in vivo* by cholesterolemia. In agreement, clinical studies indicated that middle-aged individuals, having high levels of plasma cholesterol, were at increased risk of developing AD later in life (Kivipelto et al., 2002; Notkola et al., 1998; Whitmer et al., 2005b; Whitmer et al., 2005a). Moreover, these individuals were shown to have higher levels of Aβ peptides in their brains compared to normocholesterolemia individuals (Pappolla et al., 2003). Epidemiological studies reported that reducing plasma cholesterol levels of mid-life individuals to normal levels by using cholesterol modulators such as statins decreases the risk of developing AD later in life (Haag et al., 2009; Jick et al., 2000; Rockwood et al., 2002; Wolozin et al., 2000; Wolozin et al., 2007; Zamrini et al., 2004). Furthermore, the antecedent statin administration in cognitively intact elderly revealed that over time, statin users accumulate less NFT (but not amyloid plaques) than age-matched statin-free subjects (Li et al., 2007a). However, the use of these cholesterol-lowering drugs in mild to moderate AD patients did not show beneficial effects on cognitive or global functions (Feldman et al., 2010) which suggests that a pre-symptomatic use of statins in middle-aged subjects may be a more appropriated prevention treatment for AD.

In the CNS, it has been suggested that cholesterol increases the activity of β- and γ-secretases (Cordy et al., 2003; Ehehalt et al., 2003) which reside in cholesterol-rich lipid rafts whereas low cholesterol level inhibits their activities (Grimm et al., 2008). On the other hand, α-secretase which promotes the non-amyloidogenic pathway, needs cholesterol poor membranes to process APP (Kojro et al., 2001). It has been proposed that high levels of free cholesterol alter plasma membrane composition, fluidity and increase the number of lipid rafts which prevents the α-secretase/APP interaction but favors the interaction with β-secretase. Therefore, cholesterol content modulates APP processing and a high cholesterol environment favors Aβ generation. In agreement, increased intracellular cholesterol levels negatively regulates α-secretase and stimulates Aβ production (Frears et al., 1999; Xiu et al., 2006). In addition to elevated cholesterol in lipid rafts, an increase in cholesterol esters in the form of cytoplasmic droplets has also been shown to regulate Aβ generation. Antagonists of ACAT, the enzyme catalyzing the formation of cholesterol esters, downregulate the production of Aβ (Huttunen et al., 2007; Huttunen et al., 2009; Puglielli et al., 2001).

The role of cholesterol on tau phosphorylation and NFT formation is not well defined. It has been proposed that upregulation of Aβ caused by high membrane cholesterol contents indirectly promotes the production of NFT (McLaurin et al., 2003). For instance, high membrane cholesterol levels may render neurons more susceptible to Aβ-induced calpain activation and tau toxicity (Nicholson and Ferreira, 2009). In parallel, the inherited neurodegenerative lipid storage disorder, NPC, shows intracellular accumulation of cholesterol in neurons due to abnormal cholesterol transport (Vanier, 2010). Neurons of NPC subjects display high levels of cholesterol with accumulation of NFT that resemble the tangle-bearing neurons in AD (Distl et al., 2003; Ohm et al., 2003). Another possible effect of cholesterol overload on tau pathology may be via oxidative stress. Markers of oxidative stress were associated with neurofibrillary lesions in AD brains (Markesbery and Carney, 1999).

While increasing evidences support a role for cholesterol in AD pathology, it remains unclear to what extent alterations in brain cholesterol metabolism contribute

mechanistically to the development of this disease. However, the possibility that the apparent beneficial effects of mid-life use of cholesterol-lowering drugs on the late-life risk for AD may stem from the prevention of vascular risk factors cannot be excluded.

1.3.5 Vascular risk factors and Alzheimer's disease

Currently, consensus is reached about the important contribution of vascular disorders to the development of AD. Epidemiological and neuropathological studies have revealed that several vascular-related diseases such as hypertension, hypercholesterolemia, atherosclerosis, ischemic white matter lesions, history of stroke, infarcts, diabetes mellitus and obesity are all recognized risk factors for AD (Breteler, 2000; Morse, 2006; Schneider, 2009; Skoog and Gustafson, 2006; Solomon et al., 2009; Whitmer et al., 2007). All of these vascular-related diseases lead to impairment of cerebral perfusion (hypoperfusion) indicating that neurovascular dysfunction is an integral part of AD development and this gave rise to the vascular hypothesis of AD (de la Torre, 2004). More importantly, the degree to which these factors influence the development of AD is affected by the inheritance of the APOE ε4 allele. It is known that APOE ε4 carriers display high serum cholesterol levels (Davignon et al., 1988) which in turn associate with AD and Aβ levels (Kivipelto et al., 2002; Pappolla et al., 2003). However, brain cholesterol is not influenced by serum cholesterol because of the BBB (Dietschy and Turley, 2001; Hooijmans et al., 2007; Kivipelto et al., 2002) which suggests that the apoE ε4 isoform may increase and aggravate serum cholesterol levels causing atherosclerosis and other vascular-related diseases which may subsequently cause hypoperfusion of the brain.

Hypertension is present in approximately 50% of people above 70 years of age and elevated mid-life systolic blood pressure is associated with lower brain weight, white matter lesions and greater numbers of senile plaques in the brain (Kivipelto et al., 2002; Skoog and Gustafson, 2006). Anti-hypertensive agents such as angiotensin-converting enzyme inhibitors and diuretics were shown to markedly reduce the risk of cognitive impairment and in some studies, delay onset of AD (Shah et al., 2009). Atherosclerosis is

also a process that takes decades before it manifests itself and it is therefore logical that progressive blood vessel pathology initiates dysfunctions in the BBB, cerebral hypoperfusion, and ischemia in the brain which may lead to brain pathological processes with cognitive impairment many years before the appearance of dementia symptoms.

While some investigators failed to report any correlations between serum cholesterol levels and AD, many others demonstrated higher levels of total and low-density-lipoprotein (LDL)-cholesterol in AD patients compared to control elderly (Evans et al., 2000; Lesser et al., 2001; Lesser et al., 2009). In turn, elevated levels of circulating LDL-cholesterol in AD patients was further correlated with brain Aβ levels (Kuo et al., 1998) which is consistent with the observations that cardiovascular or its risk factors associate with brain amyloid pathology. Moreover, the APOE ε4 allele, which increases the risk of AD, is also associated with higher levels of total and LDL-cholesterol (Boerwinkle and Utermann, 1988). However, carrying the APOE ε4 allele often does not increase the risk for AD in countries where people have low fat diets. The prevalence of AD in African population is very low compared to Western population of the same ethnic background (Hendrie et al., 1995) supporting the concept that lifestyle factors greatly contribute to the risk of developing AD.

Although the exact mechanism by which these lipid changes and vascular risk factors influence the incidence of AD remains unclear, growing evidences point towards the involvement of abnormal cholesterol transport, distribution and accumulation in the pathology of both vascular and Alzheimer's diseases.

1.4 PCSK9

Many modulators of lipid homeostasis in the vascular system and also present in the CNS, are now being investigated in the context of AD pathophysiology. Most of these lipid-related genes are among the most important genetic risk factors in the AlzGene database. Variants in APOE, APOJ, PICALM, BIN1, SORL1, LDLR, APOC genes, just to name those few, all relate to lipid metabolism and have shown association with AD

risk (http://www.alzgene.org) (Bertram et al., 2007). More than five years ago, one gene in particular drew my attention. This gene is the proprotein convertase subtilisin/kexin type 9 (PCSK9), a member of the subtilisin family of secretory serine proteinases.

The human genome is predicted to encode ~30 000 gene products in which approximately 500-600 genes are mammalian proteases that belong to one of the six classes of proteases; serine, metallo, cysteinyl, aspartyl, threonine and glutamyl (Seidah et al., 2006). Classification is based on the nucleophile required for their protease activity and on the identity of their catalytic subunit. These proteases are involved in the cleavage of precursor proteins (proproteins) in multiple cellular compartments including those of the secretory pathway (from the endoplasmic reticulum, golgi apparatus, endosomes, lysosomes, secretory vesicles, dense core granules to the cell surface). To date, nine subtilisin proprotein convertases (PC) have been identified; PC1/3, PC2, FURIN, PC4, PC5/6, PACE4, PC7, SKI-1/S1P and PCSK9, all of which have distinct functions in lipid metabolism. These PCs are secretory proteolytic enzymes that through the secretory pathway, cleave precursor proteins into their biological mature active forms. The relevance of activating proproteins via limited proteolysis is to generate diversity within the proteome by increasing the number of polypeptide products that can be generated from a single precursor protein. PCs are such diverse elements as their substrates are growth factors, cytokines, adhesion molecules, hormones, enzymes, receptors, transcription factors and even surface glycoproteins of infectious agents (Bergeron et al., 2000; Seidah and Chretien, 1999; Seidah and Prat, 2002).

Most of the PCs play essential role in embryogenesis and lipid homeostasis as demonstrated by apparent abnormal phenotypes with growth retardation and peripheral and CNS developmental abnormalities in PC knockout mouse models (Creemers and Khatib, 2008; Scamuffa et al., 2006). In addition to their requirement for many physiological processes, these enzymes have also been linked to various pathologies such as tumorigenesis, diabetes, lipid disorders, infectious diseases and AD (Creemers and Khatib, 2008; Khatib et al., 2002). More specifically, Furin and PC5/6 have been involved in the neurodegenerative processes of AD by their direct implications in the

proteolytic cleavage of both α- and β-secretases (Benjannet et al., 2001; Creemers et al., 2001). Those convertases along with PACE4 were also shown to activate endothelial and lipoprotein lipase and influence the development of dyslipidemia and atherosclerosis (Jin et al., 2007). Deficiency of PC2 or PC1/3 have demonstrated important neuroendocrine functions and in humans, PC1/3 have been associated with obesity (Allen et al., 2001; Jackson et al., 1997).

1.4.1 Structure, processing and regulation of PCSK9

Interestingly, PCSK9 was shown to only be expressed in vertebrates suggesting it is the result of a recent evolution. Originally characterized by Seidah and coworkers in 2003, the human PCSK9 gene is localized on chromosome 1 and is synthesized as a 692 amino acids proPCSK9 zymogen (Seidah et al., 2003). Like other convertases, proPCSK9 undergoes in the endoplasmic reticulum, an autocatalytic processing of its N-terminal prosegment. This cleaved prosegment is believed to remain attached to PCSK9 and inhibits the activity of this protease by presumably preventing potential substrates access to the active site (Seidah et al., 2003). As shown in figure 5, PCSK9 contains: a signal peptide (SP) that during the translocation process is cleaved in the endoplasmic reticulum; an inhibitory prosegment (Pro) which is critical for the integrity of the prosegment-PCSK9 complex (Cunningham et al., 2007); a subtilisin-like catalytic domain including the catalytic triad (Asp186, His226, and Ser386 residues) and the binding site of the EGF-A domain of the LDLR (Zhang et al., 2007); and the C-terminal cysteine and histidine rich domain (Cunningham et al., 2007).

This protease has been shown to be regulated at the transcription level by sterol regulatory element binding proteins, more specifically SREBP-2 (Horton et al., 2003; Maxwell et al., 2003). As mentioned previously, SREBP are transcriptional factors regulated by cholesterol levels. When intracellular sterol levels are low, SREBP-2 are activated, released in the cytosol and migrate to the nucleus where they activate transcription of genes involved in cholesterol metabolism including HMGCoAR and the LDLR (Horton et al., 2002; Horton, 2002). Like many other sterol-responsive genes,

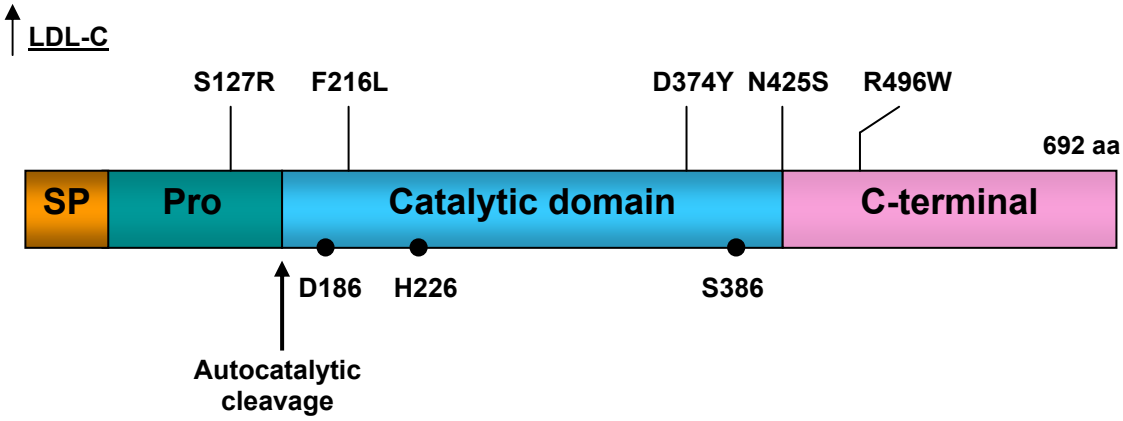

Figure 5: Structure of the human proPCSK9 protein.

PCSK9 is a precursor protein that is synthesized as a 692 amino acids (aa) proprotein. It contains a signal peptide (SP), a prosegment (Pro) that is autocatalytically cleaved through the secretory pathway, a catalytic domain containing the catalytic triad (D186, H226 and S386) and a cysteine and histine rich C-terminal domain. Mutations indicated above the chart represent some of the gain-of-function mutations in PCSK9 reported to associate with hypercholesterolemia.

PCSK9 contains a sterol regulatory element in its promoter region, the primary binding site of SREBP-2, which governs the sterol-regulated transcription of PCSK9 (Jeong et al., 2008). This protease expression was shown to be upregulated in transgenic mice overexpressing SREBP as well as in cultured cells depleted of cholesterol (Dubuc et al., 2004; Horton et al., 2003) whereas PCSK9 expression was significantly reduced in mice fed with a cholesterol-rich diet (Maxwell et al., 2003). These findings were the first clues reporting sensitive responses of PCSK9 to changes in cellular cholesterol levels and first evidences supporting a role for this protease in cholesterol metabolism.

1.4.2 PCSK9 and familial hypercholesterolemia

In the last decade, the PCSK9 gene has been recognized as the third gene associated with autosomal dominant hypercholesterolemia (ADH) (Abifadel et al., 2003). This inherited disorder causes a selective increase in serum LDL-cholesterol concentrations and gives rise to deposition of cholesterol in tissues, tendon and skin xanthomas, arcus cornea, progressive and premature atherosclerosis and cardiovascular diseases. The two other genes leading to ADH are the LDLR and apolipoprotein B (APOB), the latter being the LDLR ligand in the periphery (Goldstein and Brown, 1978; Innerarity et al., 1987). Since the discovery that gain-of-function mutations in PCSK9 associate with hypercholesterolemia, loss-of-function mutations were also described and linked to hypocholesterolemia and reduction of coronary heart diseases (Cohen et al., 2005; Cohen et al., 2006). The mechanism behind these pathologies is essentially related to one of the central role of PCSK9 to modulate the LDLR turnover in the liver (Maxwell and Breslow, 2004).

Several mutations and single nucleotide polymorphisms in PCSK9 gene have now been discovered and further to their risk of developing ADH, some have shown associations with coronary heart disease, premature myocardial infarction, ischemic stroke or intracranial atherosclerosis (Abboud et al., 2007; Chen et al., 2005). It is of interest to note that the E670G polymorphism of PCSK9 which affects plasma LDL-cholesterol levels and severity of coronary atherosclerosis (Chen et al., 2005), also

associates with increased carotid artery intima media thickness (IMT) with additive effects when stratified according to the APOE ε4 allele. A more favourable plasma lipid profile and decreased IMT was observed in carriers of the G670 PCSK9 and APOE ε2 and APOE ε3 compared to APOE ε4 carriers (Norata et al., 2009).

1.4.3 Role of PCSK9 and regulation of the LDLR

Nowadays, PCSK9 is well accepted as a major player in the regulation of plasma cholesterol homeostasis via its ability to downregulate LDLR expressed at the cell surface of the liver. Overexpression of PCSK9 in mice led to the first observation linking PCSK9 to increased plasma LDL-cholesterol levels combined with reduced LDLR protein but not mRNA levels (Maxwell and Breslow, 2004). Conversely, the absence of PCSK9 in knockout mice demonstrated ~50% reduction in circulating LDL-cholesterol due to a 3 fold increase in hepatic LDLR and higher cholesterol clearance rate (Rashid et al., 2005). Furthermore, the administration of statins to PCSK9 (-/-) animals sharply increases the LDLR expression, which causes a twofold increase in the plasma LDL-cholesterol clearance rate (Rashid et al., 2005). Although PCSK9 was found to also affect LDLR levels in extrahepatic organs such as adipose tissue, lung and kidney, its more drastic effect is observed in the liver (Schmidt et al., 2008). Interestingly, a critical role for this convertase in the early days of liver regeneration was recently proposed (Seidah et al., 2003; Zaid et al., 2008). The regenerating liver of PCSK9-deficient mice exhibited necrotic lesions which were prevented by a high-cholesterol diet, indicating that the major role of PCSK9 during liver regeneration resides in its ability to regulate cholesterol levels (Zaid et al., 2008).

The LDLR removes LDL-cholesterol from the circulation through endocytosis of LDL/LDLR complexes within clathrin-coated regions (Fielding and Fielding, 1996). Although the exact mechanism by which PCSK9 modulates LDLR protein expression remains to be fully elucidated, the latter is believed to be directed through the endosomal/lysosomal degradation pathway which prevents its recycling at the cell surface. A proteosomal degradation mechanism has been excluded as the PCSK9-induced

degradation of the LDLR was not affected by inhibitors of the proteosome or of cysteine-, aspartly- or metalloproteases (Maxwell et al., 2005). Evidences suggest that PCSK9 mediate degradation of the LDLR via both intracellular and extracellular LDLR endocytic pathways (Lagace et al., 2006; Maxwell and Breslow, 2004; Park et al., 2004; Poirier et al., 2009) while its catalytic activity would not be required (Li et al., 2007b; McNutt et al., 2007).

1.4.4 Role of PCSK9 in the brain

Although much less expressed in the brain than other organs like liver, intestine or kidney, PCSK9 expression was observed in specific brain areas such as the cerebellum, hippocampus and brain cortex (Zaid et al., 2008). Compared to the large body of literature on PCSK9 in the periphery, far fewer studies investigated its function in the brain.

In collaboration with Seidah and his group, I previously demonstrated that PCSK9 promotes cortical neurogenesis *in vitro* (Seidah et al., 2003). Its overexpression in mouse cortical progenitor cells led to enhanced recruitment of undifferentiated neural progenitor cells into the neuronal lineage whereas a mutant form (H226A) had no significant effect (Seidah et al., 2003). That was the first study supporting a putative role for PCSK9 in the brain, more specifically in neuronal differentiation. Three years later, a role for PCSK9 in the development of the nervous system distinct from that in cholesterologenic organs was suggested following neuroectodermal differentiation of P19 cells (Poirier et al., 2006). Although PCSK9 has not affected the levels of LDLR in these differentiated cells, this protease was shown to efficiently bind and degrade LDLR and its two closest family members, the ApoER2 and VLDLR, in different cell lines (Poirier et al., 2008b; Shan et al., 2008). On the other hand, controversy remains as a very recent study showed that levels of these receptors were not affected by PCSK9 in the normal CNS (Liu et al., 2010). Earlier studies revealed that another closely related transmembrane receptor, LRP1, is not affected by PCSK9 (Benjannet et al., 2004; Park et al., 2004).

LRP1, apoER2, and VLDLR are significantly expressed in the brain and involved in several signalling pathways during development, synapse formation and maintenance of neuronal functions in the adult brain (Herz, 2009; Herz and Chen, 2006). While a putative role for PCSK9 in the development of the nervous system has been suggested, it is possible that PCSK9 exerts its function independently of a receptor-mediated degradation mechanism. In addition, it should be noted that no deleterious effects were observed in knockout mice (Rashid et al., 2005) or in two women lacking functional PCSK9 (Hooper et al., 2007; Zhao et al., 2006).

Nevertheless, it is now generally accepted among experts that PCSK9 i) acts as an important regulator of plasma cholesterol homeostasis, ii) modulates the levels of LDLR protein expression at the cell surface of the liver and other peripheral organs, iii) genetic variations affect blood cholesterol levels and lead to hypo- or hypercholesterolemia, iv) gain-of-function mutations are associated with ADH, atherosclerosis and stroke and v) is involved in liver regeneration (Zaid et al., 2008).

1.5 Rationale and objectives of the research

Numerous evidences now link cholesterol homeostasis to the pathogenesis of AD. The majority of studies have focused on the relationship between apoE, amyloid metabolism and cholesterol homeostasis. Research in the cardiovascular field tells us that several proteins involved in peripheral cholesterol homeostasis are also present in the CNS and may worth some investigations in regard of AD.

The LDLR is a good example as it has a well established role in regulating circulating LDL-cholesterol as well as brain cholesterol levels during development, following brain damage or under neurodegenerative conditions. Whether levels of peripheral cholesterol are altered or brain cholesterol transport and distribution system dysfunctional, one common element for both systems is the LDLR. Consequently, defective LDLR endocytosis mechanisms which affect peripheral or brain cholesterol homeostasis may ultimately increase the risk of hypercholesterolemia and

(cerebro)/vascular disorders as well as certain type of neurodegenerative diseases. Transport of cholesterol through the LDLR endocytosis system is therefore a relevant target in the context of AD. Given that PCSK9 was recently identified as one of the key regulator of LDLR degradation in the periphery, this prompted us to formulate the following general working hypothesis;

In the brain, PCSK9 may play a role in cholesterol homeostasis by regulating the expression of the LDLR proteins under normal and especially neurodegenerative conditions.

In the **first study** (Chapter 2), we took advantage of having the Brain Bank here at the Douglas institute to investigate changes in PCSK9 expression in AD brains characterized with advanced neurodegeneration. We wanted to determine whether this protease is involved in neurodegenerative processes such as those found in AD. Using polymerase chain reaction and immunoreactivity approaches, we answered the following specific questions:

1. Is the expression of PCSK9 altered in different post-mortem brain regions of AD compared to non-demented patients?
2. Does the expression of the LDLR vary between control and AD brains and if so, does it correlate with changes in PCSK9 expression?
3. Do genetic variants in PCSK9 influence the risk of AD?
4. Do these variants or PCSK9 expression correlate with the extent of AD neurodegeneration?

In the **second study** (Chapter 3), we used the entorhinal cortex lesion model in mice to investigate whether PCSK9 is implicated in cholesterol-driven synaptic remodelling processes related to neuronal plasticity and synaptogenesis following brain damage. These questions were answered:

1. Does the expression of these lipid-related proteins change over the deafferentation and reinnervation phases occurring after ECL?
2. Do their expressions correlate with that of known modulators of cholesterol?

In the **third study** (Chapter 4), we used an *in vitro* model of reactive neuronal plasticity to determine whether PCSK9 ectopic expression regulates the protein levels of LDLR and ApoER2 during reactive synaptogenesis. Neuronal-like cells were used to investigate PCSK9 functions in lesion-induced synaptic remodelling processes.

Chapter 2: Study 1

INCREASED EXPRESSION OF PROPROTEIN CONVERTASE PCSK9 IN LATE-ONSET ALZHEIMER'S DISEASE BRAIN

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2.1 Preface to Chapter 2

The growing appreciation that there are some overlaps between pathological processes leading to vascular diseases and dementia, have highlighted the relationship that exists between disturbances in cholesterol homeostatic processes and AD pathology. This is primarily based on the discovery that apoE genotype significantly impacts the age of onset and increases the risk of developing AD (Poirier et al., 1993b; Strittmatter et al., 1993). The findings that i) elevated plasma cholesterol in mid-life associates with an increased risk of AD and ii) that intracellular cholesterol regulates Aβ peptides formation that accumulate in the brain of AD patients (Kivipelto et al., 2002; Pappolla et al., 2003) have strongly suggested that genes known to play a role in cholesterol metabolism such as lipid cholesterol biosynthesis, trafficking and intracellular metabolism, in the periphery as well as in the central nervous system, may participate in the pathogenesis of AD.

Gain of function mutations in PCSK9 gene have been associated with elevated levels of plasma cholesterol leading to familial hypercholesterolemia (Abifadel et al., 2003; Abifadel et al., 2007; Leren, 2004; Timms et al., 2004). In opposite, mutations in PCSK9 were also found to cause hypocholesterolemia as individuals with those genetic variants have significantly lower plasma LDL-cholesterol levels (Cohen et al., 2005; Cohen et al., 2006; Zhao et al., 2006). Of interest, the prevalence of these loss-of-function mutations in PCSK9 gene is higher in African populations or American individuals of African descent and carriers of these mutations show protection from cardiovascular disease (Cohen et al., 2005; Cohen et al., 2006; Hooper et al., 2007). The involvement of PCSK9 in plasma cholesterolemia most likely reflects its influence on hepatic LDLR levels (Maxwell and Breslow, 2004; Park et al., 2004).

In the following study, we investigated whether some common single nucleotide polymorphisms in PCSK9 may associate with a higher risk of developing AD. Since brain damage or neurodegeneration is known to affect cholesterol turnover in the CNS, we examined whether PCSK9 may be involved in brain lipid homeostatic processes by modulating LDLR levels.

2.2 Abstract

Increasing evidence highlights the importance of cholesterol in the pathophysiology of Alzheimer's disease (AD). The proprotein convertase PCSK9 has been recently associated with familial hypercholesterolemia. It regulates plasma cholesterol metabolism by enhancing low-density-lipoprotein receptor (LDLR) protein catabolism. Here, we demonstrate for the first time that PCSK9 expression is increased in cortical and hippocampal areas but not in the cerebellum of AD subjects. Five common PCSK9 polymorphisms were examined for genetic associations with AD and modulation of gene expression. Although none of PCSK9 variants showed associations with non-stratified AD cases, men carrying either the rs2483205-C, rs483462-A or rs662145-T allele showed a significant higher risk for AD. No correlations were detected between PCSK9 risk variants and its expression levels or with neurofibrillary tangle or amyloid plaque densities; suggesting a secondary involvement of PCSK9 in AD pathophysiology. We further demonstrated that cortical LDLR mRNA prevalence but not protein levels are increased in the AD brain. Altogether, these findings suggest that PCSK9 expression is upregulated in response to the ongoing neurodegeneration and, may be implicated in the regulation of cholesterol transport and internalization mediated by the apoE/LDLR pathway during compensatory axonal and synaptic remodelling.

Keywords: Alzheimer's disease; PCSK9; LDLR; cholesterol homeostasis; neurodegenerative processes; synaptic remodelling.

2.3 Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It is characterized by progressive brain degeneration and cognitive decline. The neurodegeneration occurring in this age-related disease is hypothesised to be the result of the progressive accumulation of insoluble beta-amyloid (Aβ) aggregates and neurofibrillary tangles (NFT) over time (Hardy and Selkoe, 2002).

In recent years, a number of findings have reported a strong relationship between lipid metabolism dysfunction and AD. The prevalent genetic association of apolipoprotein E (APOE) ε4 allele with late-onset-AD (LOAD) (Poirier et al., 1993b; Strittmatter et al., 1993) along with the function of apoE as the main cholesterol carrier in the brain have emphasised the potential importance of cholesterol transport and mobilization in AD pathophysiology. Indeed, considerable evidences have shown that changes in lipid content or distribution directly influence the neuropathological processes in AD and cognitive impairment.

Several studies in animal models indicated that hypercholesterolemia favours Aβ production (Jaya Prasanthi et al., 2008; Shie et al., 2002; Sparks et al., 1994) whereas the activity of beta and gamma-secretases, involved in Aβ generation, was inhibited by low levels of cholesterol (Grimm et al., 2008). In addition, a link between cholesterol and tangle formation was first reported in Niemann-Pick type C (NPC) disease. This neurodegenerative disorder, caused by mutations in the NPC1 and NPC2 genes, leads to the accumulation of free cholesterol in the endosomes and lysosomes of neurons due to an aberrant lipid trafficking/storage. This excessive lipid deposition was shown to affect NFT formation in both NPC and AD neurons (Distl et al., 2001; Ohm et al., 2003).

In the last decade, many studies have revealed that cardiovascular-related risk factors such as hypertension, hypercholesterolemia, atherosclerosis, diabetes mellitus and stroke also behave as risk factors for both vascular dementia and sporadic AD (Breteler, 2000; de la Torre, 2004). This, in turn, prompted the investigation of several genes involved in lipid metabolism for possible association with AD. Most of these lipid-related

genes actually belong to the top 30 list of the most important genetic risk factors in the AlzGene database (http://www.alzgene.org) (Bertram et al., 2007).

Recently, the proprotein convertase subtilisin/kexin type 9 (PCSK9) was reported to behave as a key player in plasma cholesterol homeostasis. PCSK9 is a member of the subtilisin family of secretory serine proteinases and is highly expressed in the liver, kidney, small intestine and to lesser extent, in the brain (Seidah et al., 2003; Zaid et al., 2008). Proprotein convertases are responsible for the proteolytic maturation of secretory proteins including growth factors, hormones, receptors, enzymes, transcription factors and cell surface proteins (Seidah et al., 2003; Seidah and Prat, 2002). However, no direct substrates for PCSK9 have been identified so far and this protease has the ability to function independently of its proteolytic activity (Li et al., 2007b; McNutt et al., 2007).

Genetic studies have demonstrated that PCSK9 is formally associated with an autosomal dominant form of familial hypercholesterolemia (Abifadel et al., 2003). The mechanism responsible for this pathology is related to the ability of PCSK9 to regulate plasma low-density-lipoprotein (LDL) cholesterol levels via post-translational downregulation of the cell surface LDL receptors (LDLR) (Benjannet et al., 2004; Maxwell and Breslow, 2004). It is now proposed that PCSK9 interacts directly with the LDLR, causing the formation of a protein complex that is rapidly targeted for degradation in the late endosomal/lysosomal pathway (Poirier et al., 2009; Zhang et al., 2007). Therefore, PCSK9 prevents the recycling and rerouting of the LDLR through the cell surface and indirectly regulates plasma cholesterol concentrations.

In the central nervous system (CNS), the LDLR plays a crucial role in the maintenance of brain cholesterol homeostasis as it mediates the interaction and endocytosis of apoE-cholesterol lipoparticles. Internalization of this free cholesterol can serve as an immediate source of lipids for membrane synthesis and neurites elongation. Mobilization and distribution of cholesterol to neurons via the apoE/LDLR uptake pathway is therefore central for synapse formation and remodelling during development or under neurodegenerative conditions, as well as for general maintenance of neuronal

plasticity and functions (Pfrieger, 2003a; Poirier et al., 1993a; Poirier, 1994). Absence of brain LDLR in mice causes a loss of synaptic integrity and plasticity accompanied with impairment of learning and memory functions (Mulder et al., 2004; Mulder et al., 2007). PCSK9 was shown to be markedly active during CNS development (Poirier et al., 2006; Seidah et al., 2003). However, very little is known regarding its potential function in the adult and aging brain.

Considering the major role of PCSK9 in plasma cholesterol metabolism, we hypothesised that PCSK9 play a similar role in the brain, particularly in response to injury or neurodegenerative processes such as those found in AD. In the present study, we examined the expression of PCSK9 in the brain of a large cohort of autopsy-confirmed AD cases, and investigated whether single-nucleotide polymorphisms (SNP) in PCSK9 gene display association with the risk of sporadic AD.

2.4 Materials and methods

Subjects

All AD and control subjects used in this study originate from the same eastern Canadian population isolate. For the genetic association study, 394 autopsy-confirmed AD cases and 289 age-matched control cases were obtained from the Douglas Institute Brain Bank in Montreal, Canada whereas 153 clinical AD cases and 147 age-matched control subjects were obtained from the McGill Centre for Studies in Aging, Montreal, Canada. From those, 71 AD and 40 age-matched control brain tissues were used for biochemical analyses. Neuropathological analyses were consistent with the criteria used in the classification of Khachaturian (Khachaturian, 1985) and were defined as sporadic AD because family history did not reveal any first-degree relative with AD. Age-matched clinical control cases were free of neurological or neuropsychiatric diseases and autopsy-confirmed control cases were free of brain neuropathological lesions (NFT and senile plaques (SP) $< 20/mm^3$ and $< 10/mm^2$, respectively). This study is conformed to The Code of Ethics of the World medical Association and was approved by the Ethics Board of the Douglas Hospital Research Center. All participants signed an informed consent.

RNA isolation

Total RNA was isolated from frozen frontal cortex and cerebellum tissues of 40 AD cases (50% men and women, mean age at death 78.1 ± 7.7 years) and 40 controls (50% men and women, mean age at death 70.0 ± 10.3 years) with a mean post-mortem delay of 23.1 hours. The Maxwell 16 Tissue LEV total RNA Purification kit and Maxwell 16 instrument (Promega, Madison, WI) were used for RNA extraction. Purity and quality of each RNA extract was analysed by spectrophotometric A_{260nm}/A_{280nm} ratio and by Bioanalyser (Agilent Technologies, Palo Alto, CA) according to the manufacturer's guidelines.

Real-time polymerase chain reaction (RT-PCR)

The reverse transcription (RT) reaction was performed with the high capacity cDNA RT kit (Applied Biosystems, Foster City, CA) on a Multigene thermal cycler (Labnet International Inc., Woodbridge, NJ). Total RNA (500 ng) was used for each 50 µl PCR reaction. The quantitative real-time PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) with assay-on-demand gene expression product for PCSK9 and LDLR as target genes and hypoxanthine phosphoribosyltransferase (HPRT) as an endogenous control (FAM™ reporter dye on the target gene probes, VIC™ reporter dye on the endogenous control gene probe, Applied Biosystems). TaqMan® Fast Universal PCR Master mix and 3 µl of cDNA were added in a total volume of 10 µl and thermal cycling conditions were; 2 minutes (min) at 50ºC; 10 min at 95ºC; followed by 40 cycles of 15 seconds (sec) at 95ºC and 1 min at 60ºC. All samples were analysed in triplicate and the average Ct value was used in all analyses. Relative gene expression (expressed as fold change of mRNA levels in all AD tissues relative to the average of mRNA levels in control tissues) was calculated using the $2^{-\Delta\Delta Ct}$ method.

Brain tissues and ELISA assay

Frozen human hippocampal, frontal cortex and cerebellum tissues from 71 AD cases (mean age at death 78.4 ± 9.3 years) and 38 controls (mean age at death 69.9 ± 11.9 years) with a mean post-mortem delay of 23.5 hours were used for the protein expression study. Brain tissues were sonicated on ice in bicarbonate/carbonate solution (100 mM, pH

9.6) and centrifuged at 3000 rpm for 10 min at 4ºC except frontal cortex tissues that were sonicated in a buffer containing 30 mM EDTA, 250 mM NaCl, 1 mM DTT, 50 mM K_2HPO_4 (pH 7.2) and centrifuged for 20 min at 4ºC. Protein concentration in supernatants was measured with the BCA protein dosage kit (Pierce, Rockford, Il).

Indirect ELISA was performed on Costar 96-well EIA/RIA plates (Fisher Scientific, Ottawa, ON) that were first incubated overnight at 4ºC with brain protein homogenates and purified synthetic PCSK9 peptide (CRSRHLAQASQELQ) used as standard (437 to 7500 µg/ml), applied in triplicate. The plates also included blank wells that contained the bicarbonate/carbonate buffer without brain homogenates. The next day, the primary antibody (goat polyclonal PCSK9 antibody, ab28770, Abcam, Cambridge, USA) diluted in a blocking solution (phosphate buffered saline (PBS) 10 mM and bovine serum albumin (BSA) 1%) was added to each wells for 2 hours at room temperature. Then, all wells were rinsed with washing buffer (0.1% Tween-20/ 1.0 M Tris base salt, TBS-T) and incubated with the detection antibody in blocking buffer (biotinylated goat antibody, ab6740, Abcam) for another 2 hours. After further washes, the plates were incubated with an alkaline-phosphatase streptavidin solution for 1 hour (Invitrogen Canada Inc., Burlington, ON). Following this incubation, wells were washed and an alkaline phosphatase fluorescent substrate (AttoPhos, Promega, San Luis Obispo, USA) was added to each wells for 30 min at 37ºC. Fluorescence was detected with a microplate fluorescent reader (FL600, Bio-Tek, Winooski, VT) with a 450 nm excitation filter and 580 nm emission filter. Results are expressed as the mean fold increase of protein concentrations in AD cases relative to the average concentration in control subjects ± standard error mean (SEM). For LDLR protein quantification, we used mouse liver homogenates (sonicated in PBS 10 mM) to produce a standard curve with concentrations ranging from 4 to 135 µg/ml. The primary and detection antibodies used were a rabbit polyclonal LDLR antibody (NB110-57162, Novus Biological, Littleton, USA) and a biotinylated rabbit antibody (ab6720, Abcam), respectively. Western blot analyses were used to confirm the selectivity of the antibody and the integrity of the full length protein (not shown).

PCSK9 and APOE genetic analyses

The genetic association study was performed on a cohort of 547 autopsy-confirmed AD cases (mean age at death of 78.9 ± 8.6, 38.7% male) and 436 age-matched control subjects (mean age at death of 73.4 ± 11.6, 48.3% male). Genomic DNA was extracted from brain tissues, blood or blood lymphocytes using the DNeasy kit, the QIAamp DNA blood kit (both from Qiagen, Mississauga, ON) or automated DNA extraction (NA-1000, AutoGen, Massachusetts, USA), respectively. Standard PCR reaction conditions and primer pairs that were synthesized by Eurofins MWG Operon (Alabama, USA) were used to amplify PCSK9 target sequences. Amplification was carried out in a total volume of 50 µl on a Stratagene Gradient thermocycler (Agilent Technologies) for an initial denaturation step of 5 min at 95ºC followed by 45 amplification cycles (95ºC for 45 sec; 55ºC for 45 sec; 72ºC for 1 min) and a final extension step of 5 min at 72ºC. Then, PCSK9 SNPs (rs2483205, rs499883, rs483462 and rs662145) were genotyped by pyrosequencing on the PyroMark™ Q96 ID instrument (Biotage, Sweden). Sets of primers were designed by the PyroMark assay design software (Biotage).

The PCSK9 rs2479409 SNP was genotyped using the restriction enzyme BSAH1 (New England BioLabs Inc., ON). DNA was amplified using forward primer 5′- CCA GTT GAT TTC TTG AAC ATG G-3′ and reverse primer 5′- TTG CTT GCT TTT GAT GTC CAG-3′ for an initial step of 10 min at 95ºC followed by 35 cycles (95ºC for 1min30; 60ºC for 2min30; 72ºC for 3 min) and a final elongation step of 10 min at 72ºC. Amplicons were digested with BSAH1 for 1h30 at 37ºC and digests were visualised on agarose gel. APOE genotype was determined by allele-specific extension of purified brain DNA according to standard PCR methods described elsewhere (Nalbantoglu et al., 1994; Poirier et al., 1993b).

Biochemical analyses

Aβ40 and Aβ42 peptide levels in human hippocampal and frontal cortices were measured from 24 AD and 18 control tissues using a sandwich ELISA assay as previously described (Beffert et al., 1999). NFT and SP densities in 62 AD and 14 control tissues

were determined in six brain areas (CA1 region of the hippocampus, subiculum, parasubiculum, fusiform gyrus, frontal and parietal cortex) and neuropathological staining was performed as described in (Blain et al., 2006a). Total brain cholesterol was extracted from frontal cortex and cerebellum areas of 71 AD and 19 control frozen tissues and quantified using a sensitive high-performance liquid chromatography (HPLC) technique described before (Blain et al., 2006a). The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity was measured on 59 AD and 33 control brain extracts from the frontal cortex and cerebellum regions and quantified by HPLC whereas apoE levels in human frontal cortex from 50 AD and 30 control brain tissues were determined by ELISA as described in (Beffert et al., 1999).

Statistical analysis

Comparisons of PCSK9 and LDLR expression levels in AD and controls were done by one-way ANOVA tests using the SPSS 15.0 software. Associations between PCSK9 variants and its expression were also analysed by ANOVA univariate analysis. Pearson correlations were used to assess relationships between PCSK9 variants or protein expression and NFT, SP, Aβ, cholesterol, HMGCoAR activity or apoE levels. Genetic associations between PCSK9 SNPs and the risk of AD were analysed by binary logistic regression model in the entire population or, when adjusted for gender or APOE status. Hardy-Weinberg equilibrium (HWE) was tested for each group with the HaploView 4.1 or the DeFinetti computer program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Genetic association is presented as odds ratio (OR) with the 95% confidence interval (CI). Haplotypes were estimated using the HaploView 4.1 software and blocks were delineated using the solid spine of linkage disequilibrium (LD) method. Rare haplotypes (frequency < 5%) were excluded from the analysis. All statistical tests were considered significant with a p value < 0.05.

2.5 Results

2.5.1 PCSK9 expression is increased in LOAD

In order to investigate the contribution of PCSK9 in AD, its expression was assessed in the brain of autopsy-confirmed AD cases and age/gender-matched control cases. mRNA prevalence was first assessed by quantitative-RT-PCR and the relative fold difference between PCSK9 mRNA levels in frontal cortex and cerebellum tissues of AD was compared to control cases as illustrated in figure 6. PCSK9 gene expression in the human frontal cortex was found to significantly differ in AD (2.2 fold increase, $p < 0.01$) versus age-matched control cases (Fig. 6A) but not in the cerebellum area (a low pathology control brain area; Fig. 6B). Similarly, ELISA analyses of PCSK9 protein contents revealed robust increases in the hippocampus (1.6 fold, $p < 0.001$, Fig. 7A) and frontal cortex (1.8 fold, $p < 0.001$, Fig. 7B) of AD patients compared to age-matched controls, but not in the low pathology cerebellum area (Fig. 7C).

2.5.2 LDLR gene expression is upregulated in LOAD but not its protein levels

Consistent with the PCSK9 observations, the LDLR mRNA prevalence was significantly increased in the frontal cortex of AD patients relative to age-matched controls (1.8 fold, $p < 0.01$, Fig. 8A) but unchanged in cerebellum tissues (Fig. 8B). In contrast, LDLR protein levels remained unaffected in the hippocampus, frontal cortex or cerebellum of AD as compared to control cases (Fig. 9). This observation actually agrees with the notion that PCSK9 normally acts as an enhancer of LDLR degradation in peripheral organs; the more PCSK9 available, the less LDLR detected despite quantitative compensatory upregulation of the LDLR gene expression in the same brain area.

Importantly, no significant correlations were observed between PCSK9 or LDLR relative expression with gender and the APOE ε4 allele in the whole cohort or when stratified according to the disease status (data not shown).

2.5.3 PCSK9 as a candidate gene for LOAD in men

We next determined whether common PCSK9 genetic variants (rs2479409, rs2483205, rs499883, rs483462 and rs662145) significantly associate with sporadic AD in a large case/control association study. Distribution of these five genotypes followed HWE in both AD cases and controls either in the whole population or, when stratified for gender. The global genetic association study for the five PCSK9 SNPs failed to reveal any significant link with AD risk in the whole population (Table 1). None of the five variants were found to affect the age of onset, age of death or disease duration. The APOE4 allele did not affect the PCSK9-mediated risk level in the non-stratified population AD (data not shown). However, the interaction between gender and PCSK9 variants for three of the SNPs was found to be significant in the regression model (Table 1). Logistic regression analyses revealed that males carrying the rs2483205-C allele display an increased risk for AD (OR = 1.81, 95% CI, 1.05-3.10; $p = 0.03$) whereas males having at least one copy of the rs483462-A or rs662145-T allele also showed a significant association with AD risk (OR = 2.17, 95% CI, 1.11-4.24; $p = 0.022$, OR = 2.62, 95% CI, 1.22-5.63; $p = 0.011$, respectively). Females carrying the same alleles are not at risk of developing AD (Table 1).

The degree of LD between the PCSK9 SNPs is summarized in figure 10. The rs483462 and rs662145 polymorphisms display a significant association with each other ($D' = 97$, Fig. 10) but not with AD. However, the 3-site (CAT) haplotype (rs2483205-C, rs483462-A and rs662145-T) analysis nearly revealed a significant association with AD in the male sub-population (6% increase, $p = 0.06$) (Table 2). On the other hand, the haplotype (CT) (rs2483205-C and rs662145-T) showed a significant association with AD in the male sub-group due to an increased frequency of this haplotype (increase of 8%, $p = 0.02$) in AD cases (0.53) compared to controls (0.45) (Table 2). No significant associations were observed for the other haplotype combinations.

2.5.4 Effects of PCSK9 polymorphisms on brain cholesterol metabolism and AD pathology

The association of PCSK9 polymorphisms and the extent of neurodegenerative processes were also examined in autopsied brains. The five genetic variants tested in this study failed to show any associations with Aβ40 and Aβ42 levels and with NFT and SP densities in the frontal cortex or hippocampus of AD cases when compared to age-matched controls (data not shown).

Investigations of the potential impact of PCSK9 variants on three important components of brain lipid metabolism, namely cholesterol level, transport (apoE levels) and synthesis (HMGCoAR activity) were found to be negative. Importantly, none of these PCSK9 genetic variants affected PCSK9 expression in brain tissues from the cohort or, when stratified according to disease, gender or both disease and gender status (data not shown).

To further investigate whether PCSK9 participates in the neurodegenerative processes in AD, we examined if its protein levels in the brain of autopsy-confirmed AD and control cases associates with neuropathological markers of AD. Pearson correlation analyses failed to demonstrate any associations between hippocampal and cortical PCSK9 protein contents and levels of Aβ40 and Aβ42, or NFT and SP densities in six different cortical and hippocampal brain areas (data not shown).

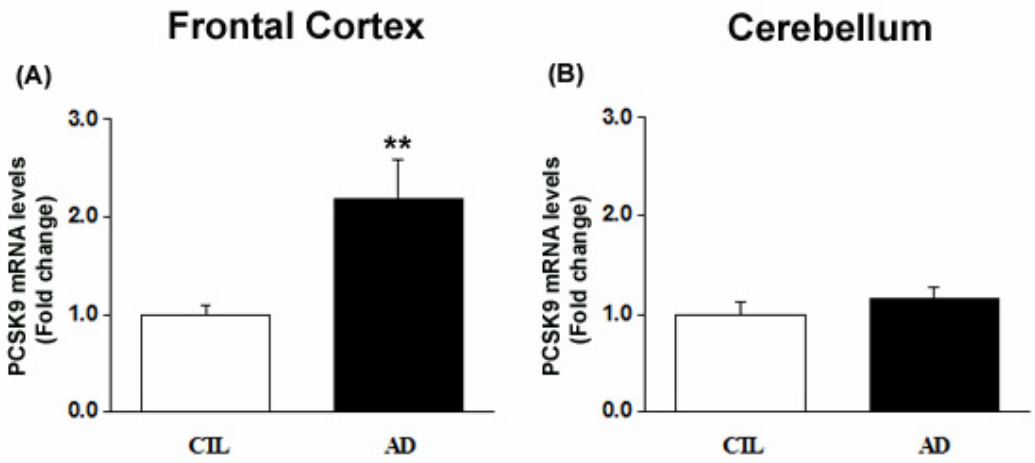

Figure 6: PCSK9 expression in AD compared to control brains.

Relative PCSK9 mRNA levels in the frontal cortex (A) and cerebellum (B) of AD and control (CTL) brains. PCSK9 mRNA levels were quantified by RT-PCR in 40 AD and 40 CTL cases and mRNA levels were normalized to HPRT mRNA expression for the same samples. Gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method and results are expressed as mean of PCSK9 mRNA fold change of each AD subjects relative to the average mRNA levels in control tissues ± SEM. Statistical differences between AD and controls were assessed by one-way ANOVA tests (** $p < 0.01$).

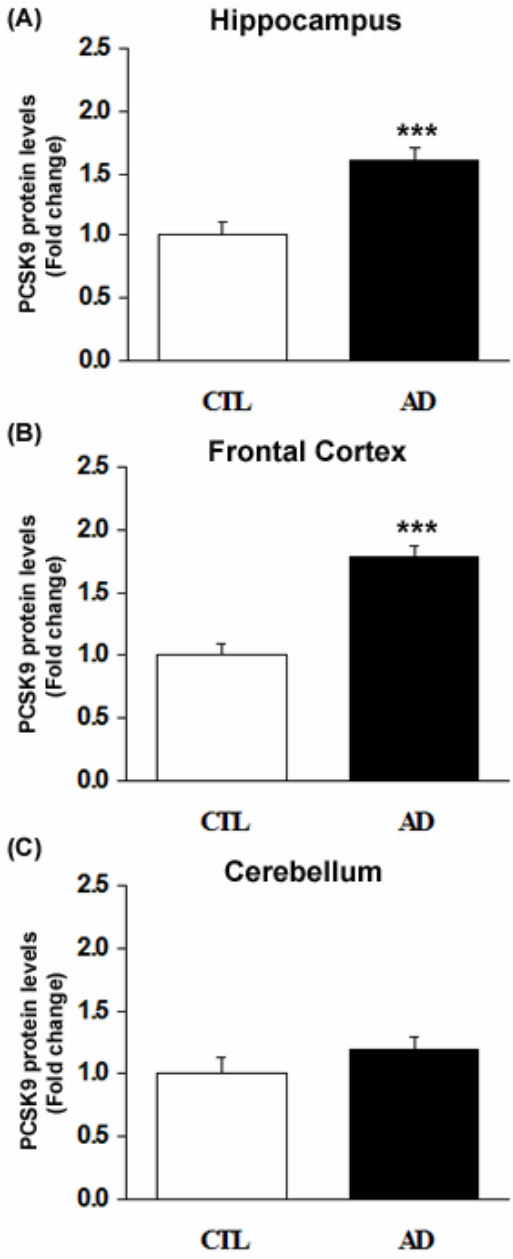

Figure 7: PCSK9 protein levels in AD and control brains.

Relative PCSK9 protein levels in the hippocampus (A), frontal cortex (B) and cerebellum (C) of 71 AD and 38 controls. ELISA assays were used to measure PCSK9 protein expression in brain extracts and results are expressed as mean fold increase of PCSK9 protein levels in each AD cases relative to the average of PCSK9 proteins in control subjects ± SEM. ANOVA analyses revealed significant increases of PCSK9 protein expression in the hippocampus and frontal cortex (**$p < 0.001$) of AD compared to control brains.

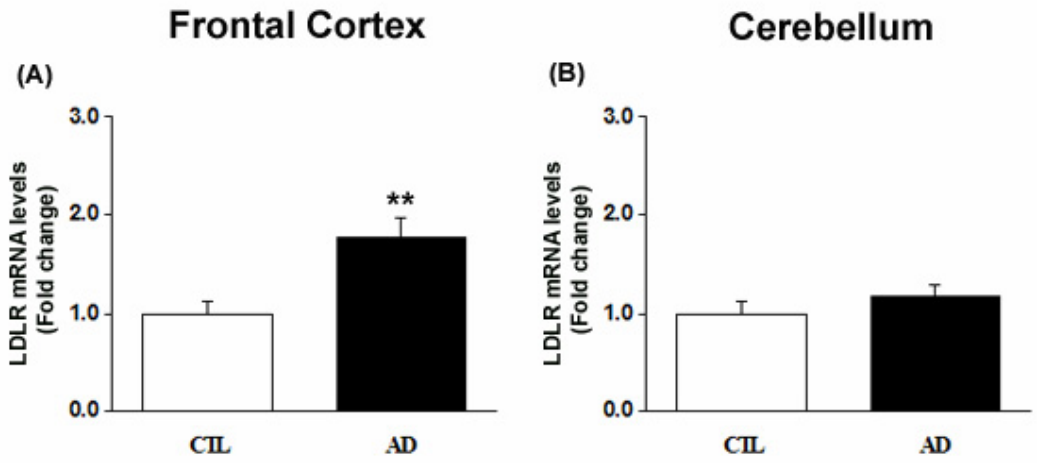

Figure 8: LDLR mRNA expression in AD compared to control brains.

Relative mRNA expression of LDLR in the frontal cortex (A) and cerebellum (B) of AD and control brains. All LDLR mRNA values were normalized to HPRT mRNA levels for the same samples. Quantification by RT-PCR was carried out in triplicate for all brain samples (40 AD and 40 CTL). Results are reported as mean fold induction of LDLR mRNA levels in AD cases relative to the average LDLR mRNA expression in controls ± SEM. Significance was assessed by one-way ANOVA statistical analyses. Transcription of the LDLR in the frontal cortex of AD cases was significantly higher than controls (**$p < 0.01$).

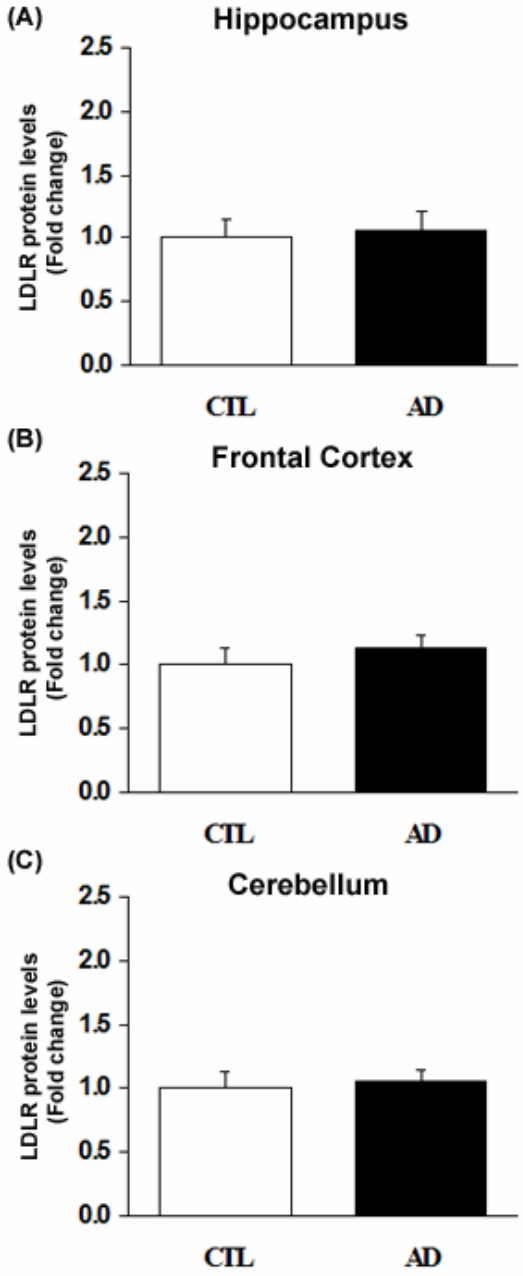

Figure 9: Protein levels of LDLR in AD and control brains.

Relative LDLR protein levels in the brain of AD and control subjects. LDLR protein levels were measured by ELISA in the hippocampus (A), frontal cortex (B) and cerebellum (C) of all brain samples (71 AD and 38 CTL). Results are expressed as a mean of LDLR proteins fold change in AD brains relative to the average of LDLR protein levels in control brains ± SEM. Statistical analyses were assessed by one-way ANOVA tests and no significant differences in LDLR protein levels were observed between AD and control cases.

Table 1: Allele frequency of PCSK9 SNPs in AD cases and controls stratified by gender.

PCSK9 Allele	Total			Men			Women		
	CTL *n* (%)	AD *n* (%)	*p*-value, OR	CTL *n* (%)	AD *n* (%)	*p*-value OR [95% CI]	CTL *n* (%)	AD *n* (%)	*p*-value, OR
rs2479409 (A/G)									
G(−) carrier	193 (44.9)	229 (43.0)	0.59, 0.90	90 (43.1)	89 (43.2)	0.46, 1.26 [0.68-2.35]	103 (46.6)	140 (42.9)	0.25, 0.74
G(+) carrier	237 (55.1)	303 (57.0)	0.57, 1.08	119 (56.9)	117 (56.8)	0.98, 0.99 [0.67-1.47]	118 (53.4)	186 (57.1)	0.40, 1.16
Total	430	532		209	206		221	326	
rs2483205 (C/T)									
C(−) carrier	84 (19.3)	105 (19.2)	0.48, 0.91	41 (19.5)	25 (11.8)	0.19, 0.76 [0.51-1.14]	43 (19.0)	80 (23.8)	0.99, 1.00
C(+) carrier	352 (80.7)	442 (80.8)	0.98, 1.01	169 (80.5)	186 (88.2)	**0.03, 1.81 [1.05-3.10]**	183 (81.0)	256 (76.2)	0.18, 0.75
Total	436	547		210	211		226	336	
rs499883 (A/G)									
G(−) carrier	175 (40.1)	214 (39.1)	0.62, 1.10	89 (42.4)	83 (39.3)	0.65, 1.14 [0.65-2.01]	86 (38.1)	131 (39.0)	0.82, 1.06
G(+) carrier	261 (59.9)	333 (60.9)	0.75, 1.04	121 (57.6)	128 (60.7)	0.52, 1.13 [0.77-1.67]	140 (61.9)	205 (61.0)	0.82, 0.96
Total	436	547		210	211		226	336	
rs483462 (A/G)									
A(−) carrier	39 (8.9)	39 (7.1)	0.40, 1.11	28 (13.3)	14 (6.6)	0.22, 1.27 [0.87-1.86]	11 (4.9)	25 (7.4)	0.97, 1.01
A(+) carrier	397 (91.1)	508 (92.9)	0.30, 1.28	182 (86.7)	197 (93.4)	**0.02, 2.17 [1.11-4.24]**	215 (95.1)	311 (92.6)	0.22, 0.64
Total	436	547		210	211		226	336	
rs662145 (C/T)									
T(−) carrier	32 (7.4)	27 (5.0)	0.69, 1.05	24 (11.6)	10 (4.8)	0.64, 1.10 [0.74-1.61]	8 (3.6)	17 (5.1)	0.85, 1.03
T(+) carrier	399 (92.6)	515 (95.0)	0.11, 1.53	183 (88.4)	200 (95.2)	**0.01, 2.62 [1.22-5.63]**	216 (96.4)	315 (94.9)	0.39, 0.69
Total	431	542		207	210		224	332	

OR, odds ratio; 95% CI, 95% confidence interval.

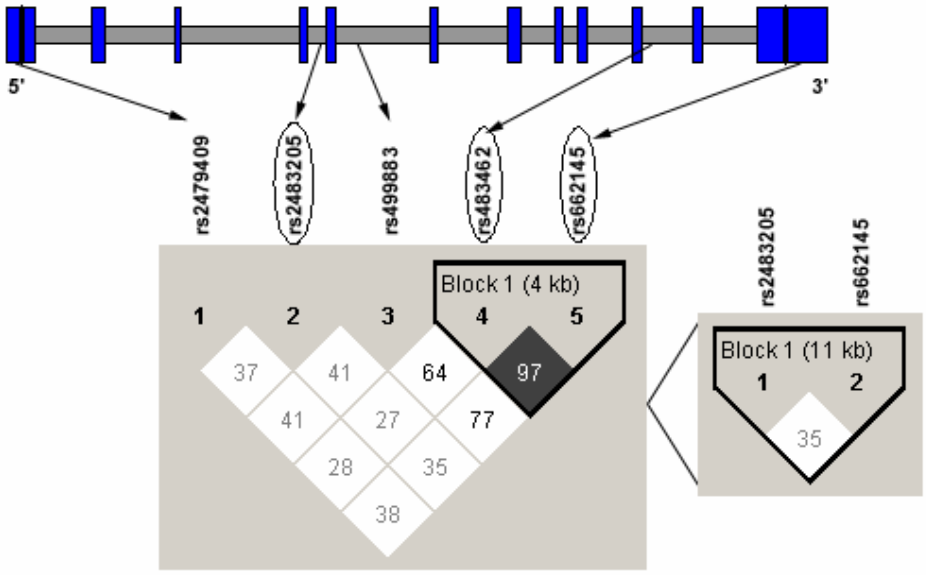

Figure 10: Genomic structure of PCSK9 and location of the five studied polymorphisms.

The black boxes indicate the 12 exons of PCSK9 gene. The rs2479409 SNP is located in the promoter region whereas SNPs rs2483205, rs499883 and rs483462 are in the introns 4, 5 and 10, respectively. The rs662145 SNP is located in the non-coding region of exon 12. Haplotype blocks were delineated using the solid spine of LD method in HaploView software. Linkage disequilibrium between the five PCSK9 SNPs in the whole cohort is indicated within each box and number represents the pairwise LD as assessed by D′.

Table 2: Haplotype association analysis between PCSK9 gene and AD risk stratified by gender.

Haplotype	Total			Men			Women		
	Frequency		*p*-value	Frequency		*p*-value	Frequency		*p*-value
	CTL	AD		CTL	AD		CTL	AD	
rs2483205 (C/T) rs483462 (A/G) rs662145 (C/T)									
CAT	0.44	0.44	0.68	0.43	0.49	**0.06**	0.44	0.41	0.38
TAT	0.28	0.26	0.49	0.27	0.21	0.06	0.29	0.30	0.74
TGC	0.15	0.15	0.87	0.16	0.15	0.75	0.15	0.16	0.59
CGC	0.09	0.08	0.55	0.10	0.09	0.59	0.08	0.08	0.90
rs2483205 (C/T) rs662145 (C/T)									
CT	0.46	0.48	0.38	0.45	0.53	**0.02**	0.47	0.45	0.53
TT	0.29	0.28	0.50	0.28	0.23	0.07	0.31	0.32	0.77
TC	0.16	0.16	0.88	0.17	0.15	0.64	0.15	0.16	0.52
CC	0.09	0.09	0.50	0.11	0.10	0.59	0.08	0.08	0.84

2.6 Discussion

Several lines of evidence support a key role for an aberrant cholesterol metabolism in the aetiopathology of late onset sporadic AD. To date, only the APOE4 and to lesser extent APOJ, APOC1, PICALM and CR1 genes have been formally associated with the disease in case control studies (Petit-Turcotte et al., 2001; Poduslo et al., 1998; Poirier et al., 1993b; Strittmatter et al., 1993) and large scale genome wide association studies (Blacker et al., 2003; Harold et al., 2009; Lambert et al., 2009). These genes are also known to directly or indirectly modulate AD pathology either via lipid trafficking, endocytosis and/or Aβ clearance.

Mobilization and distribution of cholesterol in the brain is of crucial importance for development, normal neuronal functions but also for brain remodelling following injury or neurodegeneration. In the adult brain, neurons have a limited synthetic capability but a marked need for cholesterol which is usually obtained through the LDLR uptake pathway. It is postulated that mature neurons meet their cholesterol requirement mainly through astrocytes-mediated secretion of apoE-cholesterol lipoparticles (such as HDL) and delivery via a LDLR-mediated endocytosis. ApoE serves as the main cholesterol transporter in the brain and acts as the principal ligand for LDLR. Alterations to lipoproteins trafficking in apoE and/or LDLR deficient mice severely compromise synaptic integrity, function and remodelling. In addition, mice deficient for the apoE or LDLR present an altered distribution of cholesterol (Igbavboa et al., 1997) with reduced neuronal plasticity and synapse formation, impaired long term potentiation (LTP) and cognitive deficits (Krugers et al., 1997; Krzywkowski et al., 1999; Mulder et al., 2004; Mulder et al., 2007).

In the current study, PCSK9, a modulator of plasma LDL-cholesterol levels recently associated with familial hypercholesterolemia (Abifadel et al., 2003), was shown to significantly increase AD risk level in men. It was further demonstrated that PCSK9 expression is significantly increased in the brain of autopsied AD cases. Since PCSK9 tightly regulates cell surface LDLR through degradation, it became relevant to investigate

a putative role for this convertase in the maintenance of brain cholesterol homeostasis by assessing key bioregulators of its metabolism.

It is now well established that in the periphery, PCSK9 regulates plasma cholesterol homeostasis by modulating LDLR catabolism. Both *in vivo* and *in vitro* studies demonstrated that overexpression of PCSK9 associates with a drastic decrease in hepatic LDLR protein levels (but not of its mRNA) which leads to increase plasma LDL-cholesterol levels (Lalanne et al., 2005; Maxwell and Breslow, 2004). The opposite finding was reported in PCSK9 deficient mice (Rashid et al., 2005; Zaid et al., 2008). Normally, experimental deafferentation of the hippocampus in rats was shown to cause a transient increase of apoE and LDLR expression in the deafferentated area of the brain (Poirier et al., 1991b; Poirier et al., 1993a). In contrast, results obtained in this study indicate that increases in hippocampal and cortical PCSK9 protein levels in AD are coupled with normal levels of LDLR protein, despite a significant upregulation of the LDLR mRNA prevalence in the same brain area. These findings suggest that under active neurodegenerative conditions, enhanced PCSK9 production is used to facilitate local LDLR degradation and to presumably repress lipoproteins uptake via the lipoprotein receptor.

No obvious associations were observed between PCSK9 protein concentrations and cholesterol levels, apoE levels or HMGCoAR activity in both, normal and AD brain tissue homogenates; further emphasizing the privileged PCSK9-LDLR biological interaction. Given that synaptogenesis and synaptic remodelling processes rely extensively on cholesterol bioavailability and mobilization via the apoE/LDLR pathway, it is conceivable that PCSK9 may play an active role during synaptic remodelling; particularly in the late phase of this process when the intracellular cholesterol available exceeds the cholesterol required by the synaptogenesis process. At that moment, it is imperative that the LDLR levels return to basal concentrations to prevent the toxic accumulation of free cholesterol.

In agreement, several studies in cell lines have demonstrated that high intracellular cholesterol levels increase Aβ production (Frears et al., 1999; Puglielli et al., 2001). Findings reported above clearly show that the cells do not exclusively rely on the transcriptional regulation of the LDLR to limit cholesterol-enriched lipoprotein endocytosis but it uses PCSK9 as an additional post-translational regulator of LDLR catabolism. Furthermore, reports of PCSK9 involvement during the development of the nervous system (Poirier et al., 2006; Seidah et al., 2003) and mouse liver regeneration (Seidah et al., 2003; Zaid et al., 2008) is certainly consistent with the notion that PCSK9 is actively involved in membrane remodelling. In this proposed model, PCSK9 acts as an on-site mechanism that directly regulates brain cholesterol uptake via the apoE/LDLR pathway to prevent excessive cholesterol accumulation within neuronal cells undergoing membrane remodelling and synaptic reorganisation. This working model will be further investigated in upcoming studies examining the PCSK9-LDLR connection during lesion-induced synaptic remodelling in both animal and neuronal cell culture models.

The absence of any link between PCSK9 expression and key pathological markers such as NFT and SP densities and Aβ levels tends to rule out a direct involvement of PCSK9 in the pathophysiological process underlying sporadic AD, at least, in very late-stages of the disease. However, a recent report indicated that PCSK9 affects the metabolism of BACE1 (β-site APP-cleaving enzyme 1) and the rate of Aβ generation (Jonas et al., 2008). Jonas and colleagues (2008) demonstrated that overexpression of PCSK9 in cell cultures stimulates the degradation of non-acetylated BACE1 whereas its absence resulted in increased levels of BACE1 and Aβ in the brain of PCSK9-/- mice (Jonas et al., 2008). On the other hand, studies performed on mice overexpressing LDLR in the brain clearly demonstrated the importance of this receptor molecular cascade in the prevention of Aβ deposition and extracellular clearance in transgenic models of AD pathology (Kim et al., 2009).

Because it is well established that the lack of PCSK9 results in increased number of LDLR, one can speculate that modulation of BACE1 and generation of Aβ by PCSK9 may function independently of its effect on the LDLR-mediated endocytosis process in

the early, non-symptomatic stages of the disease. When taken together, the above findings suggest that PCSK9 may have different time-dependent functions during synaptic remodelling processes. First, PCSK9 may regulate BACE1 and Aβ metabolism during the early phase of neurodegeneration to most likely limit Aβ deposition whereas, during the late phase of compensatory synaptic remodelling processes, PCSK9 may negatively regulate the LDLR levels to prevent the problematic intracellular accumulation of both cholesterol and Aβ peptides into neurons undergoing synaptic reorganisation.

Until now, mutations of the PCSK9 gene have only been associated with altered plasma cholesterol metabolism. PCSK9-mediated gain/loss of function is receiving significantly more attention these days although the population prevalence of these genetic anomalies remains extremely low. This is why in this work we focused on five relatively common PCSK9 polymorphisms that are spread throughout the gene. These variants were examined for possible association with AD risk but more importantly, for possible association with PCSK9 protein levels. While we did not observe any significant association between PCSK9 variants and AD in our eastern Canadian population, stratification by gender indicates that men carrying either the C, A or T alleles of SNPs rs2483205, rs483462 or rs662145 exhibit a much higher risk of developing AD. Furthermore, men carrying the rs2483205 and rs662145 (CT) 2-site haplotype display a significantly higher risk of developing AD compared to non-carriers (see Table 2).

Recent studies demonstrated that a PCSK9 variant associates with blood lipid levels in a similar gender specific manner whereas plasma PCSK9 levels were shown to correlate with blood cholesterol concentration in men, only (Evans and Beil, 2006; Mayne et al., 2007). The exact mechanism underlying the gender differences in PCSK9 regulation and function is unclear at the moment. Levels of brain PCSK9 protein and cholesterol are not affected by gender or PCSK9 variants in the AD and control groups, at least, in the brain areas covered in this study. Since the blood cholesterol values of our subjects were not available, we cannot exclude a possible role of blood cholesterol levels in altering indirectly the risk for AD in our population. This will be the subject of further investigations by our group.

In conclusion, this study provides the first evidence that PCSK9 appears to play an active role in the modulation of the LDLR in response to brain degenerative processes found in AD. While we could not find any direct evidence of a gene/disease or genetic variants/gene expression relationship, we identified three distinct polymorphisms in the PCSK9 gene that modulate the disease risk levels in the male sub-population.

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Disclosure statement

The authors have no conflicts of interest to declare.

Chapter 3: Study 2

PCSK9 IS INVOLVED IN POST-INJURY SYNAPTIC REMODELLING

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3.1 Preface to Chapter 3

In the previous chapter, we showed that PCSK9 expression is upregulated in the frontal cortex and hippocampus but not in the low-pathology cerebellum of AD patients compared to age-matched control subjects. This increase does not correlate with markers of AD neuropathology or known cholesterol modulators in late-stages of the disease. Moreover, the five studied genetic variants did not affect PCSK9 expression or associate with a higher risk of AD. We proposed that the increased PCSK9 expression could be a brain response to neurodegeneration in an attempt to recover and compensate from synaptic loss and neuronal death. While the mechanistic explanation for this link is unknown, a possible PCSK9-dependent modulation of LDLR protein levels seems to be at play.

Entorhinal fibers constitute the main afferent pathway to the hippocampal formation and when damaged, it causes the loss of synapses in the hippocampus (Masliah et al., 1993; Scheff et al., 2007). This circuitry is of particular importance because of its critical role in cognitive functions such as learning and memory and its vulnerability to degeneration in AD and normal ageing (Crews and Masliah, 2010; Giannakopoulos et al., 1998). In early phases of neurodegeneration, the brain maintains a compensatory response to overwhelm this synaptic loss and rebuild its neuronal network. Neuronal damage induces marked changes in lipid homeostatic processes and promotes axonal growth and collateral sprouting of granule cell neurons to generate new synapses and replace the lost ones (Pfrieger, 2003c; Poirier et al., 1993a).

To study a possible implication of PCSK9 in synaptic repair processes via modulation of cholesterol transport and delivery to neurons through the LDLR-mediated endocytosis pathway, we used the ECL model. This model maintains relevance in the context of AD as the deafferentation and reinnervation phases following ECL mimics the early pre-clinical phases of neurodegeneration found in AD. In this study, we examined the expression of PCSK9 and known modulators of cholesterol homeostasis over the plastic recovery period.

3.2 Abstract

In the brain, synthesis and distribution of cholesterol are required for generating new membranes and terminals during synaptic plasticity and remodelling. The apoE and LDLR are two key players in the regulation of cholesterol-rich lipoprotein distribution and endocytosis; deficiency in any of these proteins compromises both cell proliferation and synaptogenesis. In periphery, the proprotein convertase PCSK9 regulates plasma cholesterol levels by enhancing the degradation of the LDLR. Presumably, PCSK9 has a similar role in the normal and injured central nervous system. To this end, unilateral entorhinal cortex lesions were used to examine hippocampal PCSK9 expression in mice undergoing synaptic remodelling and terminal proliferation. A time-dependent upregulation of PCSK9 expression was observed during the early stages of synaptic remodelling which coincides with increases of apoE and LDLR levels in the deafferented area of the hippocampus. HMGCoAR expression and activity, the rate-limiting step in cholesterol synthesis, remain unchanged relative to controls. These observations suggest that during active reinnervation in the CNS, endogenous cholesterol synthesis is neglected in favour of cholesterol-lipoprotein uptake via the apoE/LDLR pathway: the latter being regulated, at least in part, by the activity of PCSK9 in the early phase of the reinnervation process.

3.3 Introduction

Cholesterol is an essential component of mammalian cell membranes. It plays a fundamental role in membrane synthesis, synaptogenesis and maintenance of neuronal plasticity and functions during development, in the adult brain or after injury (Pfrieger, 2003a). Therefore, cholesterol is critical for the proper functioning of the brain and is tightly regulated by elaborated mechanisms that balance levels of cholesterol synthesis, uptake, release and transport (Pfrieger, 2003a; Poirier, 1994). Abnormal brain cholesterol metabolism occurs in several diseases such as Niewmann-Pick disease type C, Smith-Lemi-Opitz syndrome and Tangier disease. More recently, this pathway was implicated in the risk and pathogenesis of Alzheimer's disease.

Cholesterol synthesis in the central nervous system (CNS) is mainly achieved by oligodendrocytes and astrocytes independently from plasma cholesterol contributions. *In vitro*, oligodendrocytes which produce cholesterol to form myelin sheaths display a higher capacity for cholesterol synthesis than astrocytes. Astroglia, on the other hand, synthesize 2- to 3 fold more cholesterol than neurons (Bjorkhem and Meaney, 2004). Experimental evidences suggest that once neurons have reached maturity, lipid production is progressively reduced to mainly rely on lipoprotein-bound cholesterol supplied by astrocytes (Mauch et al., 2001; Pfrieger, 2003b; Poirier, 1994). The notion that cholesterol transits from astrocytes to neurons is particularly crucial in the neuronal response to neurodegeneration and experimental lesions. In this context, astrocytes were shown to recycle cholesterol released from degenerating neurons and to redistribute this lipid-recycled pool to neurons undergoing synaptic remodelling (Pfrieger, 2003c; Poirier et al., 1993a).

Mainly synthesized by astrocytes, apolipoprotein E (apoE) mediates this lipid transport to neurons via internalization of apoE-lipoprotein-cholesterol complexes through the cell surface low-density-lipoprotein receptors (LDLR). ApoE was shown to play a pivotal role in cholesterol delivery for growth and synapses remodelling (Poirier et al., 1991a; Poirier et al., 1993a). Similarly, the LDLR was also found to be crucial for neuronal cell proliferation, axonal elongation and synapse formation (Ignatius et al.,

1987b; Mulder et al., 2007; Posse de Chaves et al., 2000). Accordingly, the absence of either apoE or LDLR results in defective distribution/internalization systems associated with impaired synaptic plasticity and cognitive deficits (Cao et al., 2006; Champagne et al., 2002; Champagne et al., 2005; Krugers et al., 1997; Masliah et al., 1995; Mulder et al., 2004; Mulder et al., 2007; Oitzl et al., 1997). These findings indicate that alteration of cholesterol influx via the apoE/LDLR uptake pathway compromises synaptic plasticity/remodelling and indirectly affects learning and memory.

Recently, the proprotein convertase subtilisin/kexin type 9 (PCSK9) has been identified as the third gene involved in familial hypercholesterolemia (Abifadel et al., 2003). It emerges as a major regulator of plasma cholesterol due to its ability to enhance the degradation of LDLR (Benjannet et al., 2004; Maxwell and Breslow, 2004). Studies addressing the functional relevance of PCSK9 in mice and humans revealed that overexpression or gain-of-function mutations of PCSK9 is associated with a dramatic decrease in liver LDLR protein levels (but not mRNA) and increased circulating low-density-lipoprotein (LDL)-cholesterol (Horton et al., 2007; Maxwell and Breslow, 2004; Park et al., 2004) which mimics the phenotype observed in LDLR-deficient mice (Horton et al., 2007). Increased number of liver LDLR was reported along with an accelerated LDL-cholesterol clearance and reduced levels of circulating cholesterol in PCSK9 deficient mice (Rashid et al., 2005).

In addition, a putative role for PCSK9 in the CNS development was recently suggested by Poirier and colleagues (Poirier et al., 2006; Poirier et al., 2008b; Seidah et al., 2003). Given the importance of cellular cholesterol uptake for neuronal growth, plasticity and synaptogenesis, we propose that PCSK9 may regulate brain cholesterol transport and internalization by modulating the apoE/LDLR uptake pathway. In this study, we investigated the expression of PCSK9 and its effect on the dynamic regulation of cholesterol metabolism by assessing biomarkers involved in synthesis, transport and internalization during injury-induced synaptic loss and repair in the adult mouse hippocampus. A time-dependent upregulation of PCSK9 expression was observed during

the early stages of synaptic remodelling, coinciding with a marked upregulation of apoE and LDLR levels in the deafferented zone of the hippocampal area.

3.4 Materials and methods

Animals

Male C57BL/6J mice age 12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed individually in an enriched environment, fed with a diet of standard laboratory chow *ad labium*, and maintained on a 12-hour light-dark cycle. All protocols are in accordance with the Canadian Guidelines for Use and Care of Laboratory Animals and have been approved by the McGill University Animal Care Committee.

Unilateral Entorhinal cortex lesion (ECL)

Unilateral electrolytic lesions to the right entorhinal cortex were performed as previously described (Blain et al., 2004). Briefly, anesthesia was induced by intramuscular injection of 1.0µl/g of body weight of a ketamine/xylazine/acepromazine mix. Skull was shaved and the mouse was placed in a stereotaxic frame in a flat skull position. Lambda 0 was taken by aligning the electrode with the suture lines. The skull was drilled to allow the electrode to pass, at an angle of 6º, to the four lesion coordinates: (1) [AP: 0mm], [L: -3.0mm], and [DV: -3.0mm, -4.0mm]; (2) [AP: 0mm], [L: -3.5mm], and [DV: -3.0mm, -4.0mm]; (3) [AP: +0.5mm], [L: -4.0mm], and [DV: -3.0mm, -4.0mm]; (4) [AP: +1.0mm], [L: -4.0mm], and [DV: -3.0mm, -4.0mm]. A current of 1mA was applied for 10 seconds (sec) at each coordinate. Then, the skull was patched with bone wax and the incision sutured. SHAM-operated animals were treated similarly however, the electrode was lowered only 1.0mm and no current was passed. All mice were given a subcutaneous injection of physiological saline to prevent dehydration. Animals were nursed until their recovery and remained under a heat lamp for 24 hours before being returned to their home cage. Food and water were not withheld prior to, or following surgery. Animals were sacrificed at different timepoints: 2, 6, 14, 21, 40 and 60 days post-lesion (DPL) following a lethal dose of anaesthetic and then, perfused transcardially with 30ml of ice-cold 0.01M phosphate buffered saline (PBS) solution.

SHAM-operated mice were sacrificed at 14 DPL. Note that the contralateral side of the brain serves as an internal control while external control is represented by SHAM-operated animals.

Tissue homogenization

Brains were removed and dissected on ice into ipsi and contralateral sides to the lesion. Hippocampal and frontal cortex regions were isolated and sonicated in 0.01M PBS containing protease inhibitors (Boehringer Mannheim, Germany). Total protein concentration was measured by BCA protein dosage kit (Pierce, Rockford, Il).

Western Blot analysis

For synaptophysin protein quantification, 12.0µg of total hippocampal protein homogenates was separated by SDS-PAGE under reducing conditions. Transfer to a nitrocellulose membrane was conducted using the iBlot^{TM} Dry Blotting System according to manufacturer's specifications (Invitrogen, Carlsbad, CA). Membranes were blocked overnight in a solution of 5% dried-milk in Tris buffered saline (TBS)-T (20mM Tris base, 137mM NaCl and 0.1% Tween-20). Then, membranes were incubated with a mouse monoclonal antibody against synaptophysin (No. 902 314, Boehringer Mannheim, Germany) diluted 1/1000 in TBS-T followed with a secondary incubation with a sheep anti-mouse horseradish peroxidase-linked antibody (Amersham, Oakville, Canada). Chemiluminescent detection was performed with an enhanced chemiluminescence kit (Perkin-Elmer, Boston, MA). Detection of α-tubulin was carried out as described above, using the mouse monoclonal anti-α-tubulin antibody (M61409M, BioDesign Int., Saco, USA) and the sheep anti-mouse horseradish peroxidase-linked secondary antibody (Amersham, Oakville, Canada). Density analyses were conducted using a Kodak 1D Image Analysis Software of the Image Station 440CF (Eastman Kodak Co., Rochester, NY). The region of interest analysis was used to determine the mean intensity of synaptophysin and α-tubulin bands. Results are expressed as mean intensity of synaptophysin normalized to α-tubulin in the hippocampus ipilateral relative to the contralateral side to the ECL location for each mouse (n=5 animals per timepoints; SHAM, 2, 14, 21, 40 and 60 DPL) ± standard error mean (SEM).

Immunohistochemistry

Whole brains used for immunohistochemical analyses were removed from animals following perfusion, flash-frozen at -40^{o}C in isopentane, and stored at -80^{o}C until use. Coronal sections of 20µm of thickness were mounted on poly-L-lysine coated slides and dessicated overnight at 4^{o}C. For immunohistochemistry, we used goat or rabbit polyclonal antibodies against either PCSK9 (1/50; Abcam, Cambridge, MA) or glial fibrillary acidic protein (GFAP) (1/4000; Dako Diagnostics Canada Inc., Mississauga, ON), respectively, and a mouse monoclonal antibody against neuronal nuclei (NeuN) (1/1000; Chemicon International, Temecula, CA). Slides were first fixed for 30 minutes (min) in 4% paraformaldehyde/PBS, incubated in a 3% peroxide/PBS solution for 15 min, permeabilized with 0.4% Triton X-100/PBS for 30 min and blocked with 2% horse serum for both PCSK9 and NeuN, and goat serum for GFAP, for 1 hour at 4^{o}C as indicated by the Vectastain ABC detection kit used (Vector Laboratories, Burlingame California, USA). Then, the slides were incubated overnight at 4^{o}C with their respective primary antibodies. The next day, slides were incubated for 90 min at 4^{o}C with a secondary antibody (anti-goat-biotinylated for PCSK9, anti-rabbit-biotinylated for GFAP or anti-mouse-biotinylated antibody for NeuN) followed with 90 min incubation at 4^{o}C with the detection reagent according to the Vectastain ABC kit guidelines. Control analyses were performed with heated-primary antibodies or by omitting the primary antibodies (not shown). Immunoreactivity was revealed with the 3,3′-Diaminobenzidine solution (in TBS 50mM) for ~8 min (Sigma Aldrich Canada Ltd., Oakville, ON). Reaction was stopped with TBS 50mM. Then, slides were dehydrated and mounted with DPX and glass coverslips. Immunohistochemistry analyses were performed with a Zeiss Axioskop 2 Plus microscope (Zeiss, Germany) using either a 2.5X/0.075 or 10 X/0.30 objective (Plan-Neofluar, Zeiss, Germany). Images were captured using the Northern Eclipse Version 6.0 Image Analysis Software.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from ipsi and contralateral sides of frontal cortices, hippocampi and cerebellum of each animal (n=5 mice per timepoints; SHAM, 2, 14, 21, 40 and 60 DPL) using the QIAGEN RNeasy Mini kit (QIAGEN Inc., Mississauga, ON).

Following extraction, 2µg of total RNA from each samples were reverse-transcribed in the GeneAmp 5700 sequence detection system (PE Applied Biosystems, Ontario, Canada) to generate cDNA in the following thermal cycle: 10min at 25ºC, 30min at 48ºC, followed by 5min at 95ºC to stop the reaction. Primer pairs for PCR amplification were as follow; mPCSK9-forward 5′-TTGGACGCTGTGTGGATCTC-3′; mPCSK9-reverse 5′-ACGCTCCGATGATGTCCTTC-3′; mLDLR-forward 5′-TGGACCGCAGCGAGTACAC -3′; mLDLR-reverse 5′-CCACCACATTCTTCAGGTTGG -3′; mHMGCoAR-forward 5′-GCCGGGACCTGACAGACTAC -3′; mHMGCoAR-reverse 5′-CCTCTCAGCTGTGGTGAA -3′; mApoE-forward 5′- TCCATTGCCTCCACCACAGT -3′; mApoE-reverse 5′- GGGCGTAGTGAGGGATGA -3′; mActin-forward 5′-TGACCGAGCGTGGCTACA -3′; mActin-reverse 5′-TCTCTTTGATGTCACGCACGAT -3′. All primers were designed using the Primer Express PE Biosystems software. Each PCR reaction was in a final volume of 35µl using the SYBR® Green method in a Gene-Amp 5700 sequence detection system (PE Applied Biosystems, Ontario, Canada) following these cycling conditions; 2 min at 50ºC; 10 min of denaturation at 95ºC; and 40 amplification cycles of; 95ºC for 15 sec and 1 min at 60ºC. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and results are expressed as ratios between ipsilateral and contralateral sides to the lesion location relative to β-actin ± SEM.

ELISA assays

Indirect ELISA was performed on Costar 96-well EIA/RIA plates (Fisher Scientific). Plates were first incubated overnight at 4ºC with either hippocampal protein homogenates from each mice (n=5 animals per timepoints; SHAM, 2, 14, 21, 40 and 60 DPL) or purified synthetic PCSK9 peptide (CRSRHLAQASQELQ) as standard (625 to 5000µg/ml) diluted in Bicarbonate/carbonate buffer (100mM, pH 9.6), applied in triplicate. All subsequent incubations were performed at room temperature on a shaker. The next day, the primary antibody (goat polyclonal PCSK9 antibody, ab28770, Abcam, Cambridge, USA; or a rabbit polyclonal LDLR antibody, NB110-57162, Novus Biological, Littleton, USA) diluted in a PBS 10mM and bovine serum albumin 1%

solution was added to each wells for 2 hours. Then, all wells were washed with TBS-T and incubated with the biotinylated detection antibody (anti-goat antibody, ab6740; or anti-rabbit antibody, ab6720, Abcam, Cambridge, USA) for 2 hours, followed by washes and a hour incubation with an alkaline-phosphatase-conjugated streptavidin solution (Invitrogen Canada Inc., Burlington, ON) to detect the biotinylated antibody. Following this incubation and further washes, an alkaline phosphatase fluorescent substrate (AttoPhos, Promega, San Luis Obispo, USA) was added to each well for 30 min at 37ºC. Fluorescent was measured by using the microplate fluorescent reader (FL600, Bio-Tek Instruments, Winooski, VT) at a 450nm/20nm exposure and 560nm/20nm emission. For LDLR protein detection, we used a mouse liver homogenate (sonicated in PBS 0.01M) as standard (4 to 135 µg/ml). A blank sample that consists of Bicarbonate/carbonate buffer was added to each plate. Relative protein levels of PCSK9 or LDLR are expressed as ratios between ipsi and contralateral side to the ECL location ± SEM.

HMGCoAR activity

The 3-hydroxy-3methylglutaryl Coenzyme A reductase (HMGCoAR) activity was measured on parieto-temporal cortices from ipsi and contralateral side to the ECL from 5 mice per timepoints (SHAM, 2, 14, 21, 40 and 60 DPL) according to the modified technique of Deutsh et al. 2002 (Deutsch et al., 2002; Plemenitas et al., 1990; Shapiro et al., 1974). Briefly, 30mg of tissues were sonicated on ice in incubation buffer (30mM EDTA, 250mM NaCl, 1mM DTT, 50mM K_2HPO_4, pH 7.2) followed with a 10 min centrifugation at 3000 rpm at 4ºC. Protein concentrations from each extracts were determined using the BCA kit (Pierce, Rockford, Il). 20µl of homogenates were completed to a final volume of 100µl with the incubation buffer and preheated at 37ºC for 10 min. Then, 50µl of cofactor-substrate solution (1.5nM NADP, 15nM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase and 150nM HMGCoA dissolved in incubation buffer) were added to each homogenates. After 60 min incubation at 37ºC, reactions were stopped by adding 150µl of K_2HPO_4 100mM containing 60nM of N-propionyl Coa as the internal standard. Samples were gently mixed and 50µl were injected on the High-performance liquid chromatography system (HPLC, Varian Inc., Palo Alto, USA). A blank sample, which does not contain mouse homogenates, is also

incorporated in the analysis so that the HMGCoAR activity is calculated by deducing the amount of HMGCoA in samples containing mouse homogenates to HMGCoA levels obtained in the blank sample:

Blank HMGCoA – mouse homogenate sample HMGCoA = HMGCoAR activity in nmoles/mg/min

The CoA, HMGCoA and N-propionyl CoA were separated using the HPLC Polaris Varian System equipped with a 5u-C18-A polaris column with a metaguard 4.6mm polaris 5uC18 (Varian Inc., Palo Alto, USA). Separation was performed with a gradient system of 75nM K_2HPO_4 and 100% Acetonitrile. The standard curve for HMGCoA ranges from 5 to 30 nM with a 10nM of N-propionyl CoA as internal control and all standards were diluted in 75nM K_2HPO_4. Again, results are expressed as ratios between ipsilateral and contralateral sides to the lesion location ± SEM.

Statistical analyses

To investigate the effects of deafferentation and reinnervation on the expression of synaptophysin, PCSK9, LDLR, HMGCoAR and apoE levels as well as on HMGCoAR activity, we performed univariate analyses (one-way ANOVA). Tukey's HSD tests were applied for all post-hoc comparisons except for synaptophysin where a Dunnett's (2-sided) post-test was applied for all timepoints. All statistical analyses were conducted using SPSS 15.0 Software and considered significant when $p \leq 0.05$.

3.5 Results

3.5.1 Time-course of synaptophysin protein levels as a marker of synaptic density

In this study, entorhinal cortex lesions (ECL) were used to map the expression of apoE, LDLR, PCSK9 and HMGCoAR activity in response to i) hippocampal deafferentation and ii) the ensuing reinnervation. It was shown that ECL causes the loss of nearly 60% of the synaptic inputs to the granule cells (GC) of the hippocampus. However, the loss of synapses is transient. A few days after the lesion, new synapses are formed virtually replacing the lost inputs within 4 weeks in rat and a bit more than 8

weeks in mice (Poirier, 1994; Steward et al., 1988). To quantify the extent of synaptic density in our study, synaptophysin levels were assessed by Western Blot analysis in the hippocampus of adult mice at different timepoints following the ECL (Fig. 11). While the exact function of this protein remains unknown, its ubiquity expression at the synapses has led to the use of synaptophysin immunostaining for quantification of synapses in rodent and human brains (Calhoun et al., 1996; Masliah et al., 1990). Hippocampal synaptophysin levels were found to be reduced by 40% at 14 DPL and to slowly return to baseline values by 60 DPL (Fig. 11): replicating similar time course reports in mice (Champagne et al., 2005; Masliah et al., 1991; White et al., 2001). These results are consistent with the different phases observed following deafferentation of the hippocampus; i.e. 0-6 days correspond to the degenerative phase while reactive sprouting and synapse replacement occur between 6-30 days, and completion of reinnervation is achieved by roughly 60 days (Champagne et al., 2005).

3.5.2 PCSK9 localization in the hippocampus of ECL mice

Analysis of PCSK9 spatial localization in the hippocampus of ECL mice indicates that it is predominantly expressed in the GC layer of the dentate gyrus (DG) and neurons of the hilar area (Fig. 12A and B). Immunopositive staining is also observed in the neuronal layers CA3 and CA1 regions and, diffused labelling in the molecular layers of the DG. PCSK9 immunoreactivity was then contrasted to that of NeuN, a specific marker of neuronal nuclei, and to that of GFAP, a specific type 1 astrocyte protein. The pattern of PCSK9 immunostaining (Fig. 12A and B) overlaps very well that of NeuN staining (Fig. 12C and D) but not that of GFAP immunoreactivity (Fig. 12E and F). PCSK9 spatial and cellular distribution is not affected by the deafferentation and reinnervation processes over the recovery period (data not shown).

3.5.3 PCSK9 expression following ECL

PCSK9 expression was subsequently assessed in a time course analysis following unilateral ECL at 0, 2, 14, 21, 40 and 60 DPL. Using quantitative RT-PCR, we found a marked increase (>2 fold) in the PCSK9 mRNA prevalence at 14 DPL (Fig. 13A)

followed by a return to baseline levels by 40 DPL. PCSK9 mRNA alterations are specific to the hippocampus as no changes were detected in the frontal cortex or cerebellum of ECL mice (data not shown). The PCSK9 mRNA increase coincides with a parallel induction of PCSK9 protein levels, with a significant peak at 14 DPL (Fig. 13B).

3.5.4 Time course effect of the ECL on cholesterol modulators

The expression of PCSK9 was then contrasted with that of HMGCoAR, the rate limiting enzyme in *de novo* cholesterol biosynthesis. No significant alterations in HMGCoAR mRNA expression or activity were observed in the deafferented parieto-temporal cortex of our ECL mice throughout the different phases of the recovery period (Fig. 14A and B): suggesting that the cholesterol transport and uptake via the apoE/LDLR pathway is used preferentially over local cholesterol synthesis. Indeed, analysis of hippocampal mRNA prevalence of apoE in ECL mice reveals a marked upregulation of the cholesterol transporter that peaked at 14 DPL (Fig. 14C): coinciding with that of PCSK9 mRNA prevalence (Fig. 13A and 14C). As it was previously demonstrated by our group, protein levels of apoE in the hippocampus of ECL mice were shown to be significantly reduced at 2 DPL followed by a significant upregulation at 14 DPL (Blain et al., 2004) as illustrated by the dashed line in figure 14C. ApoE expression throughout the deafferentation/reinnervation period is not significantly altered in the frontal cortex and cerebellum areas in ECL mice (data not shown).

3.5.5 Expression of LDLR in response to ECL

Since the transport of cholesterol by lipoproteins is enhanced during repair as indicated by apoE levels (Fig. 14C), we next investigated whether the brain LDLR expression which mediates lipoprotein-cholesterol uptake by neurons, is affected by brain injury (Fig. 15). Hippocampal LDLR expression shows a slight elevation (trend only) at 14 DPL (Fig. 15A) whereas its protein levels were found to be significantly increased at 14 DPL in the hippocampus. LDLR levels return to baseline values at 40 DPL (Fig. 15B). No differences in LDLR mRNA prevalence were detected in the frontal cortex or cerebellum areas in ECL mice (data not shown).

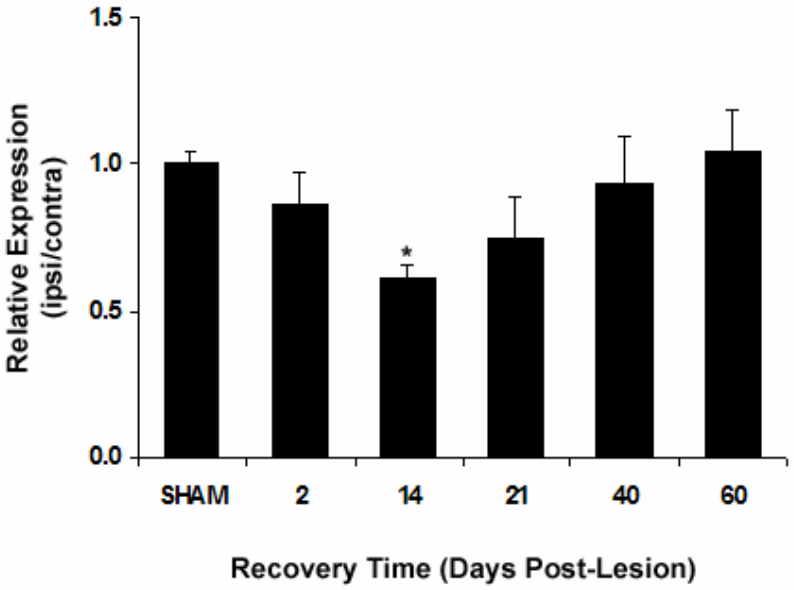

Figure 11: Synaptophysin relative protein expression in the hippocampus of ECL mice.

Protein levels were measured in the ipsilateral and contralateral sides of the hippocampus of ECL mice by Western Blot analyses. Values are normalised to α-tubulin and expressed as ratios between the ipsilateral and contralateral side of the lesion. Results correspond to mean synaptophysin intensity ± SEM of 5 animals per timepoints (SHAM, 2, 14, 21, 40 and 60 DPL). Statistical differences between lesioned and SHAM-operated animals (*p = 0.05) were assessed by univariate analysis of variance (one-way ANOVA) and Dunnett's test.

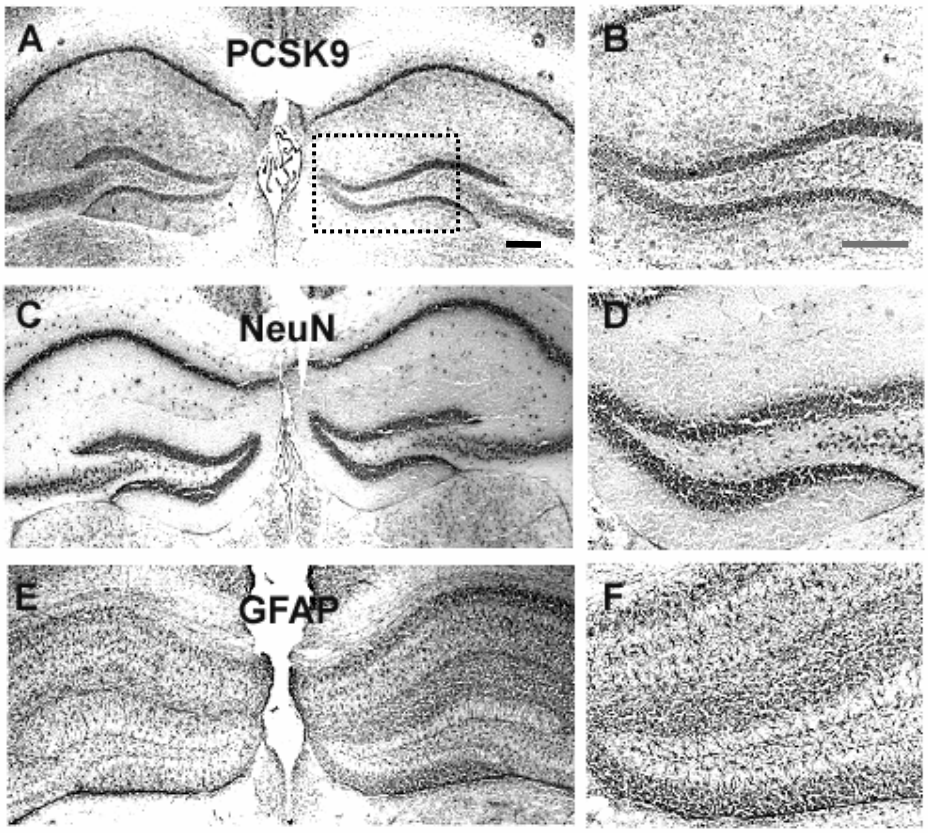

Figure 12: PCSK9 localization in the mouse deafferented hippocampus.

Hippocampal sections of DPL2 (panels A and C) and DPL6 (panel E) animals were taken with a 2.5X objective whereas panels B, D and F correspond to a 10X magnification of the ipsilateral side of the lesion of what is shown in panels A, C and E, respectively. The dashed box indicates the area that is magnified and represented in right panels. Coronal sections were subjected to PCSK9 (panels A and B), NeuN (panels C and D) and GFAP (panels E and F) immunohistochemistry. The black line represents a scale bar of 20µm for panels A, C and E whereas the grey line corresponds to a 10µm scale bar for panels B, D and F.

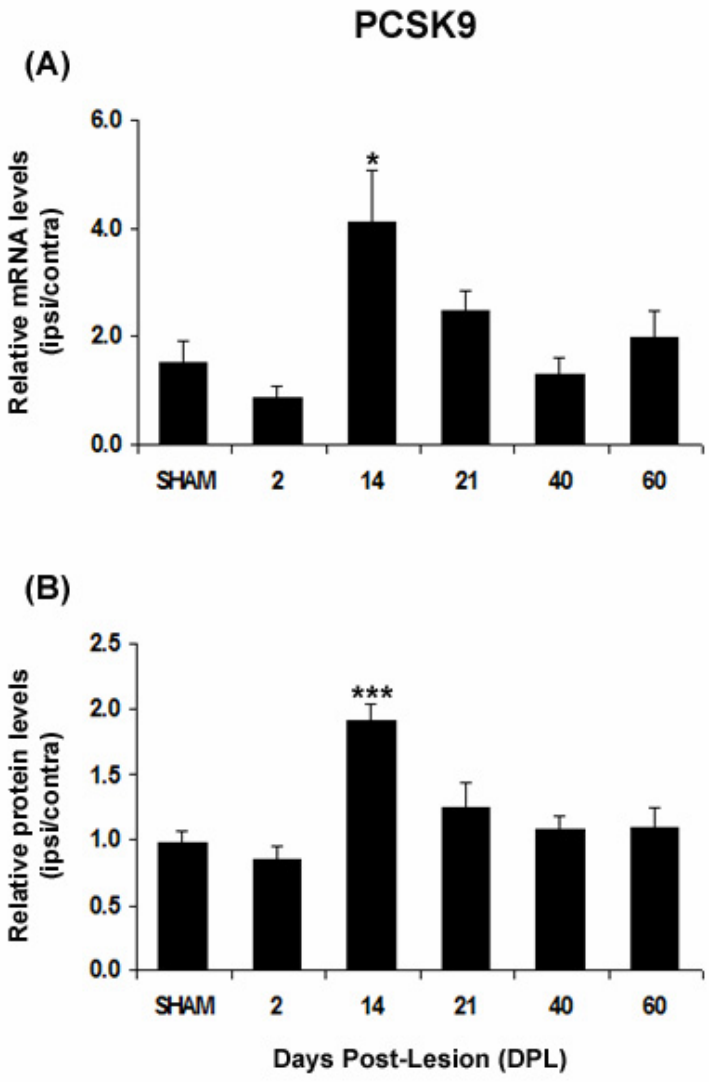

Figure 13: Time course expression of PCSK9 in the mouse hippocampus following ECL.

(A) Relative PCSK9 mRNA levels were measured by quantitative RT-PCR and values were normalized to β-actin. (B) PCSK9 relative protein levels were measured by ELISA. Results obtained from 5 animals per timepoints are expressed as mean ratios between ipsilateral and contralateral sides to the lesion ± SEM. Significance levels were determined by comparing PCSK9 signals from lesioned mice to SHAM-operated animals and analysed by univariate analysis (one-way ANOVA) and Tuckey's tests (*$p < 0.05$ and ***$p < 0.001$).

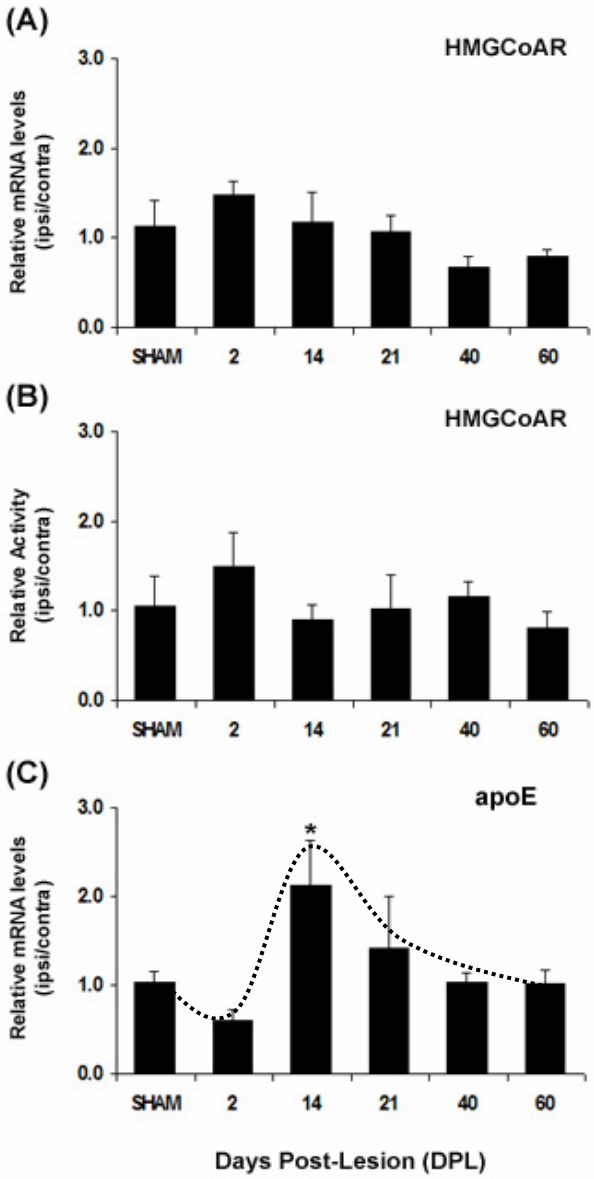

Figure 14: Time course analysis of endogenous cholesterol synthesis versus transport during deafferentation/reinnervation.

Relative HMGCoAR mRNA levels normalized to β-actin (A) and HMGCoAR activity (B) in the parieto-temporal cortex of mice after ECL. (C) ApoE mRNA levels in the hippocampus of ECL mice also normalized to β-actin. The dashed line represents hippocampal apoE protein levels following mouse ECL as previously demonstrated by our group (Blain et al., 2004). All results are expressed as ipsilateral:contralateral mean ratios (±SEM) and compared to SHAM-operated animals, from 5 animals per timepoints. One-way ANOVA and Tuckey's tests were used to determine significance (*$p < 0.05$).

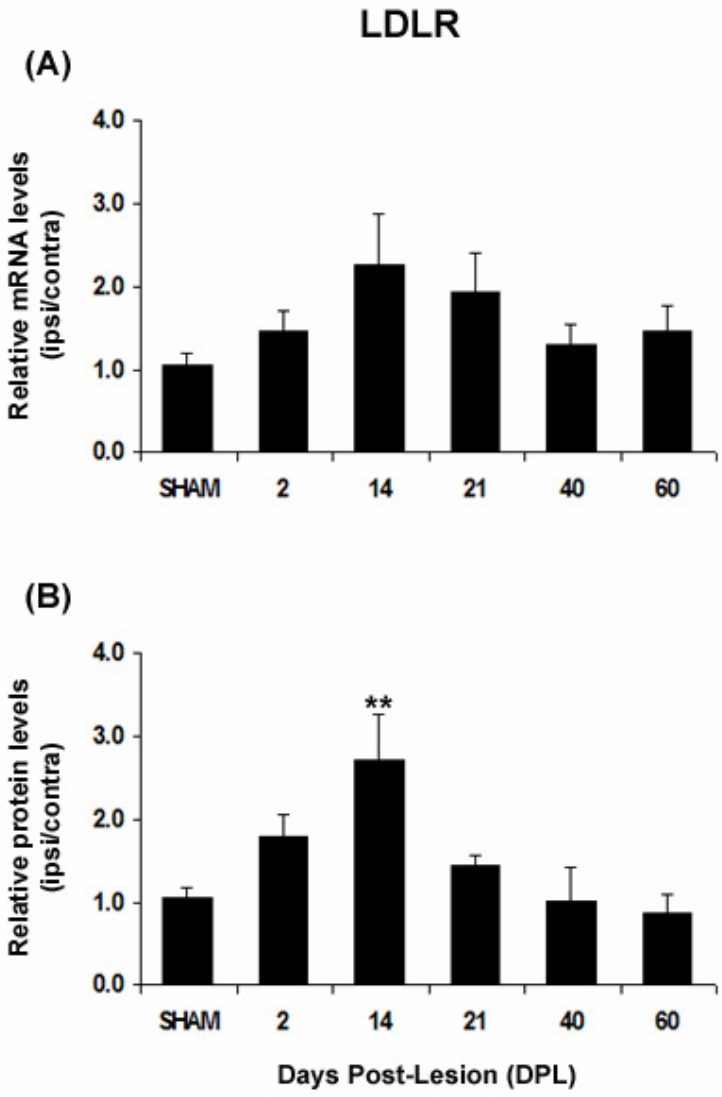

Figure 15: LDLR expression in the mouse ECL hippocampus during the deafferentation and reinnervation phases.

Relative LDLR mRNA levels were measured by quantitative RT-PCR and normalized to β-actin (A) and protein levels were quantified by ELISA (B). Results obtained from 5 animals per timepoints are expressed as mean ratios between ipsilateral and contralateral sides to the lesion ± SEM. Significance levels were determined by comparing LDLR expression from lesioned mice to SHAM-operated animals and analysed by univariate analysis (one-way ANOVA) and Tuckey's tests (**$p < 0.01$).

3.6 Discussion

In the CNS as opposed to peripheral nervous system (PNS), neurons exhibit a limited ability to regenerate. However, brain cells have the capacity of plastic responses throughout life. Following brain damage, healthy neurons compensate for the loss of synaptic inputs by inducing elongation and sprouting of their axonal terminals and by generating new synapses, a phenomenon that requires high cholesterol turnover. Therefore, rapid adaptation of cholesterol homeostasis is required for efficient synaptic remodelling processes. As it is well-established in the PNS, this sequence of compensatory responses in the CNS was shown to depend on a local lipid recycling mechanism (Boyles et al., 1989; Snipes et al., 1986).

In peripheral organs, PCSK9 has emerged as a key player in the regulation of plasma cholesterol by modulating LDLR-dependent cholesterol internalization. Evidences indicate that PCSK9 via both intra- and extracellular route, mediates LDLR degradation by targeting this receptor toward late endosome/lysosome compartments and preventing its recycling at the cell surface (Benjannet et al., 2004; Cameron et al., 2006; Lagace et al., 2006; Maxwell et al., 2005; Park et al., 2004; Qian et al., 2007). In the present study, the ECL model was used to investigate the contribution of PCSK9 in response to degeneration and reinnervation in the adult hippocampus at a moment when the cholesterol homeostasis is markedly perturbed. Results indicate that PCSK9 expression is significantly upregulated during the active phase of reinnervation and, it temporally coincides with peak expression of both apoE and LDLR.

The ECL system is an excellent model system to examine molecular mechanisms associated with degeneration and reinnervation in the CNS as it takes advantage of the laminar structure of the hippocampus. In response to deafferentation, the hippocampal cholesterol released from dead or degenerating terminals was shown to be progressively engulfed by astrocytes and redistributed to surviving neurons via LDLR-mediated endocytosis of apoE-cholesterol complexes. This cascade enables undamaged neurons to compensate for the synaptic loss by generating new membranes for axonal sprouting and synapse remodelling using lipid derived from dead or dying cells (Poirier, 2003). ECL

causes a major loss of synaptic inputs in the outer two-thirds of the molecular layer of the hippocampus which is ensued by compensatory synaptogenesis. The gradual increase in synaptophysin immunoreactivity between 6 to 30 DPL is paralleled by the heterotypic addition of acetylcholinesterase-positive terminals originating from the septal region (Champagne et al., 2005; Masliah et al., 1995).

PCSK9 gene expression and protein levels at 14 DPL coincides with the early phase of synaptic remodelling as demonstrated by synaptophysin immunoreactivity and expression levels in the deafferented zone of the hippocampal region. We also report that PCSK9 is mainly expressed in the granule cell neurons of the DG; the same cells undergoing active synaptic remodelling at the time when PCSK9 expression peaks. Based on PCSK9 role in peripheral cholesterol mobilization and LDLR degradation, it is tempting to postulate that PCSK9 may have a similar function in the brain and could modulate the core lipid homeostatic process which is associated with membrane synthesis and synaptic remodelling in the CNS. It also means that if PCSK9 plays such a pre-eminent role in the brain, it will also require its usual accessory proteins such as apoE (cholesterol transport) and LDLR (cholesterol uptake).

PCSK9 expression was found to temporally coincides with a peak in apoE and LDLR expression (Fig. 14C and 15) and levels (Blain et al., 2004). In contrast, no significant changes were observed in the HMGCoAR activity throughout the time course (Fig. 14B) of our lesion paradigm: suggesting that the cholesterol required for functional synaptic remodelling in neurons is preferentially obtained through apoE-lipoprotein/LDLR internalization pathway and not through synthesis.

One shortcoming of the present investigation is the chosen timepoints. A gap exists between the 14 and 21 DPL. We may have missed the PCSK9-dependent reduction of the LDLR expected to occur within hours or days following the peak expression of the receptor as it has been demonstrated on several occasions *in vitro* and *in vivo* in peripheral models (Benjannet et al., 2004; Maxwell and Breslow, 2004; Park et al., 2004).

Alternatively, PCSK9 may have interacted with members of the LDLR family other than the LDLR itself during the active phase of synaptogenesis. In i*n vitro* models, this convertase was implicated in the downregulation of the very-low-density-lipoprotein receptor (VLDLR) and apoE type 2 receptor (apoER2) (Poirier et al., 2008b). However, it does not affect the LDL related protein receptor (LRP) catabolism (Benjannet *et al.,* 2004). The apoE receptors have been previously implicated in signalling pathways and brain development (Benjannet et al., 2004; Poirier et al., 2008b) and PCSK9 was shown to interact with both VLDLR and apoER2 receptors to facilitate their degradation (Poirier et al., 2008b; Shan et al., 2008). However, a recent report indicated that hippocampal expression of these receptors (VLDLR, apoER2 and LDLR) under normal condition, is not affected by the deletion or overexpression of PCSK9 in mice (Liu et al., 2010). Moreover, hippocampal apoER2 and LRP were shown to be relatively unchanged in the reinnervation phase in the ECL model as opposed to the LDLR (Petit-Turcotte *et al.,* 2005; Petit-Turcotte *et al.,* 2007); highlighting the importance of apoE-mediated lipoproteins internalization (LDLR) over signalling function (LRP, VLDLR and apoER2) in the reinnervation process in the CNS. Interestingly, it has been suggested that PCSK9 may also enhance the catabolism of another cell surface receptor, CD81, which is required for the infection of cells by hepatitis C virus (Labonte *et al.,* 2009). This raises the possibility that in the CNS, other yet-to-determined receptors may be substrates for PCSK9 degradation activity.

Nevertheless, the reported involvement of PCSK9 at the onset of the neuronal differentiation process, its high expression in the developing liver and its critical role in the early phase of liver regeneration (Poirier et al., 2006; Seidah et al., 2003; Zaid et al., 2008) strongly support a role for this convertase in the regulation of cholesterol homeostasis in situations that necessitate access to substantial amount of cholesterol in a relatively short period of time.

In conclusion, our results provide evidences supporting an involvement of PCSK9 during early stages of synaptogenesis and terminal proliferation in response to brain damage. The data indicate that PCSK9 may act synergistically with apoE in regulating

cholesterol distribution and internalization to neurons via the apoE/LDLR uptake pathway. Although the exact function of PCSK9 in the injured brain remains elusive, it is postulated that PCSK9 ability to down-regulate LDLR levels in presence of excess intracellular cholesterol is at play to control the endocytosis of substantial amounts of cholesterol-rich lipoprotein complexes via the apoE/LDLR pathway used to build new sprouts and form new synapses. Given that mutations in PCSK9 gene were recently linked to familial hypercholesterolemia and that this condition is associated with significant cognitive decline in aged humans, it would be of interest to investigate if they also coincide with the development of neurodegenerative diseases such as Alzheimer's disease in which an inappropriate transport of cholesterol is proposed to be at play in the development and/or progression of this disease.

Acknowledgements

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Chapter 4: Study 3

THE PROPROTEIN CONVERTASE PCSK9 PROMOTES SYNAPTOGENESIS AND SYNAPTIC REMODELLING IN DIFFERENTIATED PC12 CELLS

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Neuroscience Letters, Submitted 2010

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4.1 Preface to Chapter 4

From our previous results, we hypothesized that PCSK9 may be involved in reactive synaptogenesis. Its concomitant upregulation with known cholesterol modulators during the reinnervation phase in ECL mice indicated that PCSK9 expression may ascribe to alterations in membrane cholesterol distribution within brain cells that are actively reorganizing their synaptic network. However, it remains unclear whether PCSK9, similarly to its role in peripheral organs, functions in the brain via a receptor-based mechanism. We demonstrated that in human brains presenting advanced-stages of neurodegeneration, elevated levels of PCSK9 maintain its ability to modulate the number of LDLR whereas such LDLR-degradation mechanism was not observed, at least with the chosen timepoints, in early phases of synaptic remodelling in ECL mice. The next set of experiments was designed to determine whether PCSK9, in response to neuronal damage, functions through a receptor-degradation mechanism. If PCSK9 is associated with cholesterol-driven synaptogenesis processes, the next relevant question became what is the effect of augmented expression of this convertase on neuronal plasticity events?

To address these issues, an *in vitro* model of neuronal reactive plasticity was used. PC12 cells which are derived from a rat adrenal pheochromocytoma have the capacity to differentiate into neuronal-like cells in presence of nerve-growth factor (Mesner et al., 1992). These cells acquire many properties of sympathetic neurons and have been extensively used to study mechanisms of neuronal development/differentiation and, neurites outgrowth following injury (Greene and Tischler, 1976; Nakayama et al., 2001; Nakayama and Aoki, 2000). Mild mechanical injury to differentiated PC12 cells generates an *in vitro* model of axonal injury which allows the investigation of reactive plasticity mechanisms compensating for traumatic alterations of neuronal processes.

In this study, we examined the proposed role of PCSK9 on synaptic plasticity and synaptogenesis and its effect on members of LDLR.

4.2 Abstract

Neurite elongation is a lipid-dependent mechanism required for both neurogenesis and synaptogenesis. Lipid availability modulates synaptic integrity and functions but also limits the formation of synapses in the developing and adult brain or, under chronic degeneration. In this study, we examined the role of PCSK9, a potent modulator of cholesterol homeostasis, on synaptogenesis and synaptic remodelling in normal or mechanically-injured neuronal differentiated PC12 cells. Synaptophysin immunoblotting was used to contrast the relative synaptic density in PC12 cells overexpressing PCSK9 or the vector alone. We found that the synaptophysin concentrations are markedly increased in differentiated PC12 cells transfected with PCSK9. In contrast, synaptophysin levels are reduced after experimental injury in control or PCSK9-transfected conditions. Finally, PCSK9 has the ability to modulate both apoE/apoB(LDL)- and apoE type 2 receptor expression during the synaptic recovery process; suggesting a receptor-based modulation of lipid homeostatic processes regulated by PCSK9 during active synaptic remodelling.

Keywords: cholesterol; LDLR; apoER2; synaptic density; in vitro model of compensatory plasticity; neuronal differentiated PC12 cells.

4.3 Introduction

It has long been assumed that the brain acts as a hardwired network structure incapable of neuroanatomical changes. This concept has been disproved when neurons were shown to maintain the ability to reorganize and remodel synaptic connections in the adult brain and following injuries or under neurodegenerative conditions (Cotman et al., 1981; Trachtenberg et al., 2002). The dynamic and continuous process of synaptic turnover consists of retraction and reformation of dendritic spines and re-routing of terminal sprouts to form new synapses on vacant post-synaptic neurons. Although neurons have a very limited capacity to formally regenerate in response to injury, they do have the ability to reorganize neuronal network using functional compensatory synaptic plasticity mechanisms. In agreement, death of neurons and loss of synapses that occur after brain damage correlates with an enhancement of axonal growth and collateral sprouting within the denervated regions (Raisman and Field, 1973).

Neurite outgrowth is a process that requires high supply of lipids for membranes buid-up (Mauch et al., 2001; Pfrieger, 2003a). Several studies demonstrated that cholesterol promotes axonal growth and synaptic remodelling via a specific mechanism of lipid endocytosis mediated by members of the low-density-lipoprotein receptor (LDLR) family (Hayashi et al., 2004; Holtzman et al., 1995; Mulder et al., 2004; Mulder et al., 2007). The transport, delivery and internalization of lipids to neurons in the brain are mediated through the production of functional apolipoprotein E-high-density-lipoprotein (apoE-HDL) particles and their internalization via LDLR uptake pathway. Mice deficient in either apoE or LDLR were shown to present marked loss of synaptic integrity and poor synaptic remodelling capacity accompanied with cognitive and learning impairments (Champagne et al., 2002; Champagne et al., 2005; Masliah et al., 1997; Mulder et al., 2004; Mulder et al., 2007).

In the periphery, the main regulator of plasma cholesterol concentrations is the LDLR. Recently, the proprotein convertase subtilisin/kexin type 9 (PCSK9) was found to regulate the degradation of the LDLR in peripheral tissues (Maxwell and Breslow, 2004; Park et al., 2004; Rashid et al., 2005). Overexpression of PCSK9 in mice results in

reductions of cell surface hepatic LDLR whereas the inverse relationship was reported in PCSK9 deficient mice (Benjannet et al., 2004; Maxwell and Breslow, 2004; Park et al., 2004; Rashid et al., 2005). Recently, we reported that PCSK9 expression is significantly increased during the hippocampal reinnervation in lesioned mice (Chapter 3) as well as in the brain of Alzheimer's disease subjects when compared to autopsied age-matched control cases (Chapter 2).

In this study, mechanically-injured differentiated PC12 cells were used as an *in vitro* model of reactive compensatory plasticity to investigate the effects of PCSK9 on the kinetic of synaptic remodelling and on key members of the LDLR family.

4.4 Material and methods

PC12 cells, neuronal differentiation and injury

PC12 cells (CRL-1721^{TM}, ATCC, USA) were cultured on collagen-coated dishes and maintained in F-12K medium supplemented with 2.5% fetal bovine serum and 12.5% horse serum. One day after seeding (1.0 X 10^6 cells/cm^2), cells were allowed to differentiate for 4 days in low serum conditions (F-12K medium with 1% horse serum containing 100 ng/ml β-NGF) and medium was changed every 2 days. After 4 days, medium was replaced and cells were mechanically dislodged from the dish with a cell scraper and slowly triturated with a pipette (~15 strokes). Cells were then plated in new collagen-coated six well plates and allowed to recover for 24 hours.

Transfection of differentiated PC12 cells

Transfection of pCMV6 vector and pCMV6-human PCSK9 plasmid (PS100001 and RC220000; Origene, USA) was performed on both uninjured and injured differentiated PC12 cells using LipoTAXI (Stratagene) according to the manufacturer's guidelines. After transfection (unlesioned cells (C), one day post-lesion/transfection (LT1), 2 days (LT2), 3 days (LT3) and 4 days (LT4)), cells were rinsed, removed from the plates and centrifuged for 5 minutes (min) at 14 000 rpm at 4^{o}C. Cells were then sonicated in lysis buffer (50mM Tris-HCL pH 7.5, 150mM NaCL, 0.5% Deoxycholate,

1.0% Triton-X-100, 0.1% SDS, and protease inhibitor). After one hour incubation at 4°C, lysates were centrifuged for another 10 min and supernatants were kept at -80°C until use.

ELISA assays

Quantification of PCSK9 and LDLR proteins was performed by indirect ELISA assays. Cell lysates were added to ELISA plates overnight at 4°C and incubated for two hours at room temperature on the next day, with the respective primary antibody (rabbit α-LDLR (NB110-57162), Novus Biological or goat α-PCSK9 (ab28770), Abcam). The biotinylated antibodies were added for another two hours (α-rabbit (ab6720) or α-goat (ab6740), Abcam) followed by one hour incubation with an alkaline-phosphatase streptavidin solution (Invitrogen). Following this incubation, an alkaline phosphatase fluorescent substrate (AttoPhos, Promega) was added to each well for 30 min at 37ºC. Fluorescence was detected with a microplate fluorescent reader (FL600, Bio-Tek, Winooski, VT) with a 450 nm excitation filter and 580 nm emission filter.

Western Blot analyses

25 ug of protein homogenates were separated by SDS-PAGE under reducing conditions. Transfer to a nitrocellulose membrane was conducted using the iBlot™ Dry Blotting System (Invitrogen) and membranes were blocked overnight in a 5% dried-milk Tris buffered saline solution (20mM Tris base, 137mM NaCl and 0.1% Tween-20). Then, membranes were incubated 1h30 with either a mouse monoclonal antibody against synaptophysin (No. 902 314, Boehringer Mannheim, Germany) or a rabbit anti-apoER2 antibody (generous gift from Dr. J. Hertz, Dallas, USA) followed by a secondary hour incubation with an anti-mouse or anti-rabbit horseradish peroxidase-linked antibody (Amersham). Synaptophysin immunoreactivity is commonly used to monitor synaptic loss and/or replacement in experimental models of deafferentation or in neurodegenerative diseases (Masliah et al., 1991; Nwosu et al., 2008). Chemiluminescent detection was performed with an enhanced chemiluminescence kit (Perkin-Elmer). Detection of α-tubulin was carried out as described above, using the mouse monoclonal anti-α-tubulin antibody (M61409M, BioDesign Int.) and the sheep anti-mouse horseradish peroxidase-linked secondary antibody. Density analyses were conducted using a Kodak

1D Image Analysis Software of the Image Station 440CF (Eastman Kodak Co., Rochester, NY). The region of interest analysis was used to determine the mean intensity of synaptophysin, apoER2 and α-tubulin bands.

Cellular Immumofluorescence

PC12 cells were seeded on four-well chamber slides and allowed to differentiate for 4 days before transfection was performed as described above. Two days after transfection, cells were fixed with 4% paraformaldehyde for 20 min. Cells were then incubated in Triton-X-100 solution for 20 min followed by 1h30 incubation in blocking solution (0.01M Phosphate buffer with 0.5% Bovine serum albumin and 4% normal goat serum). After the blocking step, cells were incubated overnight at 4ºC with the primary antibody (mouse α-DDK (for transfected PCSK9, TA50011, Origene); rabbit α-PCSK9 (for untransfected cells, 100007185, Cayman); rabbit α-LDLR; rabbit α-apoER2) and detected for one hour with the corresponding fluorochrome-tagged antibodies on the next day (α-mouse-FITC or α-rabbit-CY3). Then, slides were mounted with DAPI/Vectashield medium. Fluorescence was detected with a Nikon Eclipse E800 (Zeiss, Germany) using a 40X/0.75 objective (Plan-fluor, Nikon) and digital micrographs were obtained with the Spot advanced program Version 4.6 (Diagnostic Instruments Inc.).

4.5 Results

4.5.1 Synaptophysin immunoreactivity

Quantification of synaptophysin proteins was performed to assess lesion-induced axonal sprouting and synaptogenesis following experimental injury. Levels of synaptophysin in differentiated PC12 cells were significantly reduced following the mechanical lesion and managed to return to control levels by 3 days post-lesion; consistent with the known time course of synaptic loss and synapses build-up that ensue deafferentation (Fig. 16, black bars; $p<0.05$).

To assess the role of PCSK9 during synaptic remodelling, differentiated PC12 cells have been transfected with a vector containing the full length PCSK9 cDNA (or an

empty vector) in absence and presence of mild mechanical injury. Under normal conditions, levels of synaptophysin immunoreactivity were markedly increased in the presence of the PCSK9 vector compared to wild-type or vector-only transfected PC12 cells (Fig. 16, open bar (C), $p<0.05$).

After mechanical injury, synaptophysin immunoreactity was significantly reduced in lesioned PCSK9 transfected PC12 cells when compared to positive and negative control cells (Fig. 16, open bars (LT1 vs C), $p=0.001$). However, both intact and lesioned differentiated PC12 cells transfected with PCSK9 were found to display significantly higher synaptophysin immunoreactivity when compared to control cell conditions ($p<0.05$ for C and LT1 and $p<0.01$ for LT2); suggesting a direct contribution of the convertase in terminal and/or synapses formation. Figure 17 shows the relative PCSK9 expression profile.

4.5.2 PCSK9 cellular localisation and distribution

In an attempt to define the mechanism at play in this model, PCSK9 intracellular localisation and distribution was assessed in differentiated PC12 cells. PCSK9 immunostaining revealed that under normal conditions, it is mainly localised to the cytoplasm surrounding the nuclei although faint staining is detected in neurites (Fig. 18, panels A-C). In contrast, PCSK9 signal in transfected PC12 cells reveals strong immunoreactivity in neurites and filopodia of differentiated cells, away from the cell body (Fig. 18, D and G). Given that PCSK9 has been extensively studied and continuously demonstrated to negatively regulate the post-translational degradation of the cell surface LDLR (Maxwell and Breslow, 2004; Park et al., 2004; Rashid et al., 2005), it prompted us to further explore the PCSK9/LDLR connection in this model system.

4.5.3 PCSK9 effects on members of the LDLR family

A significant negative correlation was observed between LDLR and PCSK9 protein levels in both intact and lesioned cells ($p<0.05$, Reg. -0.563, data not shown). Furthermore, PCSK9 overexpression in transfected PC12 resulted in the significant

reduction of the apoER2 protein levels, another member of the LDLR family ($p<0.05$, Reg. -0.629, data not shown). This particular receptor is normally implicated in the development of the nervous system (Poirier et al., 2008b). The ability of PCSK9 to enhance the degradation of these two receptors may in part result from their co-localization at the cell surface of terminals (Fig. 18, D-I).

Co-localization of the LDLR and apoER2 in the plasma membrane and terminal projections of differentiated PC12 (Fig. 18, F and I) and their regulation by a well characterised PCSK9-mediated degradation mechanism (Kwon et al., 2008; Zhang et al., 2007) led us to examine both proteins in response to lesion-induced synaptic remodelling and terminal proliferation. LDLR and apoER2 levels are lower during the regeneration phase when PCSK9 reaches peak expression two days after transfection (Fig. 18, J and K, LT2).

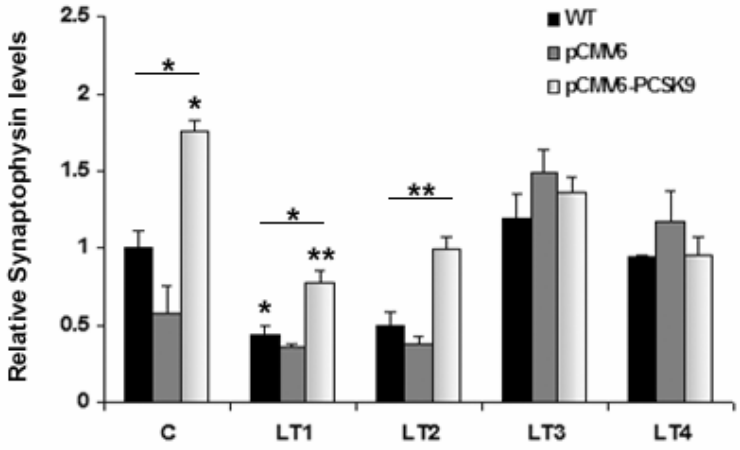


Figure 16: Levels of synaptophysin in neuronal differentiated PC12 cells under normal (C) and injured conditions (LT1-LT4).

The synaptophysin density observed in wild-type (WT), vector alone (pCMV6) or PCSK9 (pCMV6-PCSK9) transfected cells are expressed as mean fold differences relative to mean value obtained for WT unlesioned cells (C), (mean ± SEM). The synaptophysin values were normalised against α-tubulin as a loading standard for the corresponding samples. All data were generated by densitometric scans of immunoblots from three separate experiments and analysed by region of interest (ROI). Significance was determined by one-way ANOVA analysis and Tuckey's post-hoc tests for multiple comparisons (*$p < 0.05$; **$p < 0.01$).

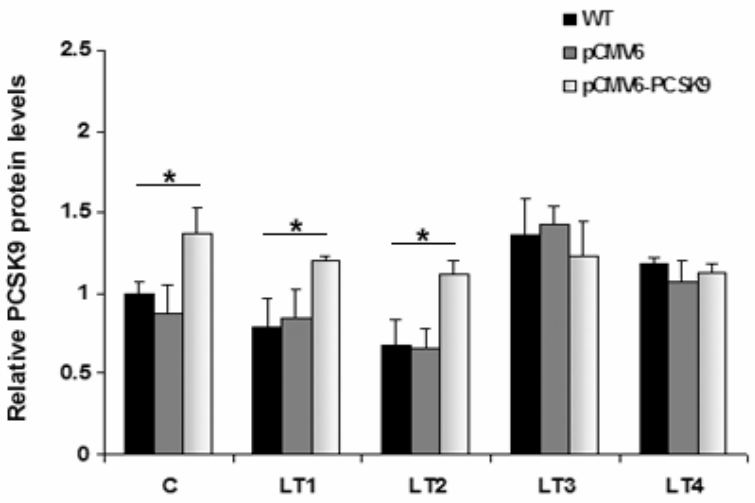
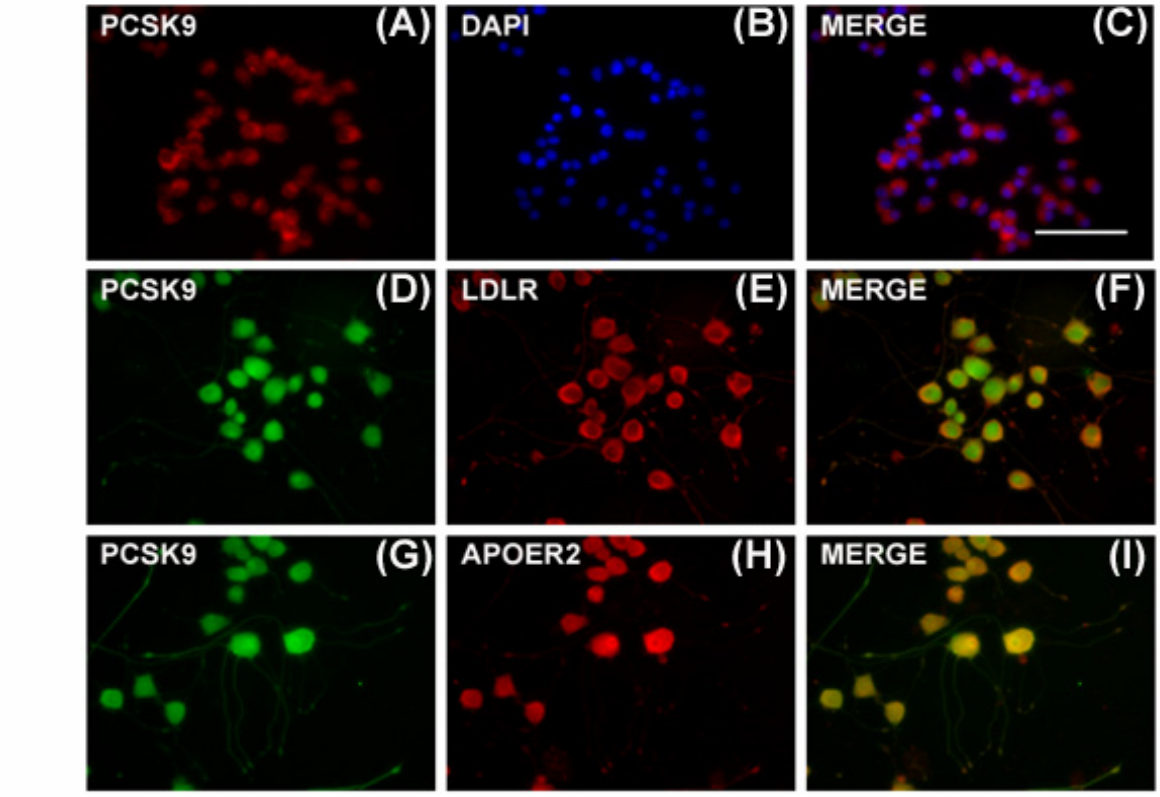


Figure 17: Levels of PCSK9 in neuronal differentiated PC12 cells under normal (C) and injured conditions (LT1-LT4).

PCSK9 protein levels observed in wild-type (WT), vector alone (pCMV6) or PCSK9 (pCMV6-PCSK9) transfected cells are expressed as mean fold differences relative to mean value obtained for WT unlesioned cells (C), (mean ± SEM). All data were obtained by ELISA assays from three separate experiments. Significance was determined by Mann-Whitney U tests with the SPSS 15.0 software (*$p \leq 0.05$) by comparing PCSK9-transfected cells to wild-type cells.


PCSK9
(A)
DAPI
(B)
MERGE
(C)
PCSK9
(D)
LDLR
(E)
MERGE
(F)
PCSK9
(G)
APOER2
(H)
MERGE
(I)

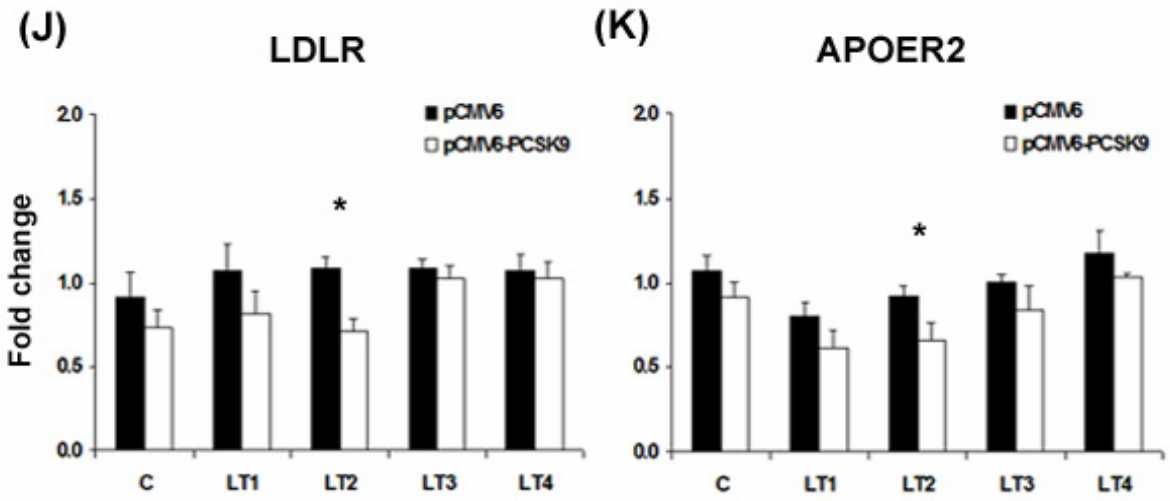

(J)
LDLR
pCMV6
pCMV6-PCSK9
Fold change
2.0
1.5
1.0
0.5
0.0
*
C
LT1
LT2
LT3
LT4
(K)
APOER2
pCMV6
pCMV6-PCSK9
2.0
1.5
1.0
0.5
0.0
*
C
LT1
LT2
LT3
LT4

Figure 18: PCSK9 cellular localisation and its effects on the LDLR and apoER2 levels in differentiated PC12 cells.

Panel A represents the endogenous staining of PCSK9 and its corresponding nuclei (DAPI) staining (B, C) in differentiated neuronal-like PC12 cells. PCSK9 distribution when overexpressed in neuronal differentiated PC12 cells is shown in green (D, G), the LDLR and apoER2 staining is represented in red (E, H) and the PCSK9 and LDLR or apoER2 co-localization is shown in merge panels (F and I, respectively). Images have been taken at a 40X magnification and the white bar represents 10 μm. LDLR (J) and apoER2 (K) protein levels are expressed as mean fold differences obtained in PCSK9 or vector transfected cells relative to the mean values obtained in WT cells for each corresponding timepoints (mean ± SEM). One-way ANOVA and Tuckey's tests were used to determine significance (*$p < 0.05$) with the SPSS 15.0 software.

4.6 Discussion

Reorganization of synaptic connections after deafferentation necessitates the growth of neurites and formation of new axonal sprouts from surviving neurons to rebuild the synaptic network. Axonal outgrowth is a process that is highly regulated by intrinsic and extrinsic molecules but firmly limited by cholesterol availability and redistribution to neurons for the generation of new membranes and terminal proliferation (Champagne et al., 2005; Mauch et al., 2001; Poirier et al., 1993a).

In the present work, we have demonstrated that a key regulator of plasma cholesterol, PCSK9, enhances the formation of new synapses in both intact and lesioned differentiated PC12 cells. This effect is mediated, at least in part, via the controlled degradation of two key apoE receptors, namely the LDL and apoE type 2 receptors. This mechanism of action is very consistent with known PCSK9 activities in brain development (Poirier et al., 2006; Seidah et al., 2003), synaptic and dendritic remodelling in the brain of adult mice (Chapter 3) as well as in liver regeneration (Seidah et al., 2003; Zaid et al., 2008).

Based on the known functions of convertase homologues, it is also conceivable that PCSK9 uses its catalytic activity to activate specific precursor proteins that in turn, govern axonal growth and synaptogenesis. For instance, several factors promoting neurogenesis/synaptogenesis such as prohormones or growth factors like nerve-growth factor (NGF), transforming growth factor TGF-β and insulin-like growth factor (IGF), are well-known substrates of different members of these convertases (Stawowy and Fleck, 2005).

In conclusion, PCSK9 transfection was shown to enhance synaptogenesis in intact differentiated PC12 cells and to catalyze the synaptic recovery in response to experimental lesions via an apoE-receptor mediated process. Further investigations are needed to fully characterize the different, yet complementary, functions of PCSK9 in the mature brain.

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Conflict of interest

The authors have no conflicts of interest to declare.

Chapter 5: General discussion

Nowadays, cholesterol has acquired a very bad reputation, and justifiably so, because elevated plasma levels associate with hypercholesterolemia, obesity and premature atherosclerosis which increase the risk of developing cardiovascular diseases and neurodegenerative disorders.

5.1 Peripheral cholesterol metabolism, PCSK9 and Alzheimer's disease

In the periphery, the reverse cholesterol transport was shown to be a major mechanism that prevents cholesterol accumulation through its transport to the liver for conversion to bile acids and its secretion into the bile for elimination (Schmitz and Grandl, 2009). Transport of cholesterol is facilitated by circulating lipoproteins which have varying effects on dyslipidemia and initiation of atherosclerosis. These lipoprotein particles are defined by their content of cholesterol, triglycerides and phospholipids and their associated apolipoproteins; high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL) and chylomicrons.

The HDL function is to remove cholesterol from peripheral tissues utilizing ATP binding cassettes (ABCA1 and G1), which results in the transfer of intracellular and dietary cholesterol to apolipoprotein A (apoA), the predominant apolipoprotein of HDL (Ragbir and Farmer, 2010). Other apolipoproteins are present on lipoproteins such as apoB and apoE which allow cholesterol clearance by receptor-mediated internalization. As these lipoproteins circulate, there are exchanges in apolipoprotein contents and transfer of cholesterol from HDL to VLDL and cholesterol-rich LDL. The latter contrary to HDL, is considered a bad cholesterol and if not removed by the liver, cholesterol from LDL progressively accumulates and associates with increased risk of atherosclerosis (Ragbir and Farmer, 2010). Therefore, the low-density-lipoprotein receptor (LDLR) expressed at the cell surface of peripheral tissues especially the liver, acts as a key regulator of plasma cholesterol concentrations as it mediates the binding, endocytosis and clearance of LDL particles from the circulation. Mutations in either LDLR or its main

ligand, APOB, result in familial hypercholesterolemia, a disease characterised by high plasma LDL-cholesterol levels, premature lipids deposition in arteries and increased risk of cardiovascular diseases (Goldstein and Brown, 1978; Innerarity et al., 1987).

Given that cardiovascular disease is one of the greatest health-related issues of our modern Western society, many efforts are given trying to reveal in addition to lifestyle risk factors, the genetic predisposition that contributes to the prevalence of this disease. In this effort, PCSK9 was identified as the third gene linked to familial hypercholesterolemia (Abifadel et al., 2003). It is involved in the metabolism of LDL-cholesterol via its negative action on the LDLR; it enhances the degradation of this receptor through a mechanism which has partially been elucidated. Some gain-of-function mutations in PCSK9 gene were reported to associate with elevated levels of plasma LDL-cholesterol which cause severe familial hypercholesterolemia whereas loss-of-function mutations give rise to the opposite results (Mousavi et al., 2009).

It is well known that high levels of LDL-cholesterol are directly correlated with the incidence of cardiovascular diseases (Lopez, 2008). In parallel, growing body of evidences is now indicating that vascular diseases and cerebrovascular dysfunctions play integral roles in the pathophysiology of neurodegenerative disorders. In fact, recent evidences from epidemiological, clinical, pathological and neuroimaging studies reveal an association between vascular-related risk factors such as hypertension, hypercholesterolemia, atherosclerosis, diabetes, obesity, myocardial infarctions, smoking, oxidative stress, ischemic white matter lesions and stroke with AD (Hooijmans and Kiliaan, 2008). It is not uncommon to find the presence of stroke and ischemic lesions in the brain of autopsied AD patients. That has led to the hypothesis that diminished cerebral perfusion from stenosed or jammed carotid arteries leading to cumulating brain damages and cognitive impairment is a process that may precede by many years the appearance of dementia symptoms. The finding that mid-life individuals who had succumbed of cardiovascular diseases or mid-life individuals having high lipid levels and died of accidental causes present AD neuropathological features (Aβ) in their brain (Kivipelto et al., 2002; Pappolla et al., 2003), is consistent with the hypothesis that neuronal damage

most likely affected by elevated plasma lipid levels begins way before the appearance of cognitive dysfunction. In agreement, no such association was found when cholesterol levels were analysed in patients later in life (Beckett et al., 2000; Tan et al., 2003). It is generally accepted that cholesterol levels are reduced in cortical and hippocampal regions but not in the low pathology cerebellum of AD patients. Much of this cholesterol reduction can directly be attributed to synaptic and neuronal loss as well as reduced cholesterol-enriched myelin (Svennerholm and Gottfries, 1994).

Taken together, these findings support one putative theory in the aetiology of AD; that early-life insults from a combination of age, lifestyle diet, vascular risk factors and genetic predisposition may all contribute to the late-life development of AD. The discovery that many lipid-related genes such as APOE, LDLR, APOB, ABCA1, LPL (Blain et al., 2004), CYP46A1 (Kolsch et al., 2009), PICALM, BIN1 and APOJ (CLU) associate with significantly higher risk of AD prompted the design of the first project of this thesis (Chapter 2). Because hypercholesterolemia is recognised as a vascular-related risk factor for AD (Kivipelto et al., 2002; Pappolla et al., 2003; Solomon et al., 2009), it was relevant to investigate whether certain genetic variants in PCSK9 gene may correlate with higher incidence of AD.

Results of this current genetic association study indicate no significant correlations between the five PCSK9 polymorphisms and the risk of AD in the general population (Chapter 2). However, only five polymorphisms have been tested in this study. The higher prevalence to AD observed in men carrying 3 of these genetic variants suggest that PCSK9 has some physiological relevance, although most likely indirect, with this disease. Furthermore, we were the first to study the expression of PCSK9 in human neurodegenerative disorders. The increased expression of PCSK9 in the frontal cortex and hippocampus of severely affected AD brains compared to age-matched control subjects further supports a possible involvement of this protease in the physiology of AD. However, the absence of any correlations between genetic variants or PCSK9 expression levels with key pathological markers of AD suggests a more likely secondary role, conceivably through compensatory processes. At least, in very late-stages of the disease

PCSK9 may be activated in response to neurodegeneration that characterises the disease in an attempt to modulate lipid metabolism and trafficking in neurons actively involved in synaptic remodelling.

5.2 Brain cholesterol metabolism

Conditions that alter neuronal integrity as in AD also affect brain cholesterol homeostasis. In the CNS, the conversion of cholesterol into 24S-hydroxycholesterol is one of the major mechanisms used by the brain to get rid of any cholesterol in excess through the BBB. Almost all 24S-hydroxycholesterol present in the human circulation is of cerebral origin and constitutes a marker for brain cholesterol homeostasis (Leoni, 2009). In the early phase of dementia, patients with AD were found to have marked increased concentrations of 24S-hydroxycholesterol in their CSF, in parallel with decreased concentrations in the circulation (Papassotiropoulos et al., 2002). Patients with active demyelinating diseases such as multiple sclerosis also present increased concentrations of 24S-hydroxycholesterol in CSF with a tendency to higher concentrations during active periods than during remission (Leoni, 2009; Teunissen et al., 2003). Together, this indicates that concentrations of 24S-hydroxycholesterol in the CSF most likely reflect a high synaptic turnover, neuronal damage and rate of neuronal loss during active neurodegenerative periods. Given that cholesterol metabolism in the brain is regulated in a similar fashion than in the periphery, it proposes that PCSK9 may participate in the maintenance of brain lipid homeostatic processes.

While the view of cholesterol as being a nasty substance that clogs arteries and causes heart diseases is widespread, the vitality of this molecule for mammalian cells is more often than not forgotten. Importantly, cholesterol constitutes an essential component of cellular membranes without which the cells cannot function. In the CNS, cholesterol is crucial for the most important feature of neurons; their ability to communicate. It is the main constituent of myelin sheats that enables efficient electrical transmission between neurons (Dietschy and Turley, 2004; Mauch et al., 2001). It is also required for membrane

synthesis, neurites outgrowth and formation of synapses during development, repair and maintenance of neuronal plasticity and function in adulthood (Pfrieger, 2003a).

Similarly to peripheral organs, cholesterol homeostasis in the brain is maintained via at least three interdependent mechanisms; endogenous synthesis, transport and endocytosis. Because the BBB prevents almost any exchange of lipids from the circulation to the CNS, it implies that all cholesterol in the brain is locally synthesized within the brain. Cells of the nervous tissue are capable of *de novo* synthesis of lipid molecules (HMGCoAR pathway) but they can also bind and take-up lipoproteins (apoE/LDLR pathway) made available in the local environment to meet their lipid requirements. Accordingly, mature neurons are believed to reduce their endogenous production of cholesterol to mainly rely on apoE-HDL-like particles produced and secreted by astrocytes. In AD brains, the activity of the HMGCoAR is reduced in cortical and hippocampal areas compared to age-matched control subjects (Appendix-2). Although, the full length HMGCoAR mRNA levels are not different between AD and control subjects, the prevalence of its transcript inversely correlates with apoE levels; suggesting a compensatory upregulation of cholesterol internalization by surviving cortical and hippocampal neurons (Appendix-2).

5.3 Reactive synaptic plasticity

During the course of ageing, several changes occur in the biology of an organism and influence cell morphology and functions leading to cumulative damages at several levels such as damage to DNA, damage to tissues and cells by oxygen radicals and cross-linking. Neurons are especially sensitive to damages caused by free radicals and the latter (oxidative damage) were also shown to increase in the normal aging and AD brain (Appendix-2). As damages accumulate, the brain reacts to neuronal damage and synaptic loss by inducing a compensatory response in order to remodel its neuronal network.

In an attempt to adapt to structural injury, uninjured nerve cells induce collateral sprouting and terminal elongation to re-build new synaptic connections. This adaptation

mechanism which is highly-cholesterol dependent implies that lipids released in the damaged area are mobilised and redistributed to neurons undergoing synaptic remodelling. In fact, astrocytes and microglia initiate this cholesterol recycling cascade as they were shown to progressively engulf synaptic terminals from degenerating or dying neurons to rapidly clear the area and allow synaptic replacement. Once internalised, cholesterol released from these terminals induce the production of apoE, apoD and apoJ. ABCA1 and ABCG1 come to play and coordinate cholesterol mobilization and efflux to these lipid-free apolipoproteins to produce functional apoE/J-HDL particles (Hirsch-Reinshagen and Wellington, 2007). Once secreted in the extracellular space, these apoE/J-HDL complexes are recognized and internalized by apoE/J receptors located in ependymal cells surrounding the ventricules or by LDLRs found at the cell surface of neurons and glia cells within the CNS. These endocytosed complexes are directed to the endosomal/lysosomal system via a mechanism involving the Niemann-Pick type C proteins 1 and 2 (NPC1 and NPC2) (Ohm et al., 2003). Within surviving neurons, the internalized cholesterol could either be esterified for storage purposes or used immediately and delivered to terminals for plasma membranes production and extension, synapse formation and remodelling. Animal models in which the lack of these aforementioned key regulators of cholesterol metabolism, (apoE, LDLR, ABCA1, NPC1) present impaired synaptic integrity and function and reduced synaptic plasticity accompanied with cognitive deficits with age (Cao et al., 2006; Karten et al., 2006; Lefterov et al., 2009; Masliah et al., 1995; Masliah et al., 1997; Mulder et al., 2004; Mulder et al., 2007; Oitzl et al., 1997; Voikar et al., 2002); which indicates that lipid trafficking and redistribution between glial cells and neurons is critical for their ability to maintain neuronal membrane integrity and function and, to remodel synaptic contacts in response to damage.

Knowing that post-injury neuronal reparative response implicates large amounts of cholesterol delivery between astrocytes and neurons for synaptogenesis, we hypothesized that PCSK9 may play an active role in these cholesterol-driven synaptic repair processes by modulating in part, the number of cell surface LDLR as previously demonstrated in peripheral cells. In agreement, PCSK9 was found to be significantly

upregulated during the intensive phase of synaptic remodelling following entorhinal cortex lesion (ECL) in mice (Chapter 3). Its upregulation observed in human AD brains may also reflect compensatory synaptogenesis occurring in a degenerating brain (Chapter 2). In addition, a recent report revealed that PCSK9 expression is stimulated by inflammation (Feingold et al., 2008) which itself (whether it is causal or end-result of brain damage) associates with numerous acute and chronic neurodegenerative disorders, including AD (Iwamoto et al., 1994; McGeer and McGeer, 2010). Of interest, the LDLR protein levels were found unaffected in the brain of AD versus control subjects even though its mRNA transcript were about 1.8 fold higher in the frontal cortex but not in the pathology-free cerebellum of AD patients (Chapter 2). This finding is consistent with the role of PCSK9 in enhancing the degradation of peripheral cell-surface LDLR proteins without affecting its mRNA levels (Maxwell and Breslow, 2004). Therefore, these results report for the first time a possible involvement for PCSK9 in synaptic remodelling in which it may regulate a significant portion of the trafficking and metabolism of brain cholesterol via a receptor-dependent degradation mechanism.

However, it is important to consider the concomitant uregulation of the LDLR and PCSK9 expression during the active phase of reinnervation in ECL mice Chapter 3). As opposed to conceivable PCSK9-induced LDLR-degradation effects occurring in late-stages of AD, the absence of a similar effect in ECL mice suggests that a reduction in LDLR levels is missed in this *in vivo* study due to limited timepoints in which a PCSK9-dependent effect on LDLR levels could take place hours or days following upregulation of PCSK9. On the other hand, PCSK9 may during the early phase of neurodegeneration/brain repair processes in AD as mimicked *in vivo* by the degeneration/reinnervation phases with the ECL model, have a receptor-independent function(s) in the brain. PCSK9 may also regulate some signalling pathways by modulating other members of the LDLR family like the VLDLR and apoER2 as previously demonstrated and observed in PC12 cells (Poirier et al., 2008b) (Chapter 4). The co-localisation of PCSK9, LDLR and apoER2 in axonal and dendritic terminals of neuronal-like cells support involvement of this protein in synaptic plasticity.

The best characterised signalling pathway mediated by apoER2 and VLDLR is the one triggerred by reelin. This signalling protein controls neuronal migration and positioning, dendritic spine development and synaptic plasticity, and in the adult brain, is mainly expressed by GABAergic interneurons (Bu, 2009). Furthermore, both apoER2 and VLDLR have binding affinity to apoE and mediate lipid endocytosis. They also interact with APP and regulate its trafficking and processing into Aβ. In the absence of common ligands, apoER2 increases the distribution of APP into lipid rafts and Aβ production (Bu, 2009). As for the LDLR, the PCSK9-mediated degradation of the apoER2 (and VLDLR) may be a mechanism that regulates intracellular uptake of lipids and Aβ peptides, or prevents the amyloidogenic pathway by limiting the apoER2/APP interaction. However, based on their signalling functions, the significance of PCSK9-mediated degradation of these receptors remains to be clarified.

It is even more interesting to emphasize here that while the expression of known modulators of cholesterol metabolism such as apoE, LDLR, ABCA1 and PCSK9 are upregulated in the active phase of synaptogenesis upon ECL, only the expression of PCSK9 is increased in late-stages AD brains compared to age-matched control subjects (Chapter 2 and Appendix-1). However, levels of apoE3 (not apoE4) and ABCA1 were previously reported to be significantly elevated at the earliest stage of dementia compared to individuals with intact cognition (Akram et al., 2010; Glockner et al., 2002).

Altogether, these results support the notion that PCSK9 play a major role in response to brain damage and fulfill separate, yet complementary functions in the ageing brain; a phenomenon clearly amplified under neurodegenerative conditions.

5.4 PCSK9 functions and possible implications in Alzheimer's disease

During development and adulthood, PCSK9 is highly expressed in the liver and small intestine, two regenerating organs involved in cholesterol metabolism (Seidah et al., 2003; Zaid et al., 2008). Interestingly, PCSK9 was found to also be expressed in brain regions where active neurogenesis takes place such as the telencephalon, rostral extension

of the olfactory epithelium, cerebellum and hippocampus (Carletti and Rossi, 2008; Seidah et al., 2003; Zaid et al., 2008).

Recent research reported a role for this convertase in neurogenesis in which the overexpression of PCSK9 has enhanced the recruitment of undifferentiated telencephalic neural progenitor cells (cells that are still proliferating) and promoted their neuronal differentiation (Seidah et al., 2003). The precise nature of the substrate(s) processed by PCSK9 is not known. However, the neurogenic effect of PCSK9 was not observed when its active site H226A was mutated; which indicates that PCSK9 requires its catalytic activity to promote neuronal differentiation of progenitor cells (Seidah et al., 2003). In agreement, the expression of PCSK9 was markedly increased in the early days of neuronal differentiation of P19 cells which did not seem to be regulated by SREBP-2. Its expression profile was very different from that of HMGCoAR and its upregulation did not affect the protein levels of endogenous LDLRs supporting a role for PCSK9 in neurogenesis distinct from a cholesterogenic function (Poirier et al., 2006). Moreover, the expression of PCSK9 in the olfactory peduncle, a location reported to contain multipotential stem cells en route to the olfactory bulb and giving rise to olfactory neurons that are renewed throughout life (Gritti et al., 2002), also supports neurogenic functions for PCSK9 in the adult brain.

Taking into consideration that the human brain, especially the hippocampus and olfactory bulb, contains neural stem cells that throughout life have the capacity to generate new functional neurons (Altman and Das, 1965; Gould and Gross, 2002), we hypothesized that in response to brain damage, PCSK9 may promote neurogenesis and synaptogenesis. The increased synaptic density observed in PC12 cells when PCSK9 is overexpressed (Chapter 4) agrees with this hypothesis.

Adult neurogenesis is actually a considerable source of structural plasticity as it involves the proliferation of progenitor cells, commitment to a neuronal phenotype, morphological and physical maturation and synaptic integration into existing neural networks. Neurogenesis is not widespread within the adult mammalian brain but is

largely restricted to the olfactory bulb, the subventricular zone of the lateral ventricule and the dentate gyrus of the hippocampus where it plays important role in synaptic plasticity and learning and memory (Crews and Masliah, 2010). Furthermore, the DG is responsive to brain insults such as excitotoxic lesion, seizures, strokes and ischemia which greatly enhances DG neurogenesis in the young and middle-aged adult hippocampus (Shetty et al., 2010). In addition, Fontana and colleagues reported enhanced neurogenesis in the deafferented hippocampus in response to ECL (Fontana et al., 2006). This neurogenesis likely reflects a compensatory response against early neurodegeneration to preserve functions and minimize damage. In agreement, stroke animal models demonstrated that neural stem cells were mildly proficient for promoting neurogenesis in aged hippocampus after injury (Jin et al., 2004b).

Prior studies established that neurogenesis occurs in the adult mammalian brain although at a reduced rate with advancing age (Kuhn et al., 1996). Within the ageing brain, the environment is different from the young adult brain in terms of intensity of signals it can produce and its ability to respond to such signals. Just as disease-related changes in the adult brain affect mature neurons and glia; neural stem cells proliferation, differentiation, maturation, and survival could as well be affected by these changes. Nevertheless, the aged brain was shown to retain the capacity to upregulate neurogenesis in response to physiological (Kempermann et al., 2002) and pathological factors (Gray et al., 2002). Data on neurogenesis in AD-like animal models remains controversial. While some have demonstrated decreased proliferation of hippocampal progenitor cells in transgenic mice (Taniuchi et al., 2007), others showed that APP/PS1 double or APP transgenic mice display increased neurogenesis in the hippocampus (Jin et al., 2004a; Lopez-Toledano and Shelanski, 2007; Yu et al., 2009). The latter finding is however consistent with an increased hippocampal neurogenesis observed in the DG and CA1 regions of AD patients (Jin et al., 2004c). Furthermore, neurogenesis was increased in neuropathological states such as ischemia or in the adult human Huntington's disease (Curtis et al., 2003) and Parkinson's disease brain (Hoglinger et al., 2004) which indicates that neurogenesis is further stimulated by neuronal damage.

Several intrinsic (expressed by the growing neurons) and extrinsic (expressed in the local environment of the growing axons) molecules can influence neurogenesis such as nearly all neurotransmitters, hormones and growth factors (Abrous et al., 2005). Epidermal-growth factor (EGF), fibroblast growth factor-2 (FGF-2), brain-derived neurotrophic factor (BDNF), stem cell factor, heparin-binding EGF, vascular endothelial growth factor (VEGF), insulin-like growth factor-I (IGF-I), glial cell derived neurotrophic factor (GDNF), nerve growth factor (NGF), neural cell adhesion molecule (NCAM L1) just to name those few, affect proliferation and neurogenesis of progenitor cells. A number of studies demonstrated that several neurotrophic factors such as those mentioned above, are enhanced in the adult injured or deafferented hippocampus (Cheng et al., 2008; Deller et al., 2006; Hagihara et al., 2005; Shetty et al., 2004; Wang et al., 2005) and serum or CSF of AD patients (Angelucci et al., 2010; Marksteiner et al., 2008; Strekalova et al., 2006). Although no such molecules have been identified yet as substrates for PCSK9, much of those are actually proteolytic targets for most of the other proprotein convertases (Bergeron et al., 2000; Seidah and Chretien, 1999; Seidah and Prat, 2002). For instance, proNGF, proBNDF, proVEGF, proNeurotrophin-3, proIGF, proTGF are all proteolytic substrates of many PCs like furin, PC1, PACE4 and PC5 (Creemers and Khatib, 2008; Lim et al., 2007; Seidah et al., 1996b; Seidah et al., 1996a) and NCAM L1 is a known substrate of PC5 (Kalus et al., 2003). Interestingly, BDNF and NGF as well as Furin, PC7, PC5 and PC1 expression are induced in lesioned peripheral nerve providing evidences that PCs are involved in the activation of neurotrophic factors during regeneration phase (Marcinkiewicz, 1999).

Therefore, it is conceivable that in response to brain damage, as seen in the brain of AD subjects, in the hippocampus of ECL mice and in injured PCSK9 overexpressing PC12 cells, PCSK9 acts as a neurogenic factor by promoting the bioactivation of neurotrophic molecules such as proBDNF or proNGF. It may use its catalytic activity to process and activate precursor proteins that directly or indirectly stimulate the proliferation and neuronal differentiation of progenitor cells. In turn, increased neuronal density may obviously lead to increased synaptic density as observed when PCSK9 is overexpressed in PC12 cells (Chapter 4). Such neurogenesis and synaptic plasticity

mechanisms are observed in several injury models like spinal cord injury (rian-Smith, 2009) and in mouse embryonic stem cells who generates mature neurons and functional synapses (Dubois-Dauphin et al., 2010). Furthermore, the fact that even following mechanical injury of PC12 cells, those in presence of overexpressed PCSK9 still present higher synaptic density compared to wild-type injured cells is consistent with a possible involvement of PCSK9 in promotion of neurogenesis and synaptogenesis during development and plasticity in the adult brain. In accordance, a very recent study reported high expression of PCSK9 in the visual cortex of mice in the first week of postnatal development (Jarvinen et al., 2010). In mice, the functional visual map formation occurs by the first postnatal week in which genes related to developmental processes such as synaptogenesis and myelination are expressed at their highest levels in the first weeks after birth. Interestingly, the visual cortex expression of PCSK9 in a progressive mouse retinal degeneration model, was elevated 4 weeks postnatal similarly to PC1 expression, which could reflect a mechanism for plasticity as the retinal degenerating mice begin compensating for visual impairment at this moment (Jarvinen et al., 2010). The authors suggested that the elevated expression of both convertases during postnatal development or under retinal degeneration could have an impact on synaptogenesis via proteolysis of neurotrophic factors such as pro-BDNF known to be upregulated during early postnatal brain development (Jarvinen et al., 2010).

Further to a neurogenic function of PCSK9 in the brain, a role for this convertase in the regulation of brain lipid homeostasis is also supported by our results. The concomitant upregulation of PCSK9, apoE, ABCA1 and LDLR during the early and active phase of synaptic remodelling in response to ECL in mice (Blain et al., 2006b) (Chapter 3 and Appendix-1) suggest synergistic effects of those cholesterol modulators in the regulation of efficient lipid trafficking and endocytosis processes. While the LDLR expressed at the cell surface of neurons are essential for the delivery and internalization of cholesterol required for membrane synthesis, neurites elongation and synaptogenesis, the degradation of this receptor by PCSK9 seems contradictory to its role in synaptic remodelling processes. However, it is known that intracellular cholesterol accumulation or excess in plasma membranes is detrimental for neuronal functions (see Appendix-2,

Fig. 29). Local increased in neuronal membranes via redistribution of brain cholesterol provides an enriched environment for Aβ production. Indeed, high intracellular and membrane cholesterol was shown to favor APP processing and Aβ production (Frears et al., 1999; Puglielli et al., 2001; Wolozin, 2001). In addition, intraneuronal accumulation of Aβ via endocytosis (extracellular Aβ clearance) could also lead to neuronal dysfunction and eventual death (Billings et al., 2005; LaFerla et al., 1997; Takahashi et al., 2002). Furthermore, intracellular misrouting and neuronal accumulation of free cholesterol as observed in Niemann Pick type C (NPC) disease correlate with elevated Alzheimer's disease-like NFT in neurons (Distl et al., 2001; Ohm et al., 2003).

On one hand, cholesterol is an essential component of the brain and on the other hand, alteration in its homeostasis affects neuronal functions and is implicated in the development of neurodegenerative disorders such as AD. A fine tuning of its concentration is therefore necessary for the proper functioning of the brain. That comes to the notion that having too low concentrations of cholesterol is not good for the organism but too high is not better! In turn, one possible mechanism to explain the PCSK9-mediated degradation of the LDLR in the brain is that PCSK9 acts as an on-site mechanism that directly regulates lipoproteins internalization via the apoE/LDLR pathway to prevent excessive cholesterol (and Aβ) accumulation in neurons undergoing membrane remodelling and synaptic reorganisation. Instead of only relying on the transcriptional regulation of the LDLR to limit cholesterol endocytosis, neurons may also depend on PCSK9 as an additional post-transcriptionnal LDLR control.

Altogether, these observations raise the possibility that PCSK9 in the brain, at least in certain pathophysiological conditions, has functions that goes beyond cholesterol metabolism. Together with previous reports, the present study suggests that brain damage (as in ageing, brain injury, ECL, AD) enhances the expression of PCSK9 to compensate for the synaptic and neuronal loss via in part promoting neural cells proliferation, differentiation, maturation and synapse formation and on the other part, regulating brain lipid metabolism by modulating LDLR-mediated lipid intracellular uptake (Fig. 19).

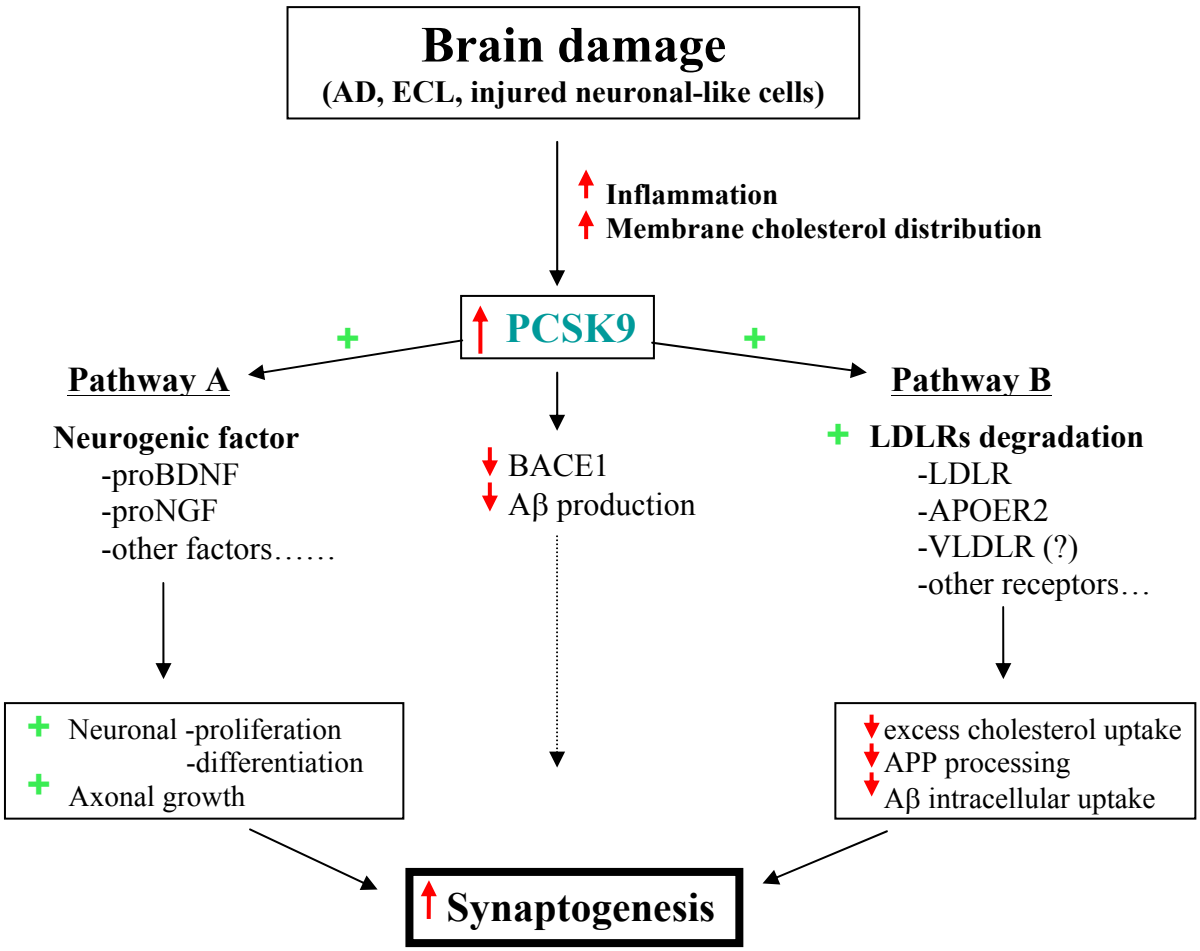


Figure 19: PCSK9 functions in the brain in response to damage

In response to brain damage or degeneration, several molecular changes occur. Increased PCSK9 expression, membrane cholesterol distribution and inflammatory response which is known to stimulate PCSK9 expression, are observed. In turn, PCSK9 may act as a neurogenic factor through the activation of neurotrophic factors such as BDNF or NGF which promotes neuronal proliferation, differentiation and maturation as well as axonal growth for synaptic remodelling. On the other hand, PCSK9 may regulate lipid distribution and endocytosis via downregulation of LDLRs which prevents excessive lipid uptake thereby limiting APP processing and Aβ production. A direct effect of PCSK9 on BACE1 and Aβ peptides was previously suggested (Jonas et al., 2008). Altogether, these changes favor functional synaptogenesis processes to recover from brain damage.

5.5 Summary and hypothetical model

The growing amount of evidences linking cholesterol to AD strongly support the idea that cholesterol accumulation or dysfunction in its metabolism, in the circulation as well as in the brain, play a major role in the development of this neurodegenerative disorder. This hypothesis is based on the original finding that the APOE ε4 allele is a strong genetic risk factor for AD (Poirier et al., 1993b; Strittmatter et al., 1993). Individuals expressing the APOE ε4 allele or mutations in other lipid-related genes such as LDLR, ABCA1 and APOJ predispose to cardiovascular disorders and act as AD risk factors. Evidences indicate that plasma LDL-cholesterol levels increase with age (Huang et al., 2009) and this could be the result of disrupted cholesterol homeostasis as a consequence of dysfunction, misrouting and/or reduced expression of these lipid-related proteins with age (Berrougui and Khalil, 2009; Marino et al., 2002; Pallottini et al., 2006; Sabaretnam et al., 2010). Furthermore, these proteins also play a direct role in the regulation of brain lipid homeostasis and amyloid metabolism. As AD is considered a multifactorial disorder in which neuronal loss begins well before the appearance of clinical symptoms, it is most likely that a contribution of diet, lifestyle and vascular genetics contribute to the age-related progressive brain damage (SP and NFT deposition, inflammation, oxidative stress, synaptic loss, hippocampal atrophy). Combination of these factors in which age is definitively the most important risk factor, followed by genetics and vascular risk factors, associates with the onset and course of neurodegeneration underlying AD or other age-related neurodegenerative disorders (Fig. 20).

However, the capacity of the brain to respond to neuronal damage largely depends on the brain cholesterol availability. It has become clear that physiological functions of apoE and other lipid modulators (ABCA1, APOJ, LDLR) are essential for the reparative and remodelling processes that occur in response to neuronal damage in both the normal ageing and neurodegenerative brain. It is of interest to note that one of the latest gene that revealed association with AD, PICALM (Harold et al., 2009), controls the assembly of clathrin-coated pits that in turn, coordinate apoE/LDLR complexes internalization. Its

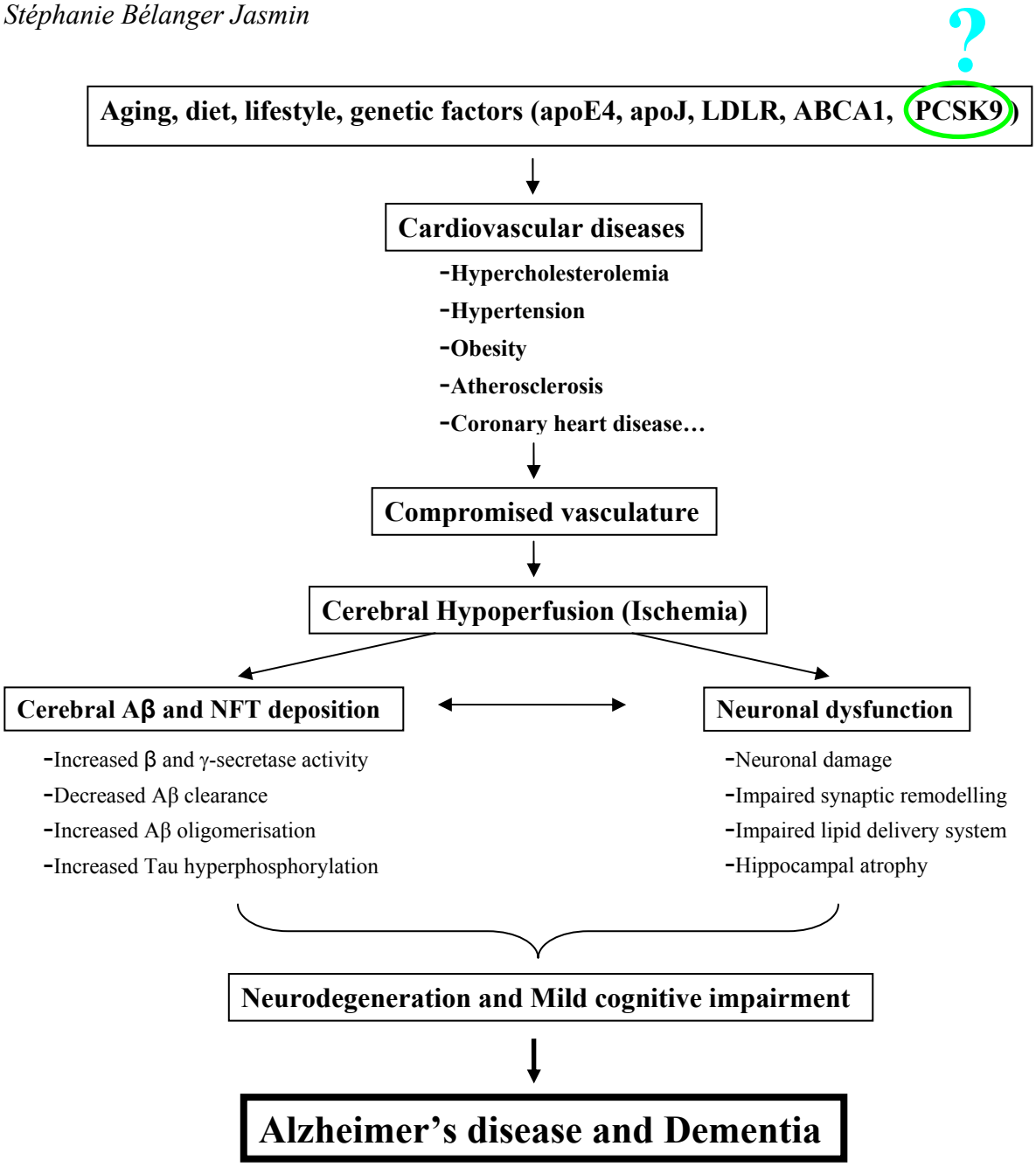


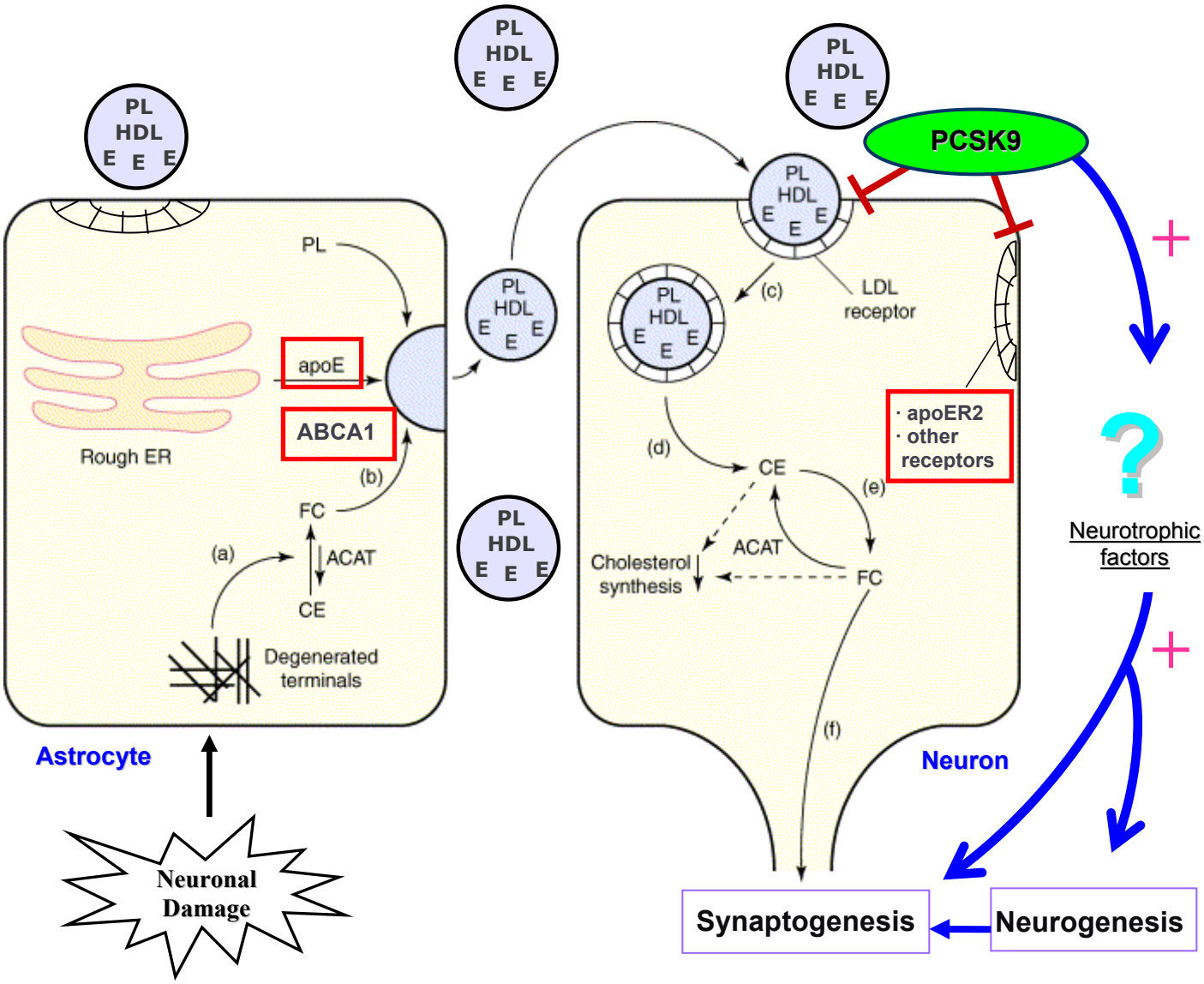
Figure 20: Peripheral cholesterol and proposed sequence of events in the development of Alzheimer's disease.

A combination of several factors such as ageing, diet, lifestyle and vascular-related genetic factors contributes to the development of vascular and cerebrovascular disorders which correlate with cerebral hypoperfusion and neuronal dysfunction. As brain damage expands, it correlates with the onset and course of neurodegeneration as found in AD.

reduction in embryonic hippocampal neurons results in dendritic atrophy, reduced endocytosis and disrupted secretory transport (Bushlin et al., 2008). The results of this PhD project has brought another player into this cascade of morphological, neurochemical and gene expression changes occurring in nerve cells in an attempt to adapt to structural injury by sprouting and remodelling.

As illustrated on figure 21, we propose a role for PCSK9 in synaptic remodelling processes in response to neuronal damage in which it stimulates neuronal differentiation and synaptogenesis either by modulating different signalling pathways (affecting the apoER2, VLDLR or other yet to determined receptors) or indirectly, by activating neurotrophic factors which in turn promotes neurogenesis, axonal outgrowth and sprouting, and synaptogenesis (Fig. 21). Although we cannot exclude the possibility that PCSK9 regulates a significant proportion of LDLR in the CNS, it is more likely that during the early pre-clinical phases of degeneration in AD, PCSK9 may function as a neurogenic factor that stimulates the generation of new neuronal cells and formation of new synapses to compensate for the lost ones. However, when nerve cells have met their lipid requirement and hold enough cholesterol to generate new membranes and extend their terminals, PCSK9 is then increased in a time-dependent manner to regulate cholesterol uptake in remodelling neurons (Fig. 21). This receptor-based regulatory mechanism most probably applies throughout the degradation process but reaches a peak in late-stages of neurodegeneration when most neurons are severely damaged, unhealthy and saturated of recycled cholesterol derived from degenerating terminals of surrounding dying neurons.

As neurogenesis in the DG of the hippocampus is known to be neurodegenerative-stage dependent (Chen et al., 2008), we propose that in the early stages of neurodegeneration, PCSK9 may be involved in the generation of new neurons as a self-repairing mechanism to compensate, at least partially, for the neuronal loss. At late stages of neurodegeneration when survival of newly generated neurons is dramatically impaired so that functional neurogenesis is reduced (Chen et al., 2008), PCSK9 may be involved in


PL
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Damage
PL
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PCSK9
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receptor
· apoER2
· other
receptors
(d)
CE
(e)
Cholesterol
synthesis
ACAT
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+
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Neurotrophic
factors
+
Synaptogenesis
Neurogenesis

Figure 21: Hypothetical model for PCSK9 in the injured central nervous system.
Degenerating terminals, produced due to neuronal damage and synaptic degeneration, are internalized by astrocytes and microglia and (a) degraded to release free cholesterol (FC). This FC can be stored as cholesterol esters (CE), formed by the acyl-coenzyme-a cholesterol acyltransferase (ACAT) or used together with other phospholipids (PL) for the assembly of apoE-cholesterol (E) complexes mediated by ATP binding cassette (ABCA1) (b). These high-density lipoprotein (HDL) complexes are either directed toward the circulation and/or to specific brain cells such as neurons that need large amount of lipids. In the latter case, apoE complexes bind and (c) are then internalized by a neuronal member of the cell surface LDL-receptor family. Once taken up by neurons, cholesterol is released in an esterified form (d) and can be converted to FC (e) for immediate use in dendritic proliferation and synaptogenesis (f). As a consequence of high cholesterol influx, transcription of genes involved in cholesterol synthesis or internalization is suppressed. In this cascade, PCSK9 may be a novel player in regulating concentration of lipids endocytosed by neurons during the active phase of remodelling. It may function as an on-site player that limits an excessive uptake of cholesterol by enhancing the degradation of cell surface LDLR. Moreover, PCSK9 may promote neurogenesis and synaptogenesis via other signalling pathways by the activation of neurotrophic factors such as growth factor and hormones or by regulating other members of the LDLR like the apoER2, VLDLR or other unknown receptors. (Picture modified from *Trends in Molecular Medicine 2003; 9 no.3; 94-101*).

the regulation of intracellular lipid endocytosis via a post-transcriptional control of the LDLR.

5.6 Therapeutic avenues and future directions

Results of this project may have significant importance from a therapeutical stand point especially when considering that PCSK9 could be used as a potent pharmacological target for reducing circulating plasma LDL-cholesterol levels.

In healthy donors, serum levels of PCSK9 were found to positively correlate with total and LDL-cholesterol but not HDL levels (Alborn et al., 2007; Careskey et al., 2008). Such correlations were confined to men and not women in another study (Mayne et al., 2007). Interestingly, the use of atorvastatin (40mg), the most widely prescribed statin to lower LDL-cholesterol, significantly increased human PCSK9 serum levels after 3 months of treatment and reduced by 42% LDL-cholesterol levels (Careskey et al., 2008). Since this study, many groups have also reported increases of PCSK9 levels in humans treated with statins in which a correlation with LDL-cholesterol levels was also disrupted (Cariou et al., 2010; Mayne et al., 2008; Welder et al., 2010). In a recent study, a dose of 80 mg of atorvastatin caused 47% increase in serum PCSK9 that was sustained for the 16 weeks of treatment. What is more striking is that while PCSK9 and LDL-cholesterol levels were highly correlated at baseline, subjects that had the greatest increases in atorvastatin-induced PCSK9 levels tended to have the largest atorvastatin-induced decreases in serum LDL-cholesterol (Welder et al., 2010); suggesting that overall, PCSK9 may have beneficial effects following statin treatment.

As much effort is made to reduce circulating cholesterol and improve health conditions, contradictory results have been obtained for the treatment of AD with cholesterol-lowering drugs. In the latest large-scale randomized controlled trial, treatment of mild to moderate AD patients with atorvastatin did not associate with significant clinical benefit (Feldman et al., 2010). However, statin use in the general population showed reduced incidence or risk of developing AD (Jick et al., 2000; Rockwood et al.,

2002; Wolozin et al., 2000; Wolozin et al., 2007; Zamrini et al., 2004). In NPC patients, a combination of lipid-lowering agent with a low-cholesterol diet has partially reduced cholesterol load in liver but did not result in neurological improvement (Schiffmann, 1996; Vanier, 2010). These epidemiological studies suggest that the appropriate treatment for AD may more likely be a preventive one in which administration of cholesterol-lowering agents should begin well before the appearance of clinical symptoms. Statins are known to inhibit cholesterol synthesis, reduce the formation and internalization of LDL-cholesterol, and upregulate LDLR at the cell surface of liver cells thereby reducing serum LDL-chlesterol and triglycerides. It is still unclear if the beneficial effects of statins in the brain are a consequence of brain penetration of those inhibitors or if the pre-symptomatic protection is mediated through plasma cholesterol alterations. It is interesting to mention that while the pre-symptomatic use of statins confers some levels of protection against AD, those agents increase by more than 40% the levels of PCSK9. A relationship with statin-induced PCSK9 levels and preclinical benefits of statin use on the incidence of AD certainly merits further investigation.

Furthermore, special attention should be paid when developing an antagonism or inhibitor of PCSK9 as a therapeutic drug. Recent studies suggested critical role of PCSK9 in liver regeneration upon hepatic damage (Seidah et al., 2003; Zaid et al., 2008), a process requiring mobilization of cholesterol and other lipids that is similar to terminal remodelling and reconstruction in the adult brain. In order to move ahead with identifying therapeutic targets for PCSK9, it is imperative that the cell biology and function of PCSK9 in the brain be better explored. Although we presented the first evidence that PCSK9 is most likely expressed by neurons than glia cells (Chapter 3), analyses of its subregional and cellular localization in brain are essential. Studies of its function, regulation, and interaction with modulators of the neuronal endocytic pathway such as caveolin and clathrin molecules, and the identification of PCSK9 substrates in the brain like growth factors, cell adhesion molecules or neurotrophins, would greatly advance our understanding of PCSK9 role in the adult and diseased brain.

Since PCSK9 knockout mice display hypersensitivity to statin, increase LDLR proteins in the liver and decrease plasma LDL-cholesterol, they could be used to study i) the implication of PCSK9 in synaptic remodelling after ECL when compared to wild-type, overexpressing PCSK9 and statin-treated mice. As ECL-mice display temporary spatial learning deficits (Hardman et al., 1997), investigating behaviour changes and cognitive functions in these differents untreated or statin-treated animals would also be relevant to clarify its putative recovery function related to synaptic remodelling further to determine the statin-induced PCSK9 significance. Moreover, generating animal models homozygous for some of the more frequent PCSK9 mutations would be of great importance to understand its brain function, its cellular biology and establishing genotype-phenotype correlation during development and in response to brain damage. Elucidating PCSK9 molecular mechanisms during neurodegeneration is also important to clarify its proposed role in neurogenesis and synaptogenesis.

In the context of AD, it would also be of interest to generate PCSK9 X APP X TAU triple transgenic mice to investigate whether PCSK9 interferes with BACE1 function and Aβ production as previously reported (Jonas et al., 2008). While we did not observe any correlation between PCSK9 and AD neuropathological markers in end-stages of the disease, we cannot exclude the possibility that in the early phase of neurodegeneration, PCSK9 affects the development and accumulation of senile plaques and neurofibrillary tangles. As it was demonstrated that PCSK9 has the ability to enhance the degradation of apoER2 and VLDLR, two signalling receptors indirectly involved in the regulation of GSK3 kinase (the main tau phosphorylation kinase) (Herz, 2009), there is a possibility that PCSK9 mediates tau phosphorylation. In fact, overexpression of PCSK9 in HepG2 cells resulted in the downregulation of mitogen-activated protein kinase and cyclin-dependent kinase 5 (Lan et al., 2010), two kinases also involved in the phosphorylation of tau. Furthermore, having access to post-mortem brain tissues from patients with mild cognitive impairment (MCI) would be of high importance to determine whether i) PCSK9 is upregulated in the early pre-clinical phase, ii) if it affects LDLR or other members of the LDLR family and iii) if it correlates with AD neuropathological markers. Also, having access to blood samples of MCI or AD patients would be of

interest to determine whether an increased circulating expression of PCSK9 is observed under these degenerative conditions and if it correlates with elevated circulating LDL-cholesterol.

The three studies presented here have shed some light on the recently discovered proprotein convertase, PCSK9, in a context of brain injury, neuronal damage and Alzheimer's disease. Although the exact role of PCSK9 in extra-hepatic organs begins to be explored, we are among the first to propose a significant role for PCSK9 in synaptic plasticity processes in the mature CNS. Results of these studies highlight the importance of PCSK9 in response to neurodegeneration and its implication in synaptic remodelling and synaptogenesis processes via at least in part, a receptor-based mechanism.

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Appendices

Appendix-1

ABC Transporters ABCA1 and ABCG1 gene Expression during Synaptic Remodelling in Response to Hippocampal Deafferentation

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Abstract

Glial-derived lipoparticles facilitate the transport of cholesterol and lipids between cells within the CNS and have been shown to support neuronal growth and synaptogenesis. Partial deafferentation of the hippocampus following unilateral entorhinal cortex lesioning (ECL) induces well-described cytoarchitectural reorganisation and reactive sprouting in the dentate gyrus. The ATP binding cassette transporters (ABC) A1 and G1 assist the generation of lipoparticles by mediating cholesterol and phospholipid efflux to extracellular apolipoprotein E (apoE), the brain's primary high density lipoprotein. To examine a role for these transporters during active synaptic remodelling, we measured the time course expression of ABCA1 and ABCG1 in the hippocampus of adult mice in response to deafferentation and subsequent reinnervation following ECL. It was observed that ABCA1, but not ABCG1, is significantly upregulated at the level of both mRNA prevalence and protein levels in the deafferented hippocampus along with apoE. The increase coincides with a period of active compensatory cholinergic sprouting in the dentate gyrus as determined by the histochemical analysis of acetylcholinesterase activity. These findings suggest a timely role for the ABCA1 transporters in the generation of functional lipoparticles and, in the dynamic regulation of cholesterol recycling observed during the early, but very active phase of dentate neurons reinnervation in response to injury.

Introduction

The brain is the most cholesterol-rich organ of the body as it contains ~25% of total cholesterol in 2% body weight (Dietschy and Turley, 2001). Regulation of cholesterol homeostasis in the central nervous system (CNS) is maintained independently from the periphery and negligible amounts of plasma cholesterol are incorporated into CNS cholesterol pool (Edmond et al., 1991; Jurevics and Morell, 1995). In turn, CNS relies on *de novo* cholesterol synthesis via the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAR) pathway, the rate limiting step in cholesterol synthesis and, on efficient lipid transport and recycling mechanisms (Dietschy, 2009; Poirier et al., 1993a). Glia and neurons provide the sterol necessary for brain development and once achieved, it is postulated that mature neurons reduce their cholesterol production to mainly depend on lipids synthesised by astrocytes (Mauch et al., 2001). This derivation of glial-cholesterol and other lipids to neurons is necessary to support the high lipid-demands imposed by synthesis of new membranes for reactive sprouting of terminals and synaptogenesis particularly observed during repair following brain damage (de Chaves et al., 1997; Mauch et al., 2001; Poirier, 1994; Posse de Chaves et al., 2000). Transport of cholesterol and lipids between cells of the CNS is facilitated primarily through the formation of lipoparticles of similar size and constitution as peripheral high-density-lipoproteins (HDL) (Illingworth and Glover, 1971; Ladu et al., 1998).

Predominantly synthesised by astrocytes, apolipoprotein E (apoE) functions as the primary protein moiety of HDL within the CNS (Mauch et al., 2001; Poirier et al., 1991a; Poirier, 1994) and has been shown to mediate the binding and internalization of glial-derived HDL by regenerating nerve terminals (DeKosky et al., 1984; Steward and Scoville, 1976). Although there is evidence to suggest that glial-derived apoE-containing lipoparticles are essential for brain development and in neural regeneration observed following injury, the mechanisms through which the assembly of HDL in the CNS is regulated are not clearly understood.

Recently, a role for members of the ATP-binding cassette (ABC) family of transporters in the regulation of cholesterol transport between cells and in the formation

of HDL has been identified. Expressed ubiquitously by microglia, astrocytes and neurons (Koldamova et al., 2003), ABCA1 has been shown to influence apoE secretion and lipidation (Abildayeva et al., 2006; Hirsch-Reinshagen and Wellington, 2007). Lack of ABCA1 in mice causes >75% reduction of brain apoE protein levels with structural and functional deficits in neurons (Hirsch-Reinshagen et al., 2004; Karasinska et al., 2009; Wahrle et al., 2004). Several lines of evidence suggest that marked alterations in CNS cholesterol homeostasis may affect brain functions such as myelination, neuronal repair and cognition, and may also contribute to the pathology of neurodegenerative disorders. In agreement, mice lacking apoE or apoE receptors present altered synaptic plasticity and learning and memory deficits (Champagne et al., 2002; Champagne et al., 2005; Krugers et al., 1997; Masliah et al., 1995; Mulder et al., 2004). Recently, genetic variations in ABCA1 have been proposed to modify the risk of Alzheimer's disease (AD) (Katzov et al., 2004; Rodriguez-Rodriguez et al., 2007; Sundar et al., 2007) although replications of the original finding has proven to be difficult (Kolsch et al., 2006; Li et al., 2004; Shibata et al., 2006).

Initially identified due to its high sequence homology with ABCA1, the half-transporter ABCG1 has since been shown to also participate in the regulation of cholesterol and sphingosine-1 phosphate efflux, and to direct the assembly of mature-HDL (Gelissen et al., 2006; Klucken et al., 2000; Vaughan and Oram, 2005). Proposed to function either as a homodimer or heterodimer in conjunction with other ABCG transporters (Klucken et al., 2000), ABCG1 has been demonstrated to facilitate the efflux of cholesterol to partially lipidated but not lipid-free lipoproteins (Kennedy et al., 2005). It is proposed that ABCA1 catalyses the initial cholesterol loading of lipid-free apolipoproteins whereas ABCG1 completes lipidation and mediates cholesterol, phospholipid and sphingosine-1 phosphate export (Gelissen et al., 2006; Vaughan and Oram, 2006). The results of *in situ* hybridization studies demonstrate that ABCG1 is expressed in the murine brain throughout development and maturity, and appears to colocalize with ABCA1 expression (Tachikawa et al., 2005). Thus, the cholesterol efflux and formation of mature HDL is proposed to be synergistically regulated by ABCA1 and ABCG1.

To determine whether these transporters function in the dynamic regulation of CNS cholesterol homeostasis, we examined the expression of ABCA1 and ABCG1 during reactive sprouting and compensatory synaptogenesis in mice following experimental hippocampal deafferentation. Here, we demonstrate that in response to brain injury, hippocampal ABCA1 but not ABCG1 expression is upregulated during the early phase of reinnervation, a time characterised by the active mobilization of lipids and their transport toward neurons undergoing remodelling. These results indicate that ABCA1 is an important player in the dynamic regulation of brain synaptogenesis, presumably through its function as regulator of local cholesterol homeostasis and transport.

Materials and Methods

Animals

Male C57BL/6J mice aged 12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were housed individually in an enriched environment and fed with a diet of standard laboratory chow *ad libium*. A 12-hour light-dark cycle was maintained with light onset at 7:00 and offset at 21:00, local time. All protocols were carried out in accordance with the Canadian Guidelines for Use and Care of Laboratory Animals, and were approved by the McGill University Animal Care Committee.

Unilateral Entorhinal Cortex Lesions (ECL)

Unilateral electrolytic lesions to the right entorhinal cortex were conducted according to the technique adapted for mice described by Blain and colleagues (Blain et al., 2004). Mice were anaesthetised by the intramuscular injection of a ketamine/xylazine/acepromazine mix (dose: 1µl/g of body weight) and placed into a stereotaxic apparatus in a flat skull position. Lambda 0 was determined and the skull was drilled to allow the electrode to pass, at an angle of 6º, to the 4 lesion coordinates: (1) [AP: 0mm], [L: -3.0mm], and [DV: -3.0mm, -4.0mm]; (2) [AP: 0mm], [L: -3.5mm], and [DV: -3.0mm, -4.0mm]; (3) [AP: +0.5mm], [L: -4.0mm], and [DV: -3.0mm, -4.0mm]; (4) [AP: +1.0mm], [L: -4.0mm], and [DV: -3.0mm, -4.0mm], and a current of 1mA was applied for a 10 secondes (sec) duration at each coordinate. The skull was then patched

with bone wax and the incision sutured. SHAM-operated animals were treated similarly, however the electrode was lowered only 1mm and no current was passed. Following surgery, mice were given a subcutaneous bolster of physiological saline to prevent dehydration. Animals were nursed throughout their recovery; remaining under a heat lamp for 24 hours before being returned to their home cage for further monitoring. Food and water were not withheld prior to or following surgery. At 2, 6, 14, 21, 40 and 60 days following surgery, mice were administered a lethal dose of anaesthetic and perfused transcardially with 30ml of ice-cold 0.01M phosphate buffered saline (PBS) solution. SHAM-operated mice were sacrificed at 14 days post-lesion (DPL).

Whole brain dissects and slide preparation

Following perfusion, whole brains from 20 mice (2, 6, 14 and 21 DPL; n=5 per group) were removed and flash-frozen at -40ºC in isopentane. Brain dissects were cryosectioned in the coronal plane producing sections of 20µm thickness and mounted on poly-L-lysine coated glass slides, desiccated overnight at 4ºC, and stored at –80ºC until use. Sections through the region of the dorsal hippocampal formation were used for histochemistry analyses.

Acetylcholinesterase (AChE) histochemistry

Cholinergic sprouting in the dentate gyrus (DG) was assessed by measuring the relative density of AChE activity in the outer molecular layer (OML) following ECL. Briefly, slides were incubated at room temperature in the substrate solution (0.0072% ethopropazine, 0.075% glycine, 0.5% cupric sulphate, 0.12% acetylthiocholine iodide, 0.68% sodium acetate; pH 5.0) for 4 hours. Following this, the slides were rinsed 3 x 5 minutes (min) in H_2O and then placed in the developer solution (0.38% sodium sulphide; pH 7.8) for 6 min. After a second series of H_2O rinses (3 x 5 min each), silver intensification was performed by placing the slides into 1% silver nitrate solution for 2 min with total light obscurity. Slides were rinsed again in H_2O and post-fixed in a 4% paraformaldehyde solution in 0.01M PBS (pH of 7.4) for 2 hours. Slides were then rinsed a final 3 times in 0.01M PBS (pH 7.4) before being dehydrated in a series of alcohol baths and cleared in xylene (2 min in each bath). Slides were cover-slipped with DPX

mounting medium and stored in total light obscurity until analysed. All products used were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Digital microphotographs of brain sections were captured using a Zeiss Axioskop 2 Plus microscope using a 2.5X/0.075 objective (Plan-Neofluar, Zeiss, Germany) and the Northern Eclipse Version 6.0 Image Analysis Software.

Assessment of cholinergic sprouting

To assess the time course and extent of cholinergic sprouting following ECL, the relative optical density (OD) of AChE staining in the DG both ipsilateral and contralateral to the lesion was evaluated using the MCDI-II image analysis system. AChE activity was measured at 6 different points along the dorsal blade of the OML of the DG on 5 sections per animal (n=5 animals per time point). Staining density was calculated as the relative OD ratio of ipsilateral to contralateral measures, thereby providing a within-section control for variations in histochemical processing.

The lamina-specific denervation of the DG following ECL has been shown to result in atrophy localised to the OML that may confound the histochemical estimation of AChE activity (Fagan and Gage, 1994). Therefore measures of OML width were assessed at 6 positions along the dorsal blade of the dentate and incorporated into the determination of the ratio of ipsilateral: contralateral AChE staining density according to the model of Fagan and Gage (Fagan and Gage, 1994) using the following equation:

$$\textbf{AChE OD Ratio} = \frac{(\text{AChE Density}_{\text{ipsilateral}}) \text{ X } (\text{OML Width}_{\text{ipsilateral}} / \text{OML Width}_{\text{contralateral}})}{(\text{AChE Density}_{\text{contralateral}})}$$

Real-time polymerase chain reaction (RT-PCR)

Ipsilateral and contralateral sides of the hippocampus and frontal cortex were isolated from 5 animals per group (SHAM, 2, 14, 21, 40 and 60 DPL) and used in the evaluation of ABCA1 and ABCG1 mRNA expression. Total RNA was extracted from these brain regions using the QIAGEN RNeasy Mini Kit (QIAGEN Inc., Mississauga, ON) according to manufacture's guidelines for animal tissues. Following extraction, 2µg

of total RNA from each sample was reverse-transcribed (RT) in the GeneAmp 5700 sequence detection system (PE Applied Biosystems) to generate cDNA in the following reaction mixture: 1X RT buffer; 5.5mM $MgCl_2$; 500µM dNTPs; 2.5µM Oligo DT; 0.4U/µl RNase Inhibitor; 1.25U/µl Multiscribe Reverse Transcriptase, in a final reaction volume of 100µl. The reverse transcriptase program included the following thermal cycle: 10 min at 25ºC, 30 min at 48ºC, followed by 5 min at 95ºC to stop the reaction.

RT-PCR was conducted using the SYBR® Green technique in the GeneAmp 5700 sequence detection system in triplicate for each RT product sample. Primer pairs used for PCR amplification were as follows: mABCA1-fwd 5'-GACCGTACTCTCGCAGGG-3' with mABCA1-rev 5'-GCGGCCTTGCCGGTAT-3'; mABCG1-fwd 5'-CCGATGTGAACCCGTTTCTT-3' with mABCG1-rev 5'-AGGCGGAGTCCTCTTCAGC-3'; and mACTIN-fwd 5'-TGACCGAGCGTGGCTACA-3' with mACTIN-rev 5'-TCTCTTTGATGTCACGCACGAT-3'. Primer pairs were generated using the Primer Express PE Biosystems software. Primer specificity was confirmed through dissociation curve analysis which demonstrated single product specific melting temperatures. No primer-dimers were observed during the 40 PCR cycles. The master-mix solution for each 35µl PCR reaction was prepared as follows: 17.5µl of SYBR Green PCR Master Mix, 3.5µl of 10pM stocks of forward and reverse primers, 7.5µl RNase-free H_2O, 3µl RT product. All reagents used were purchased from PE Biosystems (Perkin Elmer, Foster City, CA). The program applied for RT-PCR cycling consisted of 2 min at 50ºC, followed by 10 min of denaturation at 95ºC. Amplification was then achieved through 40 cycles through a two-segment RT- PCR program consisting of 15 sec at 95ºC followed by 1 min at 60ºC. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and results are expressed as ratios between ipsilateral and contralateral sides to the lesion location relative to β-actin ± standard error mean (SEM).

Mouse ABCA1 ELISA assay

Brains from 5 mice per group (SHAM, 2, 14, 21, 40 and 60 DPL) were removed and dissected on ice. The ipsi- and contralateral hippocampi were isolated and sonicated

in 0.01M PBS containing protease inhibitors (Boehringer Mannheim, Germany). Total protein concentration was measured with the BCA protein dosage kit (Pierce, Rockford, Il).

ABCA1 protein concentration was determined by indirect ELISA on Costar 96-well EIA/RIA plates (Fisher Scientific). First, plates were incubated overnight at 4ºC with either hippocampal protein homogenates from each mice (n=5 animals per time points) or rat brain homogenate (sonicated in PBS 0.01M) as standard (375 to 2812.5µg/ml) diluted in bicarbonate/carbonate buffer (100mM, pH 9.6), applied in triplicate. The next day, the primary antibody (rabbit polyclonal ABCA1 antibody, ab14146; Abcam, Cambridge, USA) diluted in a PBS 0.01M and bovine serum albumin 1% solution was added to each wells for 2 hours at room temperature. Following washes with Tris buffered saline-Tween solution, samples were incubated with the biotinylated detection antibody (anti-rabbit antibody, ab6720; Abcam, Cambridge, USA) for 2 hours. After a second series of washes, an alkaline-phosphatase-conjugated streptavidin solution (Invitrogen Canada Inc., Burlington, ON) was added to all wells for 1 hour at room temperature to detect the biotinylated antibody. Plates were finally washed and incubated with an alkaline phosphatase fluorescent substrate (AttoPhos, Promega, San Luis Obispo, USA) for 30 min at 37ºC. Fluorescence was measured with the microplate fluorescent reader (FL600, Bio-Tek Instruments) at a 450nm/20nm exposure and 560nm/20nm emission. Relative protein levels of ABCA1 are expressed as ratios between ipsi and contralateral side to the ECL location ± SEM.

Human ABCA1 ELISA assay

Quantification of ABCA1 was performed on hippocampal, frontal cortex and cerebellum tissues from 71 AD cases (mean age at death 78.4 ± 9.3 years) and 38 controls (mean age at death 69.9 ± 11.9 years). Brain tissues were sonicated on ice in bicarbonate/carbonate solution (100mM, pH 9.6) and centrifuged at 3000 rpm for 10 min at 4ºC except frontal cortex tissues that were sonicated in lysis buffer (30mM EDTA; 250mM NaCl; 1mM DTT; 50mM K_2HPO_4, pH 7.2) and centrifuged for 20 min. Protein concentration was measured by BCA protein dosage kit (Pierce, Rockford, Il). Indirect

ELISA for ABCA1 on human brain tissues was adapted from the protocol described above for the mouse. Concentrations are expressed as means ± SEM.

Statistical Analyses

One-way ANOVA analyses from the SPSS version 15.0 software were applied to investigate the effects of recovery time on the relative expression levels (expressed as ipsilateral-to-contralateral relative expression ratios) of the ABCA1 and ABCG1 target mRNA sequences following ECL in the frontal cortex and hippocampus, and to analyse ABCA1 protein expression in the ECL mouse and human hippocampi. The ipsilateral:contralateral AChE staining density ratios were also analysed using a one-way ANOVA to investigate the effect of time on AChE activity as a marker of cholinergic synaptogenesis. Tukey's HSD tests were applied for all post-hoc comparisons. Results were considered significant when $p \leq 0.05$.

Results

Time-course of cholinergic sprouting

To investigate the potential roles of ABCA1 and ABCG1 in compensatory synaptogenesis, we used the well-described unilateral ECL model. This model is characterized by a marked deafferentation of the OML of the DG followed by intensive terminal sprouting and synaptic replacement. In response to entorhinal cortex injury, nearly 60% of the synaptic inputs to the granule cell layer of the DG degenerate. This synaptic loss is transient. Within few weeks, more than 80% of enthorhinal synapses are replaced by fiber connections that originate from cholinergic septal neurons, glutamatergic commissural-associational cells of the CA3/hilar areas and to a lesser extent (~5%), from the contralateral entorhinal cortex (Matthews et al., 1976; Phinney et al., 2004; Poirier et al., 1993a; Steward et al., 1988).

Figure 22A illustrates the pattern of AChE-staining observed in the hippocampus of animals following ECL. Analysis of these results yielded a significant increase in ipsilateral AChE-staining density over time, with maximal signal intensity at 14 DPL.

AChE-activity and staining, which correlates with the number of newly formed cholinergic synapses, remains elevated at 21 DPL (Fig. 22B). This time course is consistent with the reinnervation process reported previously in the C57BL/6J mice, which is significantly delayed when compared to rats (Champagne et al., 2005; Poirier et al., 1993a).

Assessment of mRNA expression following ECL

To assess ABCA1 and ABCG1 mRNA prevalence alterations, quantitative RT-PCR analyses were performed on the hippocampus and frontal cortex of ECL mice at specific time points. These time points represent i) the degenerative phase (0-6 DPL) and ii), the reactive sprouting and synaptic remodelling phases (6-30 DPL) of the process. The reinnervation phase is completed at roughly 30-40 DPL. mRNA prevalence levels are expressed as ratios between ipsilateral and contralateral side to the lesion. Figure 23A shows a significant three-fold increase in ABCA1 mRNA expression in the hippocampus of mice at 14 DPL, with transcript levels returning to baseline by 21 DPL. ABCA1 upregulation is specific to the hippocampus as no differences in mRNA levels were detected in the frontal cortex of lesioned mice which serves as positive control in this study (Fig. 23B).

In contrast, ABCG1 mRNA prevalence remains unchanged in the deafferented hippocampus. Figure 24 illustrates the mRNA expression pattern of ABCG1 in the hippocampus (A) and frontal cortex (B) of SHAM-operated and ECL animals throughout recovery.

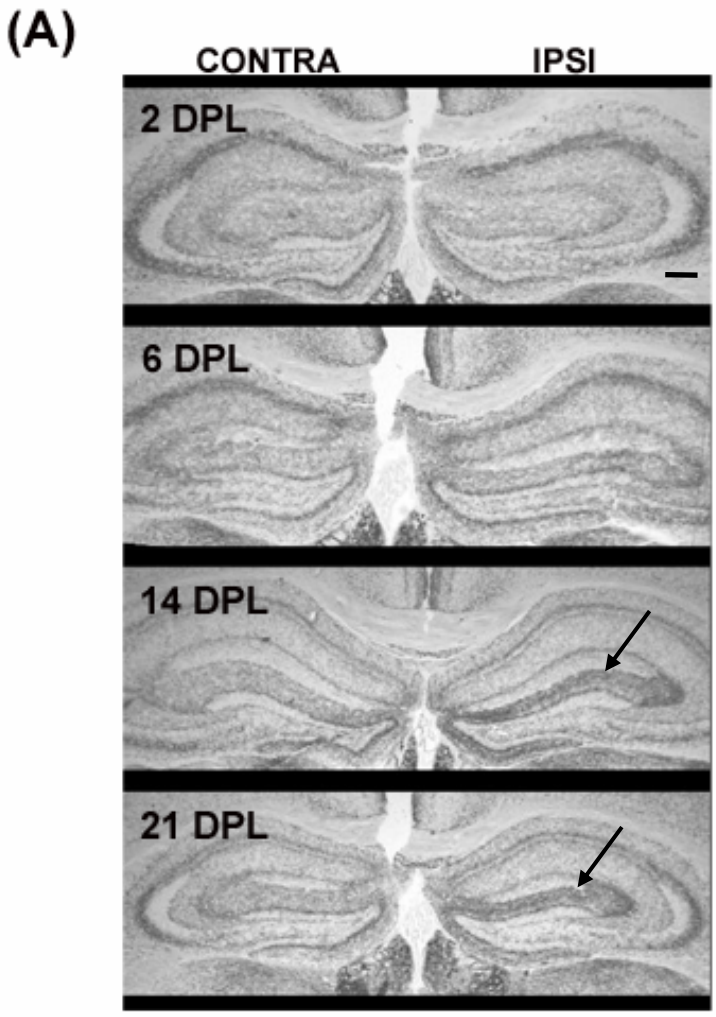
Hippocampal ABCA1 protein levels

Analyses of ABCA1 protein levels in the deafferented hippocampus reveal a significant upregulation at 14 DPL ipsilateral to the lesion (Fig. 25A). ABCA1 protein levels are not significantly altered in the contralateral hippocampus (Fig. 25B). Normalisation of the data relative to the contralateral side reveals a marked ~2.8 fold increase at 14 DPL in the hippocampus of lesioned mice when compared to SHAM-operated animals (Fig. 25C). Selectivity of the changes was confirmed by western

blotting analyses where a similar pattern of expression was observed for the 254 kD band (data not shown). As for the ABCA1 mRNA prevalence in this region, the protein levels of this transporter were back to control levels by 40 DPL (Fig. 25C).

ABCA1 protein expression in Alzheimer's disease

Since the hippocampus in AD is characterized by a marked loss of enthorinal cortex projections over the course of the disease, ABCA1 protein content was determined in the hippocampus and in frontal cortex as well as in the pathology free cerebellum of 71 end-stage, autopsy-confirmed AD cases (mean age at death 78.4 ± 9.3 years) and 38 age-matched controls (mean age at death 69.9 ± 11.9 years). In contrast to the deafferented rodent hippocampus, ABCA1 protein concentrations in AD are unchanged relative to age-matched control subjects in the three brain regions tested (Fig. 26). Neither gender nor APOE genotype were found to significantly affect ABCA1 protein levels in AD patients (data not shown). There were no significant correlations either between ABCA1 protein levels, apoE or amyloid beta (1-40 and 1-42) concentrations in human hippocampi in AD (data not shown).


(A)
CONTRA
IPSI
2 DPL
6 DPL
14 DPL
21 DPL

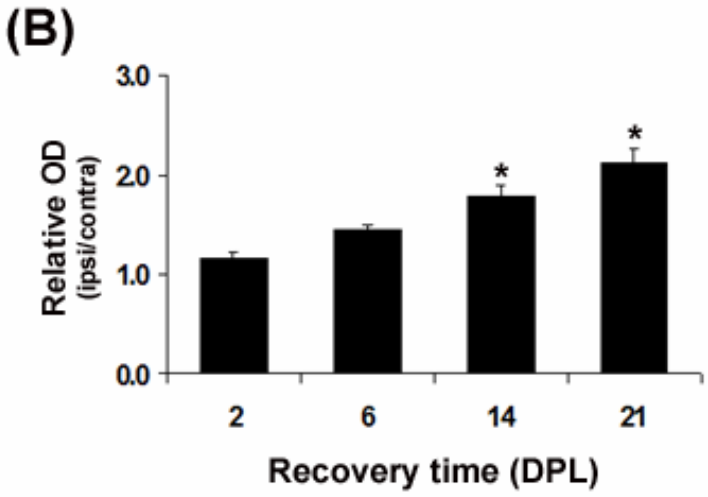

(B)
Relative OD
(ipsi/contra)
3.0
2.0
1.0
0.0
*
*
2
6
14
21
Recovery time (DPL)

Figure 22: Pattern of acetylcholinesterase (AChE) histochemical staining of hippocampal cholinergic terminals in response to entorhinal cortex lesioning.

(A) Representative photomicrographs of the AChE staining density in the dorsal region of the hippocampal formation, ispilateral (right) and contralateral (left) to lesion site at 2, 6, 14 and 21 DPL. As indicated by the arrows, a significant increase in AChE staining in the outer portion of the molecular layer of the DG is observed at 14 and 21 DPL; coinciding with the replacement of entorhinal cortex projections by septal-hippocampal ones. The black line represents a scale bar of 20μm in a 2.5X magnitude photomicrographs. (B) Quantification of AChE staining density, corrected for laminar shrinkage is presented in panel B. Values are expressed as relative OD measures of AChE staining with respect to dentate molecular width. ($p < 0.05$).

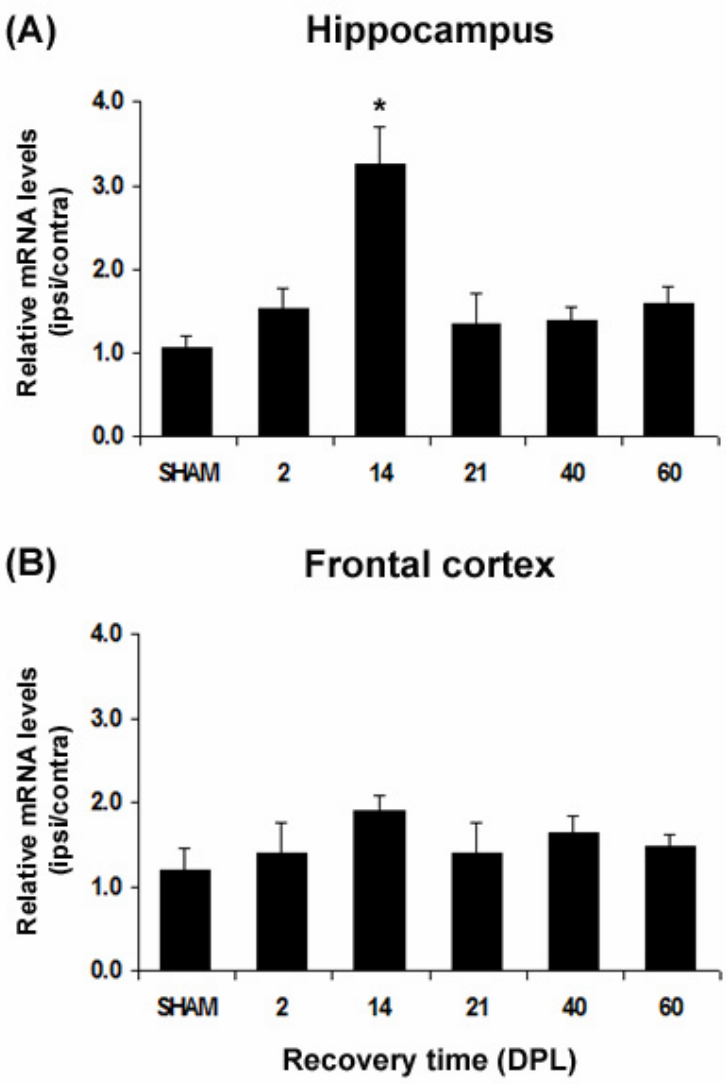


Figure 23: Quantification of ABCA1 mRNA expression in the hippocampus and frontal cortex of SHAM-operated and lesioned-mice following ECL.

(A) A significant increase in ABCA1 mRNA levels was observed in the hippocampus at 14 DPL when compared to SHAM-operated mice (*p <0.001). (B) In the frontal cortex, ABCA1 mRNA expression was not observed to significantly deviate at any of the time points studied following ECL when compared to SHAM-operated expression levels. The ABCA1 mRNA levels are expressed as ipsilateral:contralateral ratios relative to β-actin (±SEM). (n=5 mice per group).

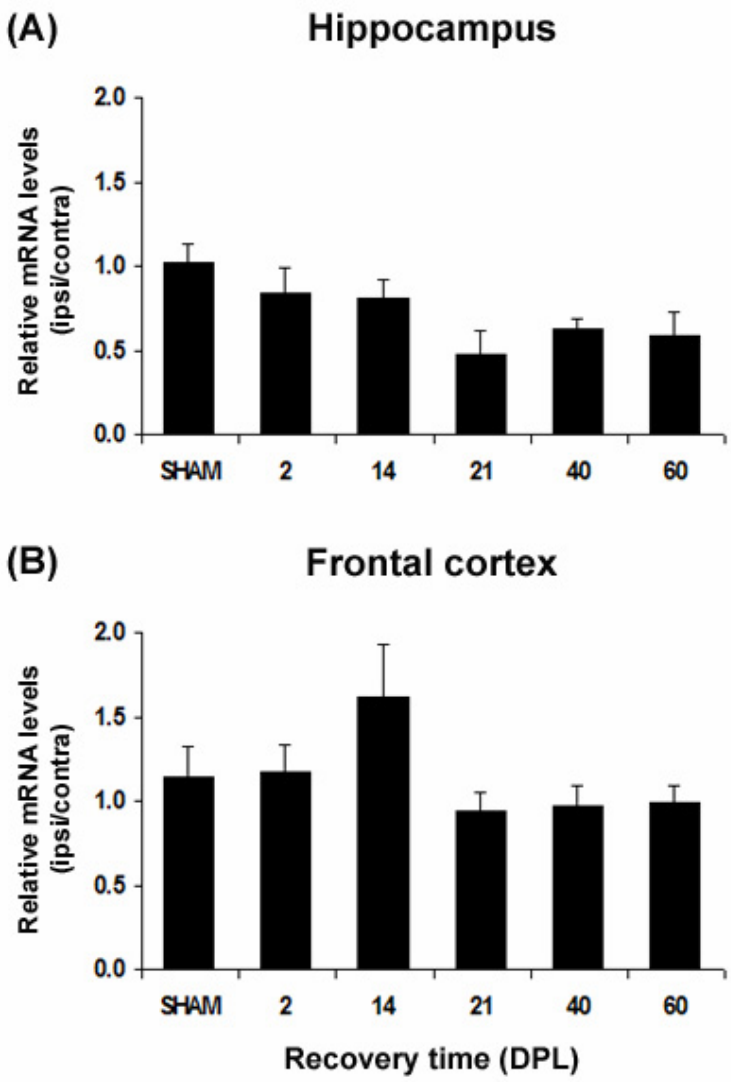


Figure 24: Quantification of ABCG1 mRNA expression in the hippocampus and frontal cortex of SHAM-operated and lesioned-mice following ECL.

ABCG1 mRNA levels are expressed as ipsilateral:contralateral ratios relative to β-actin (±SEM). The relative expression of ABCG1 mRNA in the hippocampus (A) and frontal cortex (B) of lesioned-mice were not significantly different from expression levels observed in SHAM-operated mice. (n=5 mice per group).

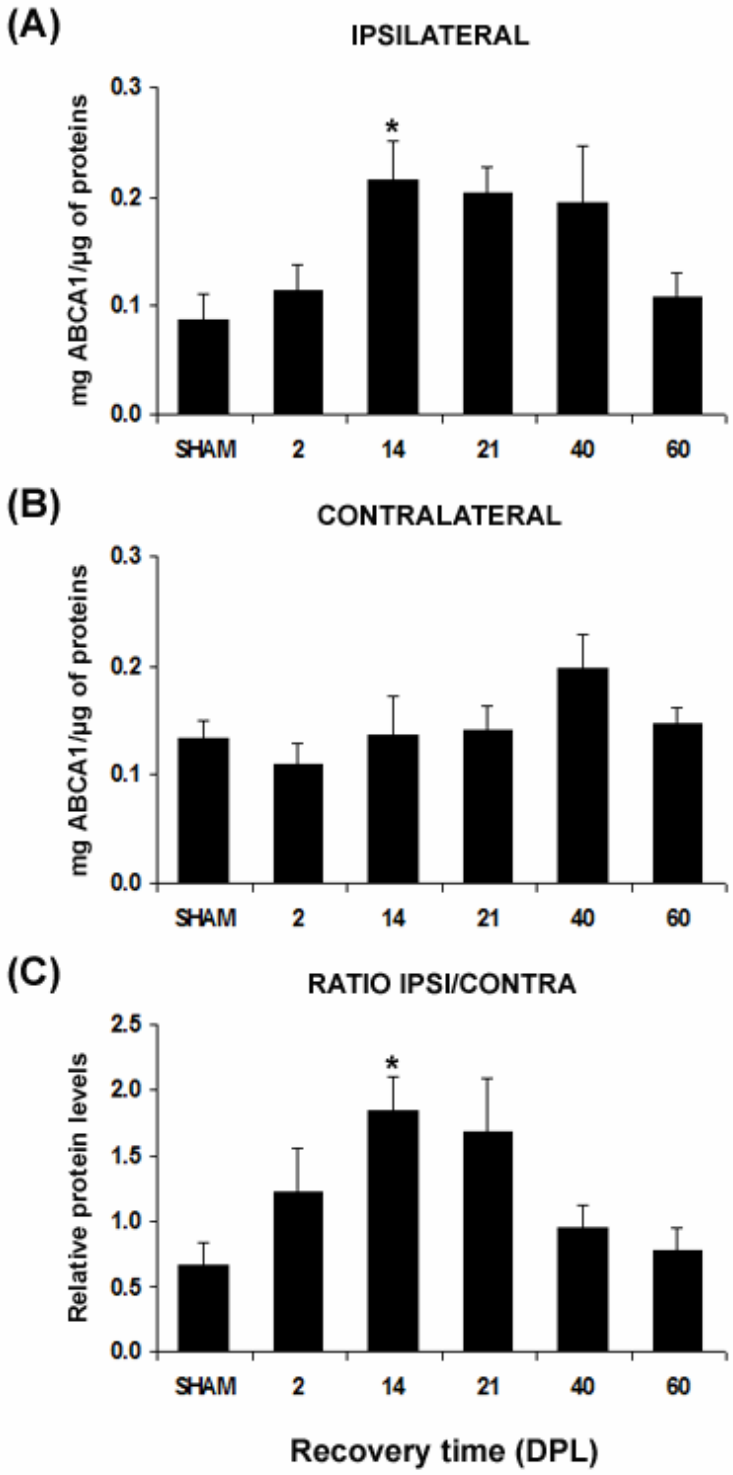

(A)
IPSILATERAL
mg ABCA1/µg of proteins
0.3
0.2
0.1
0.0
*
SHAM
2
14
21
40
60
(B)
CONTRALATERAL
mg ABCA1/µg of proteins
0.3
0.2
0.1
0.0
SHAM
2
14
21
40
60
(C)
RATIO IPSI/CONTRA
Relative protein levels
2.5
2.0
1.5
1.0
0.5
0.0
*
SHAM
2
14
21
40
60
Recovery time (DPL)

Figure 25: Time course analysis of ABCA1 protein levels in the hippocampus of SHAM-operated and ECL mice.

Measures of ABCA1 protein levels, detected by ELISA assays, were performed in the hippocampus ipsilateral and contralateral to the lesion respectively. (A) A significant (approximately 2 fold) increase in the expression of ABCA1 proteins was observed ipsilateral to the ECL location at 14 DPL (*$p<0.05$) while no significant changes were observed in the contralateral side (B). All results are obtained from 5 animals per group and the relative ABCA1 protein levels expressed as ratios between ipsilateral and contralateral side to the lesion are shown in panel (C) ± SEM. A significant increase was observed in the relative ABCA1 protein levels at 14 DPL (*$p=0.05$). Significance was determined by comparing ABCA1 signals from lesioned mice to SHAM-operated animals and analysed by univariate analysis (one-way ANOVA) and Tuckey's tests.

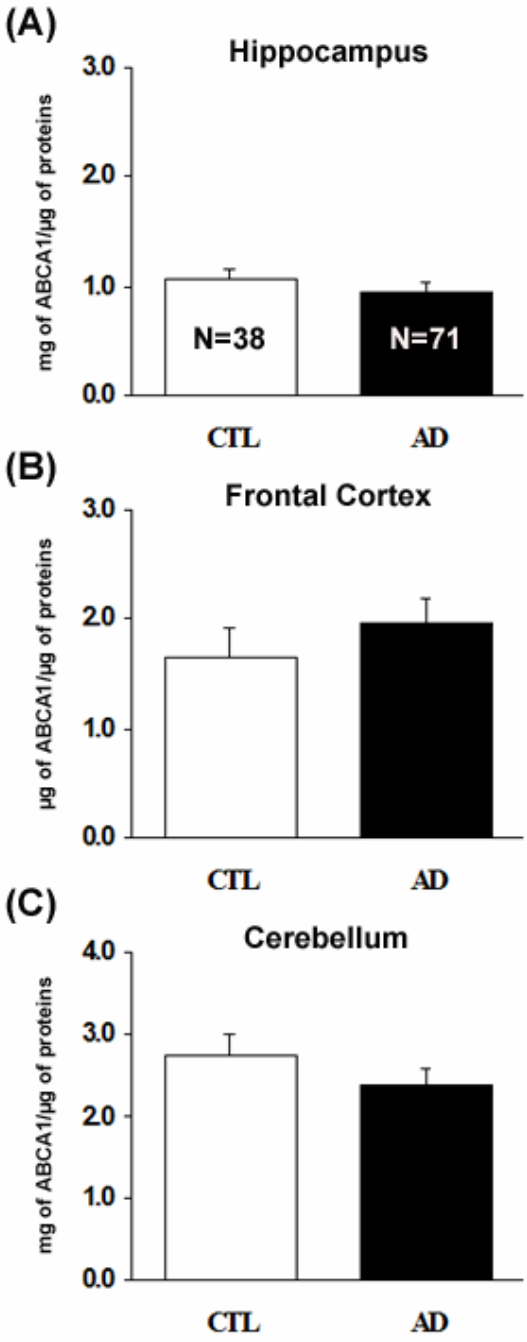


Figure 26: ABCA1 protein levels in the hippocampus (A), frontal cortex (B) and cerebellum (C) of controls (CTL) and AD patients.

ABCA1 protein levels were measured by ELISA and results are expressed as means of ABCA1 proteins/µg of total proteins in brain extracts from 71 AD patients compared to 38 controls ± SEM. No statistical differences in ABCA1 protein levels were observed in the brain of AD cases compared to controls. Significance was assessed by one-way ANOVA analysis.

Discussion

While both neurons and glial cells retain the ability to synthesise cholesterol once reaching maturity, neurons require access to additional sources of cholesterol to support the intensive lipid requirements imposed by plasticity and synaptic turnover (Mauch et al., 2001; Posse de Chaves et al., 2000). Studies conducted in cultured neurons have demonstrated that following inhibition of cholesterol synthesis, axonal growth is supported by the addition of lipoprotein-associated cholesterol (Goodrum et al., 2000; Posse de Chaves et al., 2000). Furthermore, apoE-associated cholesterol derived from glial cells is sufficient to promote axon extension and synaptogenesis (Hayashi et al., 2004; Mauch et al., 2001).

The accumulating evidences suggesting that ABC transporters promote efflux of cellular cholesterol, phospholipids and sphingosine-1 phosphate to apoE-lipoparticles raise the question of whether ABCA1 and/or ABCG1 play active roles in CNS lipid mobilization during compensatory synaptogenesis. We demonstrate here that ABCA1 and ABCG1 are differentially regulated during synaptic repair. ABCA1, but not ABCG1, is significantly (but transiently) upregulated during the early phase of synaptic replacement. The transporter levels were found to return to control values after 3 or 4 weeks. Interestingly, this phenomenon was not observed in the hippocampus of autopsy-confirmed AD cases; suggesting that if ABCA1 has any role to play in AD, it probably occurs in the pre-symptomatic phase of the disease or shortly after onset while the neuronal loss and damage are spreading.

Several studies have demonstrated that after injury, the neuronal binding capacity for apoE-containing lipoparticles, as well as apoE proteins itself, increase sharply during the reinnervation process (Blain et al., 2004; Ignatius et al., 1987a; Poirier, 1994). The concomitant and timely upregulation of ABCA1 and apoE during the early phase of the reinnervation process confirms previous works showing a functional biological interaction between ABCA1 activity and apoE levels/secretion (Hirsch-Reinshagen et al., 2004; Hirsch-Reinshagen and Wellington, 2007; Wahrle et al., 2004; Wahrle et al., 2008). These findings are also consistent with the work of Fukumoto and colleagues who

reported a delayed activation of ABCA1 and apoE mRNA expression in response to excitotoxic lesions of the hippocampus, one to two weeks after the lesion (Fukumoto et al., 2002). Furthermore, the absence of ABCA1 in mice correlated with lower brain apoE levels accompanied with behavioural impairment and synaptic abnormalities or memory deficits with age in an APP23/ABCA1$^{+/-}$ transgenic mouse model (Karasinska et al., 2009; Lefterov et al., 2009); consistent with the disregulated cholesterol trafficking reported in apoE and LDLR deficient mice (Cao et al., 2006; Champagne et al., 2002; Champagne et al., 2005; Krugers et al., 1997; Masliah et al., 1995; Mulder et al., 2004; Mulder et al., 2007; Oitzl et al., 1997).

Recently, genetic variations in the human ABCA1 gene have been associated with a higher risk for common AD in some studies (Katzov et al., 2004; Rodriguez-Rodriguez et al., 2007; Sundar et al., 2007), but not in others (Kolsch et al., 2006; Li et al., 2004; Shibata et al., 2006). Increased susceptibility to AD was also reported in APOE4 carriers born with specific ABCA1 polymorphisms (Rodriguez-Rodriguez et al., 2007). While the possible contribution of specific variants on ABCA1 expression has been invoked in the past, we did not find any significant alteration in ABCA1 expression levels in the three brain regions examined in our AD and control subjects. Either, ABCA1 does not play an active role in the pathophysiology of AD or, by the time patients reach the end-stages of the disease, levels of ABCA1 have returned to baseline values as observed in the lesion mice.

In contrast to ABCA1, ABCG1's function in the regulation of CNS sterol homeostasis remains more controversial. While some studies failed to detect any effect of ABCG1 overexpression or deficiency on brain lipid levels, others report strong correlations between ABCG1 and lipid biosynthesis (Burgess et al., 2008a; Burgess et al., 2008b; Kennedy et al., 2005). Moreover, ABCA1 has been repeatedly reported to act sequentially with ABCG1 in promoting sphingosine-1 phosphate and cholesterol efflux, as well as apoE lipidation (Burgess et al., 2008b; Gelissen et al., 2006; Kennedy et al., 2005; Vaughan and Oram, 2006) whereas Karten et al. (2006) demonstrated that cholesterol loading *in vitro* enhances ABCG1 but not ABCA1 expression and correlates

best with cholesterol efflux in astrocytes (Karten et al., 2006). While it is difficult to reconcile the *in vivo* observations with the *in vitro* reports, the fact that i) ABCG1 overexpression did not influence cognition, learning and memory or hippocampal synaptic plasticity (Parkinson et al., 2009) nor ABCA1 or apoE levels *in vivo* in transgenic mice (Burgess et al., 2008a; Burgess et al., 2008b) tend to suggest a modest contribution, if any, in lipid mobilization and in the maintenance of synaptic integrity or plasticity; also consistent with unaltered ABCG1 expression observed in our lesioned-mice during synaptic remodelling processes.

Finally, it is of interest to note that members of the ABCG family such as ABCG4 which is also expressed in neural tissues (Tachikawa et al., 2005), can dimerise with ABCG1 to form functional heterogeneous transporters. Therefore the examination of the expression of other ABCG members during reactive sprouting and their interactions with ABCG1 may provide insights into the functional role of these transporters during cholesterol recycling and synaptic remodelling.

In conclusion, results of this study suggest that during active remodelling, there is an increase in cholesterol efflux to nascent lipid-poor apoE and the generation of pre-HDL is mediated by the activity of ABCA1, apoE, but not ABCG1 transporters. The absence of alteration in ABCA1 protein levels, as it is the case for apoE (Beffert et al., 1999) , in the brain of advanced-stages AD subjects suggests that the plastic response reported by many in the hippocampal area in AD most probably occurs in the pre-symptomatic or the early phase of the disease.

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Disclosure statement

The authors have no conflicts of interest to declare.

Appendix-2

ApoE and cholesterol homeostasis in the pathophysiology and treatment of common Alzheimer′s disease

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Abstract

Converging evidences from clinical and pathological studies indicate the presence of important relationships between the ongoing deterioration of brain lipid homeostasis, vascular changes and the pathophysiology of sporadic Alzheimer's disease (AD). These associations include a) recognition that the cholesterol transporters apolipoprotein E, C1 and J are acting as major genetic risk factors for common AD in traditional and genome wide association studies and b), observations linking cardiovascular risk factors such as high mid-life plasma cholesterol, diabetes, stroke, obesity, metabolic disease and hypertension to dementia. Moreover, recent clinical findings lend support to the notion that progressive deterioration of brain cholesterol homeostasis in AD is both a central player in the disease pathophysiology and, a potential therapeutic target for disease stabilization and prevention.

Alzheimer's disease and Cholesterol Homeostasis

Alzheimer's disease (AD) is considered today to be a multi-factorial disease with a strong genetic component. It is generally agreed that the disease can be subdivided into two distinct categories: the so-called familial and sporadic forms of the disease. The discovery of genetic linkages and the identification of genes responsible for AD have certainly revolutionized our understanding of this increasingly common disorder. The identification of specific mutations in genes that have been linked to AD has certainly changed how we perceive the nature of the molecular changes responsible for the pathophysiological process that characterizes AD. Three different genes have been identified so far as disease causing genes for the rare early-onset forms of familial AD: the amyloid precursor protein (APP), the presenilin 1 (PS-1) and presenilin 2 (PS-2) (Hardy, 1997). However, coding mutations in these genes do not contribute to the risk of the far more prevalent late-onset disease which accounts for more than 95% of all AD cases worldwide. Apolipoprotein E (apoE) is the only locus known to contribute to the risk of developing the late-onset form of the disease, with the E4 and E2 alleles respectively increasing and decreasing the risk level (Corder et al., 1994; Poirier et al., 1993b). Recently, several independent genome wide association studies (GWASs) have been performed in homogenous and heterogenous population of AD and age-matched control cases in North America, Europe and Asia (Carrasquillo et al., 2009; Harold et al., 2009; Lambert et al., 2009; Li et al., 2008; Reiman et al., 2007). The apoE4 allele was found to be associated with AD in all these independent studies using genome wide statistical criterions. Surprisingly, the rest of the genetic associations uncovered in these different GWASs failed to replicate except for the apolipoprotein J (apoJ) gene, which normally serves as an accessory protein to apoE in the maintenance of cholesterol homeostasis. It is markedly over-expressed in the brain in AD and highly expressed in glial cells (May et al., 1990).

Cardiovascular Contribution to AD Pathology

The prevalence and incidence of degenerative and vascular dementias increase almost exponentially with age, from 70 years onward. In view of the increasing longevity

of humans in the 20th century, both types of dementias have progressively evolved into major public health problems worldwide. The integrity of the cerebral vasculature is crucial for the maintenance of cognitive functions during aging. Prevailing evidence suggests that cerebrovascular functions decline during normal aging, with pronounced effects in AD (Kalaria et al., 2008). The causes of these changes remain largely unknown although recent evidence suggests a significant contribution of the apoE4 allele in blood flow disturbances in AD (Thambisetty et al., 2010). Brain imaging and permeability studies show no clear functional evidence to support the structural and biochemical anomalies, but it is plausible that local and transient breach of the blood-brain barrier occurs during aging and more notably during the early stages of AD (Schneider, 2009). Upon close examination, apparently clinically normal apoE4 carriers aged 50 and older are more likely to show significant cognitive impairment and lower cerebral glucose metabolism by position-emisson tomography (PET) (de Leon et al., 2001). Even relatively young adult carriers of apoE4 (31 $\pm$ 5 years) are showing lower cerebral glucose metabolism, despite a complete absence of clinical impairments. Interestingly, the apoE4 allele dose was shown to drastically impact the age of onset in both familial and sporadic cases (Corder et al., 1993; Poirier et al., 1993b). These intriguing findings raise the possibility that apoE4 carriers, which are genetically "at risk" of developing AD, are developmentally different from their non-carrier counterparts.

The association of decreased cerebral blood flow and hypertension as major risk factors for sporadic AD has also been the target of growing interest from a therapeutic angle (Forette et al., 1998; Hoffman et al., 2009). Epidemiological and neuropathological studies have reported associations between common AD and several vascular risk factors, such as hypertension, myocardial infarctions, diabetes, obesity, hyper- or dyslipidemia, ischemic white matter lesions, generalized atherosclerosis and of course inheritance of the apoE4 allele. These findings may reflect an over-diagnosis of AD in individuals with silent cerebrovascular diseases or alternatively, cerebrovascular diseases may affect the clinical expression and onset of AD. Further possibilities include that AD may increase the risk of vascular diseases or that vascular diseases may silently stimulate AD processes. Similar mechanisms may also be involved in the pathogenesis of both

disorders, such as disturbances in lipid homeostatic processes, abnormal cholesterol transport, distribution or accumulation.

According to several longitudinal studies, hypertension appears to predispose individuals to the development of cognitive impairment and ensuing dementia, after a period varying from a few years to several decades. The presence of the apoE4 allele was shown to drastically enhance the dementia risk level in hypertensive subjects (Peila et al., 2001). Conversely, anti-hypertensive drug treatments such as angiotensin-converting enzyme (ACE) inhibitors and diuretics were shown to markedly reduce the risk of developing cognitive impairment and, in some studies, delay onset of AD itself (Shah et al., 2009). Estrogen, which is known to lower serum cholesterol levels and the risk of coronary heart disease, lessens the incidence of dementia in cross-sectional epidemiological studies (prevention?), but fails to do so with disease progression (Mulnard et al., 2000) in diagnosed mild-to-moderate AD cases.

Cholesterol Synthesis, Transport and Degradation in AD Pathophysiology

Cholesterol levels in the different brain areas in AD have been carefully documented over the past 25 years. It is generally agreed that brain cholesterol levels (total, free and esterified) are significantly reduced in hippocampal and cortical areas in AD, but not in the pathology-free cerebellum (Mulder et al., 1998; Svennerholm and Gottfries, 1994). Cholesterol plays a crucial structural role in the brain and local synthesis covers almost all the requirements. To maintain local homeostasis, the excess of cholesterol is converted into the more hydrophilic oxysterol 24S-hydroxycholesterol by the neuron-specific enzyme CYP46A1, also refer to as cholesterol 24S-hydroxylase. About 99% of the total excretion of this oxysterol by the brain occurs across the blood-brain barrier directly into the bloodstream. Almost all the 24S-hydroxycholesterol present in human circulation has a cerebral origin and its concentrations depend on the number of metabolically active neurons located in the grey matter (Leoni, 2009). Neurodegeneration combined with brain atrophy are associated with significantly reduced concentrations of oxysterols in the circulation. Patients with AD have thus been found to have marked increased concentrations of 24S-hydroxycholesterol in cerebrospinal fluid, in parallel with

decreased concentrations in the circulation (Papassotiropoulos et al., 2002). Genetic association studies examining the link between the CYP46A1 gene and sporadic AD reported conflicting results, with many positive and negative associations. On the other hand, reductions in the phospholipid content in membrane structures have been reported in AD brains. ApoE, which acts as a potent phospholipid carrier in the mature central nervous system (CNS), was shown to affect phospholipids concentrations in an apoE4 dose-dependent manner (Igbavboa et al., 2002; Klunk et al., 1998).

Interestingly, the cholesterol-to-phospholipids ratio in cortical plasmic membranes was shown to be markedly decreased in the temporal and frontal cortices of AD subjects (Mason et al., 1992), consistent with pronounced thinning of the plasma membrane in cortical areas. These pathophysiological findings further suppport clinical and epidemiological evidences suggesting that individuals with elevated mid-life plasma cholesterol have increased susceptibility to dementia and AD when compared to normo-cholesterolemic subjects (Giubilei et al., 1990; Moroney et al., 1999).

Cholesterol homeostasis in the brain is carefully maintained through a series of interdependent processes that include synthesis, storage, degradation and transport. Cholesterol and other lipids are used for membrane synthesis and for many other anabolic or catabolic activities by cells throughout the body including those of the CNS, a site of high lipid turnover. Although cells composing the nervous tissue are capable of *de novo* synthesis of lipid molecules, they can also bind and take-up lipoproteins made available in the local environment to meet their lipid requirements. Brain cells, particularly astrocytes and neurons, cultured *in vitro* synthesize cholesterol at a rate which is inversely proportional to the cholesterol content in the growth environment. Cholesterol requirement of most mammalian cells are met by two separate but inter-related processes. One process is the endogenous synthesis of cholesterol. This synthesis pathway involves over 20 reactions and is regulated primarily by the activity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) that catalyzes the formation of mevalonate, the key precursor molecule in the synthesis of cholesterol (Fig. 27). Analysis of the HMGR activity in the brain of autopsy-confirmed AD cases revealed a

significant reduction of the enzymatic activity in cortical and hippocampus areas when compared to age-matched control subjects (Poirier et al., 2008a). The reduction of HMGR enzymatic activity was found to be apoE genotype independent. HMGR mRNA levels are not different in AD versus control subjects but are inversely correlated with apoE protein levels, suggesting a compensatory up regulation of cholesterol internalization by surviving cortical and hippocampal neurons (Poirier et al., 2008a). The loss of brain HMGR activity is certainly consistent with the loss of cholesterol reported in many brain regions in AD. The other central process regulating cholesterol levels in the brain involves the utilization of lipoprotein-derived cholesterol following internalization of the lipoprotein bound to its surface receptor (usually, an apoE-rich high-density-lipoprotein (HDL)-like lipoprotein complex). ApoE has been shown to coordinate the mobilization and redistribution of cholesterol in repair, growth and maintenance of myelin and neuronal membranes during development or after injury in the peripheral nervous system. In the CNS, apoE in partnership with apoJ and apoC1 plays a pivotal role in the cholesterol delivery during membrane remodelling associated with synaptic turnover and dentritic reorganization (May et al., 1990; Poirier et al., 1991a). The near complete absence of other key plasma apolipoproteins such as apoA1 and apoB in the brain further emphasizes the critical and unique role of apoE for cholesterol transport in the normal or injured CNS. We cannot rule out the possibility that during blood-brain barrier leakage in areas of severe neurodegeneration, apolipoproteins originating from the periphery could play an active role in the local pathological processes.

Ultrastructural studies have shown that loss of neuronal input to the hippocampus prompts astrocytes and microglia to progressively engulf both pre-synaptic terminals and pre-terminal axons (Fig. 28, #1) to rapidly clear the area to allow synaptic replacement. Once metabolized, these terminal-derived ovoids generate a large glial store of lipids readily available for the synthesis of membrane components necessary for new synapses and dendrites (Fig. 28, #1, 2) in surviving adjacent neurons. The local accumulation of high concentration of cellular cholesterol in astrocytes induces apoE and apoJ synthesis and secretion in combination with cholesterol (Fig. 28, #2). As the intracellular concentration of cholesterol raises in glial cells, cholesterol synthesis is progressively

repressed at the level of HMGR (Poirier et al., 1993a). At this stage, the so-called ATP-binding cassette transporter type A1 and G1 (ABCA1/G1) come into play and coordinate the mobilization of cholesterol from the cytoplasmic pools to the cell surface membrane (Hirsch-Reinshagen et al., 2005), where it is eventually combined with apoE, apoJ and phospholipids to produce a functional HDL-like particle through the action of the lipoprotein lipase (LPL) (Blain et al., 2004). This newly synthesized lipoprotein particles is then released into the extracellular space where it migrates, in a gradient-driven fashion, toward both apoE/J receptors located on ependymal cells surrounding the ventricules or apoE receptors located on neuronal and glial cells.

Deafferented granular cell neurons in the hippocampus were shown to exhibit marked increases of low-density-lipoprotein receptors (LDLR) in the early and middle phases of the reinnervation process (Fig. 28, #3) (Poirier et al., 1993a). Both the apoE/LDL receptor related protein (LRP) and apoE receptor type 2 expressed by neurons were also shown to indirectly modulate terminal proliferation and axonal extension (Nathan et al., 1994), primarily through a signalling pathway (Beffert et al., 2003) rather than lipoproteins internalization. Following binding of the apoE-lipoprotein particle to neuronal surface LDLR, the apoE-lipoprotein/LDL receptor complex is internalized and degraded, releasing cholesterol that will be use for synapse formation and terminal proliferation (Fig. 28, #4). In response to the increase internalization of cholesterol via the apoE/LDL receptor pathway, neurons of the injured hippocampal formation reduce their HMGR activity (Fig. 28, #7), the rate limiting step in the intracellular synthesis of cholesterol. The apparent contradiction of depressed cholesterol synthesis and increased apoE expression in the presence of active synaptogenesis can thus be reconciled by postulating a specific salvage and re-utilization of cholesterol from degenerating terminals through the apoE transport/LDL receptor-uptake pathway (Poirier et al., 1993a; Posse de Chaves et al., 2000). The absence of either apoE or LDLR in deficient (knockout) mice was shown to compromise synaptic integrity and compensatory synaptogenesis, and cause age-dependent cognitive deficits, presumably due to an impaired ability of the brain to produce or bind to functional HDL-like particles (Champagne et al., 2002; Mulder et al., 2004).

Cholesterol Homeostasis and APP Metabolism: A Role for apoE-mediated beta-amyloid (Aβ) Transport and Degradation

Since Sparks' initial observation that feeding rabbits a cholesterol-enriched diet for eight weeks leads to an accumulation of intracellular Aβ immunoreactivity in hippocampal neurons (Sparks et al., 1994), studies linking cholesterol and Aβ metabolism have overrun the field of AD research. The finding that cholesterol enrichment enhances Aβ production has been widely replicated in animals (Ghribi et al., 2006; Refolo et al., 2000) and cultured cell models strongly suggesting that lipids, especially cholesterol, modulate Aβ production. Accordingly, lowering cholesterol levels apparently decreases Aβ production. Reduction in the amyloid load was observed in cultured cells and/or mammals following exposure with cholesterol-lowering agents such as probucol (Champagne et al., 2003; Poirier, 2003), statins (Fassbender et al., 2001), BM15.766 (Refolo et al., 2001) and cyclodextrin (Bodovitz and Klein, 1996; Fassbender et al., 2001). On one hand, the mechanisms by which probucol and statins reduce Aβ load could be pleitropic. Probucol was shown to affect cholesterol metabolism through multiple mechanisms of action, but most likely through the induction of apoE synthesis (Champagne et al., 2003). On the other hand, BM15.766 inhibits the enzyme catalyzing the final step of cholesterol biosynthesis, namely 7-dehydrocholesterol reductase, suggesting that the amyloid-reducing effect may involve sterol depletion (Refolo et al., 2001). Accordingly, the physical removal of cholesterol from the membranes by cyclodextrin is sufficient to inhibit Aβ production, pinpointing lipid rafts as potential crucial element for its production. In agreement with this concept, lipid rafts – membrane micro-domains rich in lipids and cholesterol – have been shown to modulate APP processing. Both γ-secretase and β-secretase BACE-1 activities have been isolated from lipids rafts (Riddell et al., 2001), whereas the majority of α-secretase ADAM10 activity occurs in non-raft regions of the membrane. Accordingly, increased intracellular cholesterol level, which favors lipid rafts formation, negatively regulates α-secretase activity (Xiu et al., 2006), but stimulates both β- and γ-secretase activities, resulting in an increase in Aβ production.

This raft/non-raft model of APP processing for explaining cholesterol-dependent regulation of Aβ production may have put one new piece in the puzzle that is AD, but the piece does not fit perfectly. This is emphasized by the findings of a paradoxical acceleration of β cleavage and Aβ production by a modest reduction in cellular cholesterol (bad-Rodriguez et al., 2004). In line with these findings, evidence suggests that it may not be total cholesterol that regulates these secretase activities, but rather the ratio of free cholesterol (FC) to cholesterol esters (CE), regulated by acyl coenzyme A:cholesterol acyltransferase (ACAT) (Puglielli et al., 2001). Moreover, ACAT activity was correlated with changes in α-, β- and γ-secretase-cleaved APP products, indicating a direct effect on APP processing. Whether ACAT activity modulates Aβ generation by directly modulating the activity of the secretases or the availability of APP for cleavage through changes in cholesterol distribution and compartmentalization remains to be clarified. Nevertheless, because these effects were only observed when cells contained physiological or supra-physiological levels of FC, the involvement of FC in Aβ modulation cannot be ruled out (Puglielli et al., 2001). The pharmacological inhibition of ACAT, which causes a concomitant increase in FC levels in the cytoplasm and a reduction in both CE levels and Aβ production (Pani et al., 2009), bears a similarity to the findings that apoE, which promotes cholesterol transport and internalization via the LDLR family in an apoE2 > apoE3 >> apoE4 fashion, strongly modulates Aβ burden (Bales et al., 2009; Beffert et al., 1999; Riddell et al., 2008).

Numerous studies demonstrate a positive correlation between amyloid burden and apoE4 allele dose, the least efficient cholesterol transporter and the greater genetic risk factor ever identified for developing AD. Indeed, increased plaque density in humans (Rebeck et al., 1993) and human apoE-transgenic mice (Bales et al., 2009; Holtzman et al., 1999; Riddell et al., 2008) have been associated with the presence of the apoE4 allele. The exact mechanism by which apoE4 may promote Aβ production and accumulation remains elusive although the isoform-dependent modulation of the local steady state levels of apoE is the primary suspect (Bales et al., 2009; Poirier, 2008). Furthermore, numerous *in vitro* studies have demonstrated that human apoE facilitates cellular Aβ uptake and degradation (Beffert et al., 1999; Yang et al., 1999). Additionally, apoE

promotes brain to blood removal of Aβ peptides by transport across the blood-brain barrier, a clearing process that follows an apoE2 > apoE3 > apoE4 gradient (Deane et al., 2008). While the predominant role of apoE in the scavenging of Aβ in the CNS is generally accepted and well documented, the presumed effect of apoE on Aβ aggregation is much more controversial. *In vitro* studies suggest that apoE isoforms, with apoE4 being the least effective, might decreased Aβ fibrillogenesis by interfering with Aβ nucleation (Evans et al., 1995). These observations contrast with animal studies, where the knock-down of the murine apoE gene resulted in a drastic decrease in Aβ deposition, along with a marked loss of thioflavin-S-positive amyloid plaques. However, human apoE-transgenic mice show a marked delay in Aβ deposition and plaques formation relative to mice expressing their murine apoE or no apoE (Holtzman et al., 2000). This suggests that human apoEs exhibit physiological properties which are different from murine apoE in regard to amyloid metabolism, removal and/or deposition in the mature brain. Furthermore, recent work indicates that many of the proteins involved in cholesterol homeostasis actually contribute to the modulation of Aβ metabolism or catabolism.

Cholesterol Metabolism: A Potential Therapeutic Target for the Treatment of Sporadic AD

As reviewed in the previous section, under normal circumstances, cholesterol synthesis via the HMGR pathway (Fig. 27 and 28) is required only when lipoprotein internalization by apoE/LDL receptor pathway is insufficient to meet the cholesterol requirement of the cell. To maintain cellular cholesterol homeostasis, there exists a rather potent negative feedback system on the HMGR activity (Fig. 27) and gene expression which results in a decrease of cholesterol synthesis in response to excess intracellular sterol internalization via members of the LDL receptor family. This first and most important feedback regulation of the HMGR activity is through decrease in gene transcription or modulation of the translation efficiency of mRNA for HMGR, resulting in decreased or increased reductase protein and activity.

Interestingly, a hereditary cholesterol storage disorder known as Niemann Pick C (NPC) shows Alzheimer-like tau pathology in youth or adolescence without the amyloid

plaque deposition. NPC is due to a mutation in either of two genes, NPC1 or NPC2/HE1, and associated with an intracellular misrouting and accumulation of FC (Morris et al., 1999). Tangle-bearing neurons in NPC subjects display high levels of cholesterol that resemble the tangle-bearing neurons in AD (Distl et al., 2001; Ohm et al., 2003).

Administration of HMGR inhibitors (statins) in NPC mice, which normally carry high concentrations of phospho-tau in the brain, causes a marked reduction of brain phospho-tau to near normal levels (Ohm et al., 2003). Figure 27 illustrates the geranylgeranylphosphate cascade believed to be responsible, at least in part, for the coordinated phosphorylation of tau in neurons (Chauhan, 2006; Ohm et al., 2003) (dotted box). Evidence obtained from multiple prospective epidemiological studies suggesting that the pre-symptomatic use of statins in middle-age subjects confers some levels of protection against sporadic AD later in life (Haag et al., 2009; Jick et al., 2000; Wolozin et al., 2007), provides us with a potentially interesting biochemical target for prevention studies. Furthermore, a recent population-based study examining the effect of statin administration in cognitively intact elderly revealed that over time, statin users accumulate significantly less neurofibrillary tangles (but not amyloid plaques) when compared to age-matched statin-free subjects (Li et al., 2007a). These evidences from the signalling and pharmacological fields point toward HMGR as a potential key player in the pathophysiology of tau phosphorylation and tangles deposition in the aging and diseased brain.

Statins are known to competitively inhibit the synthesis of cholesterol by preventing the conversion of HMG CoA to mevalonate, thereby inhibiting HMGR activity. This family of inhibitor reduces the formation and internalization of LDL-cholesterol particles into the circulation, and up-regulates LDL receptor activity at the cell surface of liver cells; thereby reducing serum LDL cholesterol and triglycerides and increasing HDL cholesterol concentration. It is unclear at this point in time whether the beneficial effects of statins observed in the brain are mediated through blood-brain penetration or via alterations of plasma cholesterol levels. In an attempt to clarify the issue, two large prospective population studies examined the effects of lipophilic versus

lipophobic statins on AD risk levels. One detected preferential benefits from lipophilic compounds, such as simvastatin, over lipophobic ones (Wolozin et al., 2007), whereas the other did not observed such dichotomy (Haag et al., 2009). Interestingly, however, both reconfirmed the association that exists between statin use and AD risk reduction.

In addition to the statin-mediated LDL receptor upregulation, statins may induce apoE synthesis and secretion, thereby facilitating both lipoprotein delivery and internalization in neurons and Aβ removal from the extracellular space. The potential use of apoE inducer drug in the treatment of AD has been reviewed elsewhere (Poirier, 2008). Briefly, two major approaches have been proposed to facilitate cholesterol mobilization and transport in AD, particularly in E4 allele carriers. One consists in the induction of apoE synthesis and secretion in the brain by probucol (Champagne et al., 2003; Poirier, 2008), a cholesterol-lowering agent used in the mid-80's to treat familial hypercholesterolemia (Enjalbert et al., 1980) whereas the other strategy proposes to administer liver X receptor (LXR) agonists which stimulate ABCA1, apoE and the LDL receptor production through a central common signalling pathway (Liang et al., 2004; Riddell et al., 2007).

These independent yet complementary results indicate that modulation of brain cholesterol homeostasis may interfere with disease onset in subjects exposed to cholesterol-lowering agents prior to a diagnosis of AD. Unfortunately, the most recent large scale randomized double blind placebo-controlled clinical trials with atorvastatin (Feldman et al., 2010) and simvastatin (Arvanitakis and Knopman, 2010) failed to slow down disease progression in mild-to-moderate AD. This suggests, as it was proposed for the anti-inflammatory approach to AD prevention, that statins may provide beneficial effects only in the pre-symptomatic stages of the disease. It is still unclear at this time whether the risk-reducing effect is specific to dementia of the Alzheimer type or, to any forms of dementia, including vascular. Alternatively, it is possible that the beneficial effect of cholesterol-lowering agents stems from a completely distinct pathway involving an independent cardiovascular risk factor which normally modulates disease onset in “at risk” individuals such as the E4 allele carriers. In this scenario, cholesterol-lowering

agents would act indirectly to prevent the effect of vascular risk factors such as circulating levels of cholesterol or atherosclerotic plaque deposition from modulating the age of onset in AD. While it remains difficult at this point in time to determine the exact mode of action by which cholesterol-lowering agents affect the pathophysiology of AD, recent findings on the presence of polymorphic genetic variants in the HMGR gene (Poirier, 2002) provide us with a possible explanation as to what could be the molecular basis and target for the beneficial effect against AD**.**

Is there a unifying hypothesis of Alzheimer's disease pathophysiology?

We have reviewed the apparent role of cardiovascular diseases and cholesterol and their potential role in the development of dementia in Alzheimer's pathology. While age is a key determinant that modulates the expression of AD pathology, genetic risk factors appear to play a central role in this process. Genes such as the apoE4, ACAT, apoJ, LRP, lipoprotein lipase or paraoxonase 1 are all known modulators of cholesterol homeostasis that also behave as genetic risk factors for common AD. Figure 29 illustrates the key elements discussed above and attempts to integrate the contribution of each key biochemical pathway involved in the pathophysiology of AD under one unifying hypothesis. We have used the original amyloid hypothesis of Hardy and Higgins (Hardy and Higgins, 1992) as a canvas and incorporated physiologically relevant contribution of apoE, ACAT and HMGR while highlighting the possible link between cholesterol and APP metabolisms in AD.

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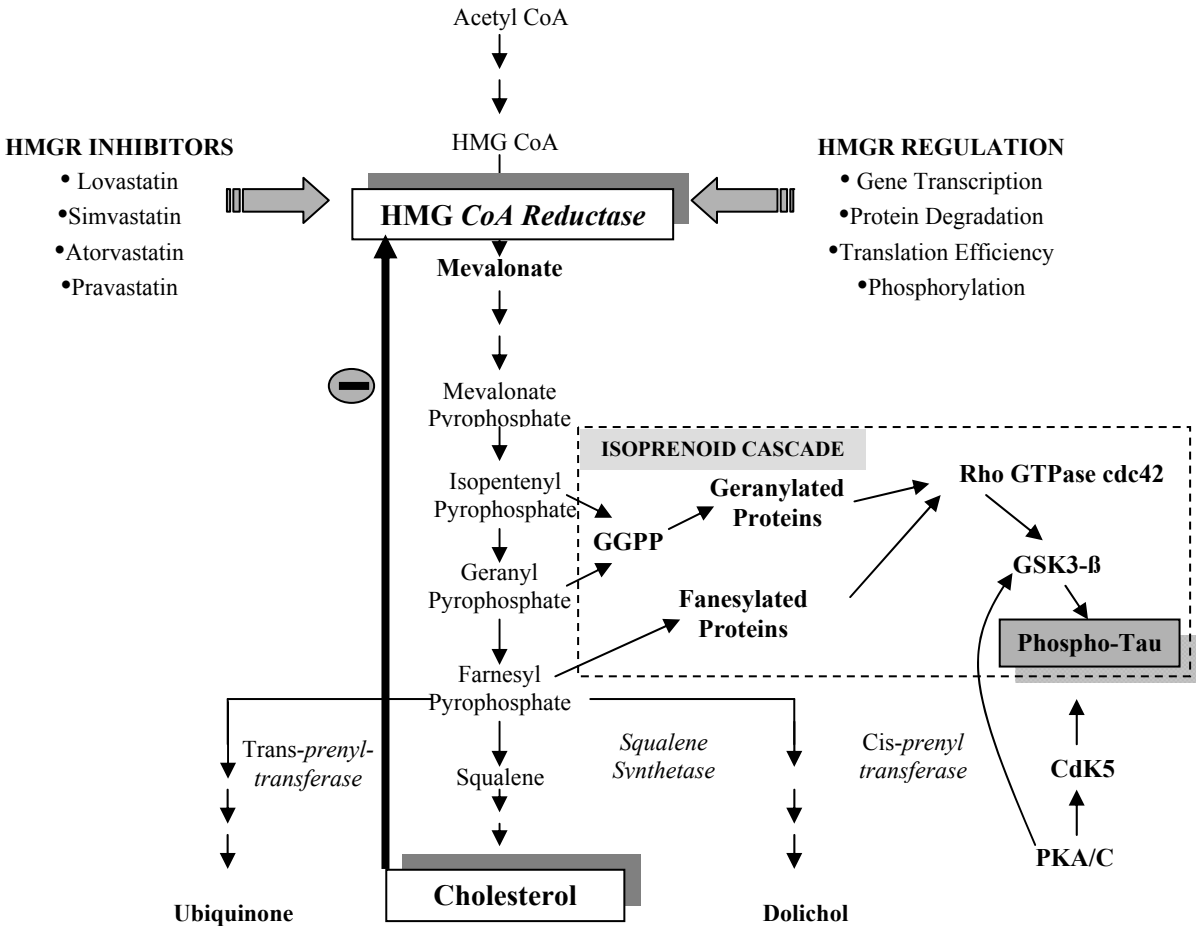

Figure 27: Cholesterol synthesis in eucaryotic cells.

Schematic representation of the mevalonate pathway and key enzymes regulating cholesterol synthesis and esterification. The isoprenoid pathway provides a clear biochemical link by which cholesterol synthesis cascade can indirectly modulate production of phosphorylated tau in the CNS. GGPP: Geranylgeranyl diphosphate, CdK5: Cyclin-dependent kinase 5, PKA: Protein kinase A, GSK3B: Glycogen synthase kinase 3 beta, HMG CoA: 3-hydroxy-3-methyl-glutaryl CoA.

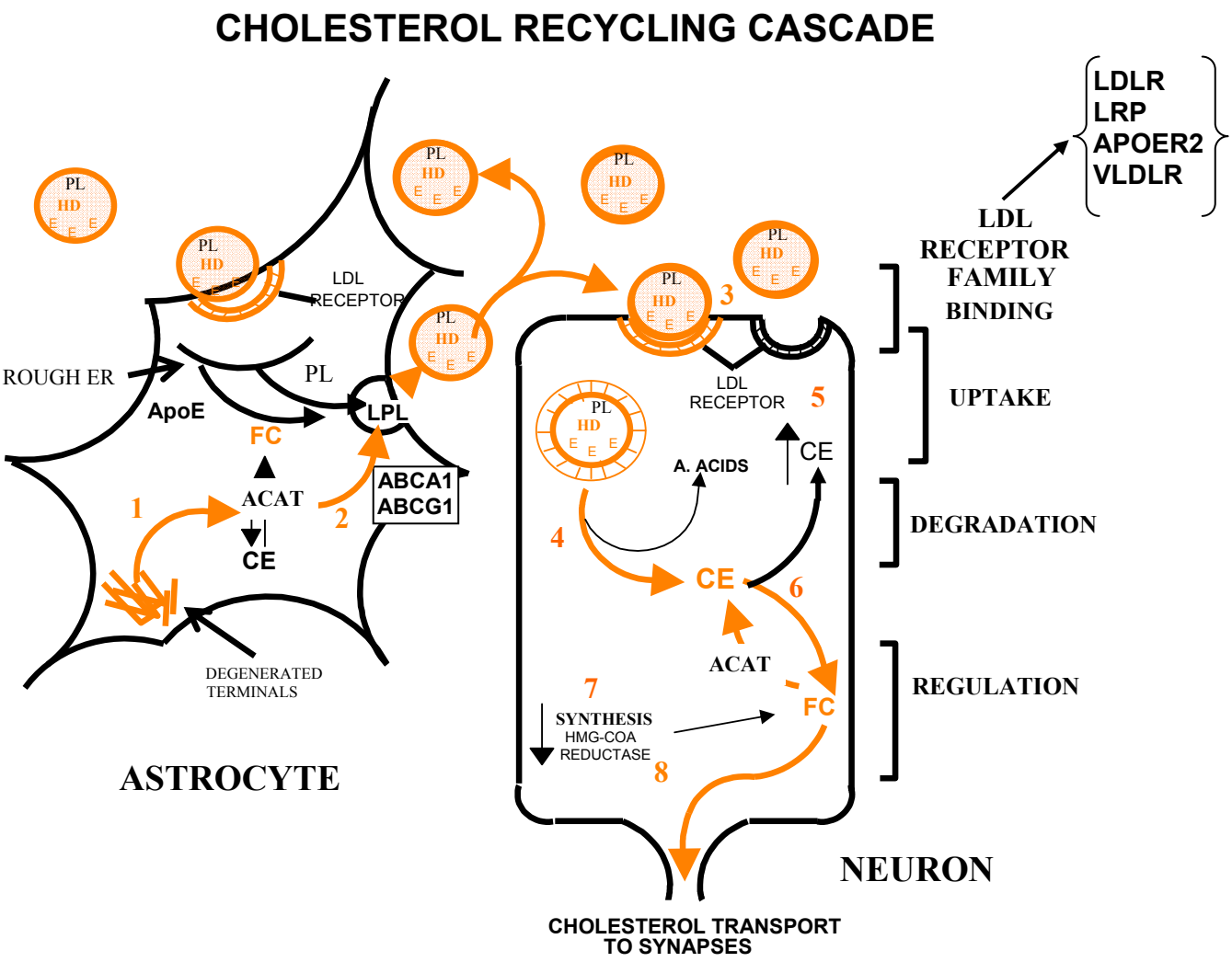

Figure 28: Schematic representation of postulated cholesterol/phospholipid recycling mechanism observed in the injured central nervous system.

Degenerating terminals are initially internalized and degraded. The non-esterified cholesterol (1) is used as free cholesterol (FC) for the assembly of an apoE+J/cholesterol/lipoprotein complex (2) or converted into cholesterol esters (CE) for storage purposes. The newly formed apoE/cholesterol/lipoprotein complexes are then directed a) toward the circulation presumably through the ependymal cells surrounding the ventricles and/or b) to specific brain cells requiring lipids. ApoE complexes are apparently internalized by the neuronal LDL receptor pathway (3) and the cholesterol released (4) for dendritic proliferation and/or synaptogenesis (8). As a consequence of the internalization process, cholesterol synthesis in neurons (via the HMGR Pathway (7)) becomes progressively repressed. E: ApoE; J: apoJ; PL: Phospholipids; CE: Cholesterol Ester; FC: Free Cholesterol. ACAT: acetyl co-enzyme A: cholesterol acyltransferase; ABCA1/G1: ATP binding cassette transporter A1 and G1; LPL: lipoprotein lipase.

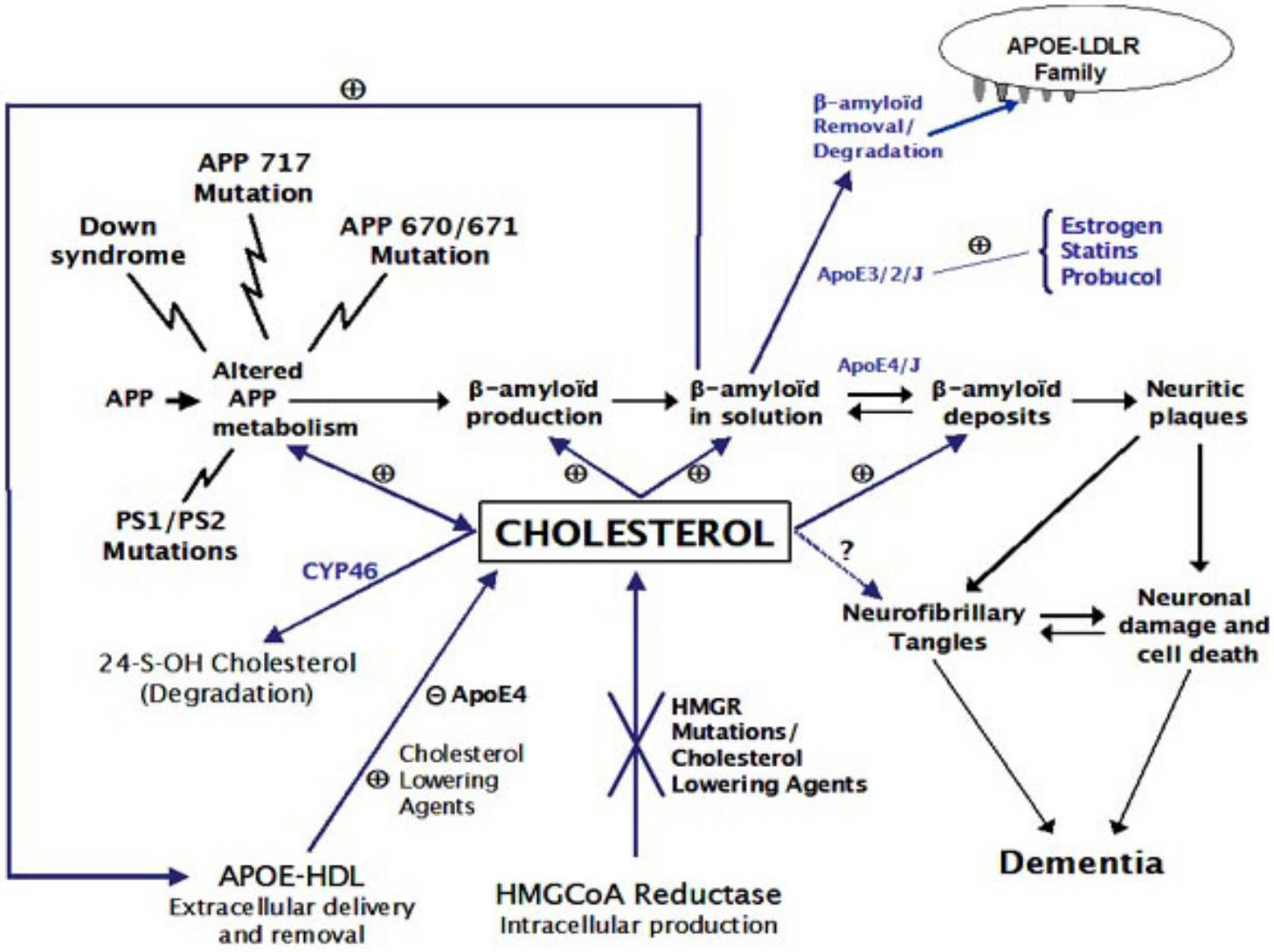

Figure 29: An apoE4/amyloid hypothesis of Alzheimer's disease pathophysiology.

The sequence of pathogenic events leading to neuronal cell loss and synaptic damage is based on the well established amyloid cascade hypothesis which proposed that accumulation of beta amyloid in the brain is the primary influence driving AD pathology. The different modulators of beta amyloid metabolism which were shown to affect lipid homeostasis such as apoE, ACAT, HMGR and apoJ have been added to the cascade. Finally, the emerging roles of cholesterol and the HMG CoA reductase (the rate limiting step in cholesterol synthesis) have been put in perspective in relation to their respective contribution to the pathophysiology of AD. +: Enhancer, -: Inhibitor.