INVESTIGATING A ROLE FOR THE ATP-BINDING CASSETTE TRANSPORTERS A1 AND G1 DURING SYNAPTIC REMODELING IN THE ADULT MOUSE

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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 by unilateral entorhinal cortex lesioning (uECL) induces well-described cytoarchitectural reorganisation and reactive sprouting in the dentate gyrus (DG). Previous studies have demonstrated a dynamic regulation of cholesterol homeostasis in the hippocampus following deafferentation, and suggest that mechanisms facilitating cholesterol transport are important during reinnervation. Furthermore, there is growing evidence that statins, a family of cholesterol-lowering drugs which inhibit the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA-R), may confer neuroprotection following trauma.

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 lipoprotein. To examine a role for these transporters in the regulation of cholesterol efflux during synaptic remodelling, and the effects of low-dose pravastatin (a potent HMGCoA-R inhibitor) on such intercellular transport mechanisms, we measured the expression of ABCA1, ABCG1, APOE, apoE(LDL)R and HMGCoA-R in the hippocampus of saline and pravastatin treated mice over time following uECL. It is shown here that ABCA1 and not ABCG1 is up-regulated at the level of mRNA and protein expression, along with APOE, in the hippocampus during active regeneration (14DPL) as determined by histochemical analysis of acetylcholinesterase staining density in the DG. While pravastatin treatment was observed

to differentially influence the expression of ABCA1 mRNA and protein over time, no effects on APOE or ABCG1 mRNA expression were observed following uECL. Additionally, HMGCoA-R mRNA expression was significantly down-regulated at 21 DPL in the deafferented hippocampus in pravastatin-treated animals. While the low-dose pravastatin treatment applied here was sufficient to inhibit HMGCoA-R activity in the liver, enzymatic activity was unaffected in the cortex.

These findings suggest that ABCA1 and not ABCG1 may be important in the APOE-mediated cholesterol recycling observed during the active phase of neural reinnervation in response to uECL. In addition, the results presented here suggest that the administration of clinically-relevant statin therapy may be sufficient to influence the regulation of cerebral cholesterol homeostasis following trauma in the adult mouse brain.

RÉSUMÉ

Dans le système nerveux central, les lipoprotéines libérées par les cellules gliales facilitent le transport intercellulaire du cholestérol et des lipides en plus de contribuer au développement neuronal et à la synaptogénèse. Une déafférentation partielle de l'hippocampe suite à une lésion unilatérale du cortex entorhinal (uECL) entraîne une réorganisation cytoarchitecturale bien définie, ainsi qu'une reconstruction synaptique active du gyrus denté (DG). Des études précédentes ont démontré qu'en réponse à la déafférentation, l'homéostasie du cholestérol dans l'hippocampe est étroitement régulée par l'activation de mécanismes facilitant le transport et la recapture du cholestérol durant le processus de réinnervation. De plus, un nombre croissant d'évidences supporte la possibilité que les statines, composés pharmaceutiques réduisant le taux de cholestérol sanguin en inhibant l'enzyme responsable de sa biosynthèse, la 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA-R), confèreraient une certaine forme de neuroprotection en réponse à des dommages cérébraux.

Les transporteurs membranaires ABCA1 et G1 (ATP-Binding Cassette Transporteur) contribuent à la formation de lipoprotéines en contrôlant l'efflux de cholestérol et de phospholipides disponibles pour l'apolipoprotéine E (apoE) située à l'extérieur des cellules. D'ailleurs, la principale apolipoprotéine produite par le cerveau est l'apoE. Or, le but de cette recherche consiste à étudier le rôle de ces transporteurs dans l'efflux de cholestérol cellulaire et sa modulation par l'administration de concentrations pharmacologiques de pravastatine (une statine liposoluble). Pour ce faire, nous avons mesuré l'expression génique et protéinique des ABCA1, ABCG1, APOE et HMGCoA-R dans l'hippocampe de souris traitées à la saline ou à la pravastatine suivant une lésion

unilatéral du cortex entorhinal. Cette étude nous a permis de démontrer que les niveaux d'expression d'ARN messagers (ARNm) et de protéines d'ABCA1 et APOE mais non ABCG1 sont augmentés dans l'hippocampe durant la phase active de ré- innervation (14 jours post-lésion). Par contre, l'administration de pravastatine a permis d'inhiber l'expression d'ARNm et de protéines d'ABCA1 sans toutefois affecter l'expression d'ARNm d'APOE et ABCG1. De plus, les niveaux hippocampiques d'ARNm de l'HMGCoA-R de souris traitées à la pravastatine sont réduits de façon significative 21 jours après la lésion entorhinal.

L'ensemble des résultats obtenus suggère que l'ABCA1 plutôt que l'ABCG1 serait impliqué directement dans le transport du cholestérol menant à la formation d'une lipoprotéine fonctionnelle contenant l'apoE et ce, durant la phase active de réinnervation neuronale. Cette étude suggère également qu'une thérapie basée sur l'administration d'une dose cliniquement pertinente de statine pourrait faciliter le transport intracellulaire du cholestérol cérébral et possiblement stimuler le processus de réparation neuronal suite à un traumatisme ou une lésion cérébrale.

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INTRODUCTION

Afflicting more than thirty percent of individuals aged 85 and above, Alzheimer's disease (AD) represents the most common form of senile dementia worldwide. The most prevalent form of AD, presenting with a sporadic, late onset after 60 years of age (LOAD), has been described as a convergence syndrome of diverse aetiology (Poirier, 2005). Although the results of epidemiological studies have identified a number of potential risk factors—both environmental and genetic—there is accumulating evidence to suggest perturbations in cholesterol metabolism may contribute, at least mechanistically, in the onset and progression of the LOAD pathology (Poirier, 2003; Blain & Poirier, 2004, Wellington, 2006).

Indeed, it has been noted that systemic disorders in cholesterol regulation, such as are observed in atherosclerosis, hypertension, and elevated concentrations of plasma LDL-cholesterol, may be identified as positive risk factors for LOAD (Stampfer, 2006). To contrast, cholesterol-lowering statin therapy, functioning through the inhibition of cholesterol biosynthesis, has been reported to decrease LOAD prevalence in some (Wolozin, et al., 2000; Jick, Zomberg & Jick, 2000; Rockwood, et al., 2002), but not all (Wagstaff, et al., 2003), populations. Perhaps the most compelling evidence supporting a direct role of cholesterol dysregulation in the aetiology of LOAD has been the reliable correlation of apolipoprotein E (APOE) genotype with disease onset and progression (Saunders, et al., 1993; Corder, et al., 1994; Poirier, et al., 1995; Myers & Goate, 2001). Originally identified in the context of cardiovascular disease (CVD) as a contributing factor in the development of atherosclerosis and hyperlipidemia (Uterman, et al., 1984; Miltiadous, Cariolou, & Elisaf, 2002; Smith, et al., 2006), APOE has since been shown to

function as a key mediator in the intracerebral transport and recycling of cholesterol and other essential lipids (Elshourbagy, et al., 1985; DeKroon, & Armati, 2001; Poirier, 2005).

Transport of cholesterol between cells of the CNS is facilitated through the formation of lipoparticles of similar size and constitution as peripheral high-density lipoproteins (HDL) (Illingworth & Glover, 1971). Predominantly synthesised by astrocytes, APOE functions as the primary protein moiety of HDL within the CNS (Diedrich, et al., 1991; Poirier, et al., 1991) and has been shown to mediate the binding and internalisation of glial-derived HDL by regenerating nerve terminals (Poirier, et al., 1994; Mauch et al., 2001). Although there is evidence to suggest that glial-derived APOE-containing lipoparticles are essential in the neural regeneration observed following injury, the mechanisms through which the assembly of HDL in the CNS is regulated are not clearly understood.

Recent studies have identified a crucial role for members of the ATP-binding cassette (ABC) family of transporters in the regulation of cholesterol transport from cells and in the formation of HDL in peripheral tissues. The results of *in situ* hybridisation experiments conducted by Tachikawa and colleagues demonstrate widespread expression of ABCA1 and ABCG1 mRNA throughout the murine brain thereby suggesting a function for these transporters in neural physiology (Tachikawa et al., 2005). The results of culture experiments suggest that ABCA1 and perhaps ABCG1 participate in the generation of lipoproteins by glia through the regulation of cholesterol and phospholipid efflux to extracellular APOE (Fujiyoshi, et al., 2007; Kim, et al., 2007).

During the first phase of this study, a potential role for the ABC transporters A1 and G1 in the regulation of cholesterol recycling in response to hippocampal

deafferentation was examined in the mouse. As glial-derived lipoproteins have been shown to mediate reactive sprouting and synaptogenesis in cell culture (Mauch et al., 2001; Göritz, et al., 2002) it was hypothesised that ABCA1 and ABCG1 may facilitate the formation of this lipoparticles and that the expression of these transporters would be up-regulated, paralleling the reported increase in APOE expression observed during the regenerative phase following lesion-induced injury (Poirier, et al., 1991, Poirier, Baccichet, et al., 1993). Thus, ABCA1 and ABCG1 may function to facilitate the recycling of cholesterol from dead or dying cells towards neurons undergoing compensatory synaptogenesis and terminal proliferation.

Increasing evidence suggesting that the dysregulation of cholesterol homeostasis may compromise neural function has fuelled interest into the effects that the pharmacological manipulation of cholesterol regulation has on neural integrity and recovery from trauma. Statins represent the most commonly prescribed family of drugs for the treatment of hypercholesterolemia and function through the competitive inhibition of HMGCoA-R. In this action, statins reduce the synthesis of both cholesterol and its derivatives, thereby exerting pleiotropic influence on cell physiology. While not without controversy (Orsi, Sherman, & Woldeselassie, 2001; Wagstaff et al., 2003), there is evidence from epidemiological, clinical and experimental studies to suggest that therapeutic statin treatment may have cognitive benefits, decreasing the prevalence of AD (Wolozin et al., 2000; Jick et al., 2000; Fassbender et al., 2001) and increasing recovery from cerebral ischemia (Daimon et al., 2004; Yrjanheikki et al., 2005; Nagaraja, et al., 2006). In addition to its plasma cholesterol lowering properties, the potential for statin treatment to interfere with isoprenoid synthesis has been demonstrated to influence microglial inflammatory responses (Kiener et al., 2001; Bi et al., 2004). In this manner,

the neuroprotective actions of cholesterol inhibition may function by promoting the clearing of lipid debris in response to trauma while impeding additional damage induced by inflammation. Furthermore, by virtue of its ability to enhance apoE(LDL) receptor expression in the brain (Posse De Chaves, et al., 2000), it is conceivable that statins actually facilitate the internalization and utilisation of lipoprotein particles transported by apoE to stimulate (and may be enhanced) synaptic turnover and dendritic remodelling..

While the mechanisms through which statin therapy exerts its neuroprotection are not fully understood, inhibition of cholesterol biosynthesis has been shown to influence regulation of cholesterol efflux, resulting in decreased ABCA1 and G1 mRNA expression and APOE secretion proportional to deficits in cellular cholesterol status (Naldu et al., 2002; Wong, Quinn, & Brown, 2004; Zanotti et al., 2006) although some studies have demonstrated the opposite effect (Argmann, et al., 2005). However, many studies examining the effects of statin therapy on the promotion of neurite extension are limited by the extremely high statin doses used and the in vitro culture systems employed (Sato-Suzuki, & Murota, 1996; Tanaka et al., 2000). Therefore, the effects of clinically relevant statin therapy (0.6mg/kg/day) on markers of cholesterol and reactive sprouting responses observed *in vivo* following deafferentation, were also be investigated. The second phase of this study examines the effect of low-dose, systemic pravastatin treatment on the expression of ABCA1, ABCG1 and APOE, involved in cholesterol transport, and HMGCoA-R, involved in cholesterol synthesis and the pharmacological target of pravastatin activity. It was hypothesised that the application of low-dose statin treatment would be sufficient to influence these mechanisms regulating cholesterol efflux within brain, and might modulate the extent or time-course of reactive sprouting and synaptogenic responses observed in the deafferented rodent hippocampus.

CHAPTER 1

LITERATURE REVIEW

1.1 ALZHEIMER'S DISEASE

First described by the Bavarian neurologist Alois Alzheimer in 1906, the progressive neurodegenerative condition which bears his name represents the most common cause of dementia in the elderly. Now recognised as the fourth leading cause of adult death, it has been estimated that 1 in 13 Canadians over the age of 65, and 1 in 3 over the age of 85, currently suffer with Alzheimer's disease (AD) (Canadian Health and Aging Working Group [CHAWG], 1994). As the 'baby-boom' generation enters their sixth-decade, the prevalence of this disease may be expected to increase, with an estimated 750,000 Canadians being diagnosed with AD and related diseases by 2031 (CHAWG, 1994). In light of the enormous economic cost of patient care, currently estimated near 5.5 billion dollars annually (Østbye & Crosse, 1994; Johnson, Davis, & Bosanquet, 2000), and the ageing demographics of the Canadian population, extensive efforts are being made in attempt to gain understanding of the disease aetiology and the mechanisms regulating its progression.

1.1.1. CLINICAL FEATURES OF ALZHEIMER'S DISEASE

Insidious in its onset, the decline in cognitive function associated with AD is progressive and pathological. While great heterogeneity exists in the clinical presentation of the AD pathophysiology, anterograde deficits in episodic memory—those memories of people, places, dates and times—are commonly the earliest neuropsychological symptom observed (Oppenheim, 1994; Greene et al., 1996; Knopman et al, 2001). Typically this amnesic phase extends for many years prior to clinical diagnosis (Linn et al., 1995) with

individuals, apparently unaware of the extent of their impairments (Correa, Graves & Costa, 1996), eventually being brought for assessment by a concerned third party who has noted changes in behaviour or ability to function either socially or professionally. With time, the disease course generally progresses to include various deficits in language, motor, attention, and executive functioning (Price, et al., 1993; Helmes & Østbye, 2002). While there exists great heterogeneity in the time-course of disease progression between individuals (Förstl & Kurz, 1999; Perry & Hodges, 1999; Lambon Ralph, et al., 2003), the life expectancy of patients with AD is notably shorter than that of matched non-demented individuals (Knopman et al., 1988), averaging between eight and twelve years following initial clinical diagnosis (Man, et al., 1992; Feldman & Kertesz, 2001).

Although a definitive diagnosis requires the histological analysis of brain pathology, the identification of possible or probable AD through differential diagnosis is quiet reliable with concordance studies suggesting that careful clinical examination may accurately differentiate between AD and other forms of dementia in eighty to ninety percent of cases (Lim, 1999; Knopman, 2001).

1.1.2. NEUROPATHOLOGICAL FEATURES OF ALZHEIMER'S DISEASE

A positive diagnosis of AD requires the combination of clinical and histological examination, with progressive dementia being considered symptomatic of the degenerative pathology associated with the disease syndrome. Characterised by the presence of extracellular β-amyloid (Aβ) plaques, intracellular neurofibrillary tangles (NFT), and significant neuronal loss, the anatomical progression of the AD pathology has been suggested to parallel the observed cognitive decline. The entorhinal and perirhinal

regions, which function as a neural interface between the hippocampal formation and associational cortices, are often the first structures affected in AD and generally demonstrate the most pronounced atrophy (Arnold, et al., 1991; Van Hoesen, Hyman, & Damasio, 1991). This degeneration typically progresses to include the hippocampus proper and temporal lobes (Van Hoesen, & Hyman, 1990), the nucleus basalis of Meynert (Tagliavini, & Pilleri, 1983), and finally the forebrain and parietal association cortices (Harasty, et al., 1999; Tiepel et al., 2005), generally sparing the cerebellum (Karas, et al., 2003). Although the mechanisms regulating the generation of the AD pathophysiology remain elusive, great advances have been made in the characterisation of the neuropathological hallmarks, neuronal cell loss, and the accumulation of Aβ and NFT.

1.1.2.1. Amyloid Plaques

The presence of extracellular neuritic amyloid plaques are required for the definitive diagnosis of AD and are therefore considered central to the general disease pathology. Classically, mature plaques are multicellular lesions; composed of a central core of tightly aggregated filamentous Aβ peptides surrounded by dystrophic axons and dendritic processes (Ferrer, et al., 1998), and closely associated activated microglia and reactive astrocytes (Lue, Walker & Rogers, 2001; Mrak, & Griffenbc, 2001; Atwood, et al., 2002). In addition more diffuse plaques of non-fibrillar Aβ peptides that are not associated with glial activation or neurite atrophy are observed extensively in the AD brain pathology (Tagalavini, et al., 1988; Yamaguchi, et al., 1988).

Following its description in meningeovascular plaques (Glenner & Wong, 1984), Aβ was identified as the core component of neuritic plaques (Masters, et al., 1985; Selkoe, et al., 1996). This peptide, varying between 40 and 42 amino acid residues in

length (Miller, et al., 1993; Roher, et al., 1993), was later determined to be derived from the cleavage of the larger, transmembrane, amyloid precursor protein (APP) (Kang, et al., 1987). While the physiological function of APP remains unclear, there is increasing evidence to suggest that it may be involved in the mediation of cellular signalling cascades, perhaps functioning as a growth factor or receptor (Rossjohn et al., 1999) or as a moderator of apoptotic sensitivity (Paradis et al., 1996).

The central role of Aβ in the AD pathology has fuelled much research into the mechanisms regulating the processing of APP and subsequent plaque formation and are now well characterised. Processing of APP and the subsequent generation of Aβ is mediated through the proteolytic activity of the α, β, and γ-secretases via either an Aβ-generating amyloidogenic pathway or the alternative non-amyloidogenic pathway (Esch, et al., 1990; Golde et al., 1992). The non-amyloidogenic pathway is mediated through the cleavage of APP within the Aβ region, by an α-secretase, thereby excluding Aβ generation and producing instead a soluble, non-amyloidogenic fragment sAPPα,. To contrast, generation of Aβ through the amyloidogenic pathway is mediated through the concerted activity of β and γ secretases, and results in the production of the soluble fragment sAPPβ in addition to Aβ. The beta-site APP-cleaving enzyme (BACE), a transmembrane aspartyl protease, has been reported to mediate the initial cleavage of APP (Hussain, et al., 1999; Vassar, et al., 1999; Yan, et al., 1999), enabling the generation of Aβ peptides by subsequent γ-secretase cleavage. Two members of the presenilin family of proteins, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) have been identified to display γ-secretase activity and mediate the cleavage of Aβ within the transmembrane domain. Two γ-secretase cleavage sites have been identified in APP yielding either the 40 ($A\beta_{1-40}$) or 42-residue ($A\beta_{1-42}$) long Aβ peptide. Both the

amyloidogenic and non-amyloidogenic pathways have been demonstrated to proceed under physiologic conditions (Esch, et al., 1990; Sisodia, et al., 1990, Haas, et al., 1992; Seubert, et al., 1992).

Evidence supporting a central role of APP processing and Aβ generation in the pathophysiology of AD has been derived from the identification of causative missense mutations in the genes encoding APP (Chartier-Harlin et al., 1991; Murrell et al., 1991; Hendriks et al., 1992; Mullan et al., 1992; Eckman et al., 1997; Ancolio et al., 1999), and the γ-secretases PSEN1 and PSEN2 (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). Each of these mutations has been demonstrated to influence APP cleavage, promoting Aβ production (Cai, Golde, & Younkin., 1993; Haass, et al., 1994; Borchelt, et al., 1996; Duff, et al., 1996), possibly increasing the ratio of $A\beta_{1-42}$ relative to $A\beta_{1-40}$ (Lansbury, 1997), and are associated with an earlier disease onset, prior to the age of 60.

1.1.2.2. Neurofibrillary Tangles

Although observed in other neurodegenerative conditions, NFT are typically found in high concentrations in the entorhinal cortex, hippocampus and amygdala of AD patients (Spillantini and Goedert, 1998). Unlike amyloid plaques, NFT are primarily intracellular lesions localised within the neuronal perikarya, axons or dendrites (Kosik, Joachim & Selkoe, 1994) and may be deposited extracellularly as the disease course progresses (Mena et al., 1995).

The early investigations using electron microscopy uncovered the NFT ultrastructure to be comprised of two intertwined filament subunits, termed paired-helical filaments (PHF) (Kidd, 1964; Terry, Gonatas, & Weiss, 1964). Later studies

demonstrated that the microtubule-associated tau protein, involved in the stabilization of axonal microtubules (Maccioni & Cambiazo, 1995), when abnormally hyperphosphorylated, aggregated to form both soluble and insoluble PHF and NFT (Ishiguro et al., 1992; Maccioni & Cambiazo, 1995, Schweers, 1995). A resultant destabilisation of microtubule networks has been proposed to compromise synaptic function through the impairment of axonal transport (Kowall & Kosik, 1987; Mandelkow, et al., 1995). Although several protein kinases have been demonstrated to regulate the phosphorylation of tau, (Ishiguro, 1992; Takashima, et al., 1993; Tsai, et al., 1994; Michel, et al., 1998; Patrick, et al., 1999), there is growing evidence that the disregulation of cyclin-dependent kinase Cdk5 and subsequent hyperphosphorylation of tau by the glycogen synthase kinase GSK3β are central to the neural degeneration associated with NFT formation (Alvarez, et al., 1999; Patrick , et al., 1999; Alvarez, Muñoz, & Maccioni, 2001). While there is evidence to suggest that Aβ may induce NFT formation (Alveraz, Muñoz, & Maccioni, 2001), the presence of degenerative NFT in the absence of Aβ suggests other mechanisms may regulate the development of PHF and NFT (Ahlijanian et al., 2000). Thus the relevance of NFT in the context of AD pathophysiology remains unclear (Takashima, 1993; Alvarez, et al., 1999).

1.1.2.3. Neuronal/Synaptic Loss

While gross neural atrophy is considered to be a hallmark feature of the AD brain pathology, studies indicate that certain subpopulations of neurons may be selectively affected by the disease. Investigations conducted by Terry and colleagues have estimated a thirty percent neuronal loss in the neocortex, hippocampus, and entorhinal cortex of AD-confirmed individuals (Terry, et al., 1981), and a forty-five percent reduction in

neocortical synapses (Terry, et al., 1991). In addition, decreased synaptic density in the frontal lobes has been correlated with cognitive deficits (DeKosky & Scheff, 1990; Terry et al., 1991). Significant loss of the cholinergic basal forebrain neurons, including those of the septal/diagonal band complex and the nucleus basalis of Meynert are also observed in the AD brain (Tagliavini, & Pilleri, 1983).These neurons provide extensive cholinergic innervation to the hippocampus, olfactory bulbs, amygdala and cortex, and their loss has been proposed to contribute to the AD symptomology (Samuel, et al., 1994; Iraizoz, et al., 1999). Although cholinergic neurons appear to be particularly sensitive to degeneration in AD, they are not exclusively affected. Significant losses to the serotonergic and noradrenergic systems have also been noted in the AD brain and have been proposed to contribute to non-cognitive behavioural symptoms observed in some AD patients (Palmer & Gershon, 1990).

1.1.3. DISEASE AETIOLOGY

Inherited in an familial autosomal dominant pattern, the causative mutations in APP and the γ-secretases PSEN1 and PSEN2 previously discussed only account for an estimated five percent of AD cases worldwide and are typically associated with an earlier onset prior to the age of 65 (Rocchi, et al., 2003).While the sufficiency of perturbations in amyloid metabolism to induce the AD pathology lends support for the importance of Aβ in the disease mechanism, the low prevalence of these mutations in sporadic cases suggests that other risk factors must contribute to the common disease aetiology. The vast majority of AD patients present later in life (after the age of 65), and do not demonstrate any clear Mendelian pattern of disease inheritance (Lee, et al., 1986). This

late-onset AD (LOAD) is believed to represent a convergence syndrome (Martins, et al., 2006), with a number of factors, both environmental and genetic, contributing to the risk of disease development.

1.1.3.1. Age

Originally believed to represent a condition of pathological ageing, the risk of developing AD increases substantially with increased survival into later life (Mann, Yates, & Marcynuik, 1984). After the age of 65, the estimated prevalence of AD is approximately 1 in 13 individuals. By the eighth decade, the risk increases five-fold, afflicting thirty percent of individuals over the age of 85 (Graham, et al., 1997). While no mechanisms involved in the ageing process have been shown to directly influence the risk of developing AD, it is generally believed that decreases in neural plasticity and redundancy (Flood, et al., 1987) which may be associated with normal ageing processes, instigate a sensitivity to the generation of disease pathology by additional risk factors, both environmental and genetic (Ardent, et al., 1997; Levi, et al., 2005).

1.1.3.2. Gender

The results of epidemiological studies suggest women are at an increased (two-fold) risk for the development of AD, independent of their propensity to live longer (Corso, et al., 1992; Manubens, et al., 1995). Although the link between gender and AD risk remains unclear, the identification of estrogen as a potent modulator of neurite outgrowth and synaptic plasticity has led some researchers to propose that postmenopausal estrogen deficiency may contribute to the development of disease pathology (Yue, et al., 2005; Bran, et al., 2007). However, studies investigating the

effects of estrogen replacement therapy (ERT) on cognition have yielded conflicting results with some demonstrating a maintenance of cognitive ability (Horgervorst, et al., 2000) and decreased prevalence of AD (Tang, et al., 1996; Baldereschi, et al., 1998; Waring, et al., 1999), while others failed to find an effect (Mulnard, et al., 2000) or found negative effects of ERT on cognition (Kang, Weuve, & Goldstein, 2004). However recent findings suggesting effect that the long-term use of estrogen in post-menopausal women may be strongly associated with heart disease and breast cancer (Humphries & Gill, 2003) has somewhat dampen the interest in this therapeutic avenue.

1.1.3.3. Apolipoprotein E Genotype

Apolipoproteins function in the regulation of intercellular transport of cholesterol and lipids, and apolipoprotein E (apoE), as the primary lipoprotein expressed in the CNS (Elshourbagy, et al., 1985), is believed to be central in the regulation of lipid homeostasis in the brain. Three allelic variants, termed epsilon (ε) 2, 3, and 4, have been identified in the human apoE gene locus to encode three protein isoforms, apoE2, apoE3 and apoE4, respectively (Utermann, et al., 1980; Zannis & Breslow, 1981; Utermann, Steinmetz, & Weber, 1982). The apoE2 isoform, present in approximately eight percent of the population (Utterman et al., 1980; Rall, Weisgraber, & Mahley, 1982), was found to include a cysteine residue at positions 112 and 158 (Utermann, et al., 1980). Far more common, the apoE3 isoform is present in seventy-eight percent of the population (Utermann et al., 1980; Rall, Weisgraber, & Mahley, 1982) and contains a cysteine at position 112 and an arginine residue at position 158 (Utermann, et al., 1980). To contrast, the apoE4 isoform, present in approximately fourteen percent of the population (Utermann et al., 1980; Rall, Weisgraber& Mahley, 1982), includes arginine residues at

both positions. (Weisgraber, Rall, & Mahley., 1981; Rall, Weisgraber, & Mahley, 1982). Thus six possible genotypes are possible in the human population—the homozygous ε2/ε2, ε3/ε3, ε4/ε4 and heterozygous ε2/ε3, ε2/ε4, ε3/ε4.

To date, the only genetic risk factor that has been reliably associated with the development of LOAD is the presence of the ε4 allelic variant (Poirier, Davignon, et al., 1993; Strittmatter, et al., 1993). The results of epidemiological studies suggest that an estimated forty percent of LOAD patients carry at least one copy of the ε4 allele (Poirier, Davignon, et al., 1993; Saunders, et al., 1993). In addition, the apoε4 genotype had been associated, not only with an earlier disease onset (Corder et al., 1993; Locke et al., 1995), but also with increases in amyloid and NFT pathology (Schmechel, et al., 1993; Strittmatter et al., 1993; Nagy, et al., 1995; Ohm et al., 1999), and cholinergic deficits (Poirier, et al., 1995; Soininen et al., 1995). In comparison, it has been proposed that the ε2 allele is protective; associated with longevity and delaying AD onset (Chartier-Harlin, et al., 1994; Corder, et al., 1994), even in the early-onset familial forms of AD (Sorbi, et al., 1995).

In light of this association, researchers have been working to identify the mechanisms through which apoE genotype may contribute to the AD pathology. Previous studies have demonstrated that apoE is able to form stable complexes with Aβ peptides, impairing polymerization and fibril formation (Strittmatter, et al., 1993; Evans, et al., 1995; Yang et al., 1997) and neural toxicity *in vitro* (Whitson et al., 1994; Puttfarcken, et al., 1997). In agreement with a possible role in the clearing of extracellular Aβ, Beffert and colleagues have shown that apoE-containing liposomes facilitated the clearing of extracellular Aβ through receptor-mediated endocytosis (Beffert et al., 1998). Differences in the binding affinity of lipidated apoE3 and apoE4 with Aβ

have led to the proposition that deficits in the mediation of Aβ-clearance by apoE4 may contribute to the disease pathology (Zhou, et al., 1996; Yang, et al., 1997; Tokuda et al., 2000).

In addition to a role in the regulation of Aβ metabolism, apoE has been shown to play a key role in the mediation of neural plasticity (Poirier et al., 1991; Champagne, Rochford & Poirier, 2005) and recovery from injury (Chen et al., 1997; Teasdale, et al., 1997; Fagan, et al., 1998). The results of *in vitro* cell studies have demonstrated an isoform-dependent promotion of neurite outgrowth by apoE, such that stimulation by apoE3>apoE4 (Nathan, et al., 1994). Studies conducted in targeted-replacement mice expressing the human apoε3 or ε4 alleles suggest that the presence of the ε4 allele may be associated with impaired reactive sprouting (White, et al., 2001), and astroglial organization (Blain, Sullivan, & Poirier, 2006) in response to deafferentation, when compared to apoE3 transgenic mice. In agreement with these results, Arendt and colleagues have demonstrated that the presence of the apoε4 allele was associated with more severe degeneration and reduced neural remodeling in the brains of AD patients (Arendt, et al., 1997). While the mechanisms through which apoE contributes to the onset and progression of the AD pathology remain the subject of continued research, the results of these studies suggest a possible role for the dysregulation of mechanisms regulating lipid transport in the disease aetiology.

1.1.3.4. Vascular Disease

In support of a potential link between the regulation of lipid homeostasis and the AD pathophysiology, growing evidence suggests that incidence of vascular disease may increase the risk of LOAD. Originally believed to represent two distinct disease

processes, studies have demonstrated an association between the presence of cerebrovascular infarcts (Snowdon, et al., 1997) and microvascular ischemic lesions (White, et al., 2002), typical of vascular dementia, with the risk for LOAD. Furthermore, the presence of coronary artery disease has been correlated with increased amyloid pathology in the brains of elderly individuals (Sparks, et al., 1990), whereas a history of myocardial infarction has been reported to increase the risk of AD in females (Aronson, et al., 1990). In addition, the results of prospective studies suggest that hypertension and hypercholesterolemia during mid-life may significantly increase the risk of developing LOAD (Launer, et al., 2000; Whitmer, et al., 2005), however measures of blood pressure or plasma-cholesterol taken later in life show no association with disease risk (Kuller, et al., 2003).

In addition to the epidemiological results suggesting an association between hypercholesterolemia and risk for AD, the results of animal studies provide additional support of a link between systemic lipid homeostasis and CNS integrity, demonstrating that the consumption of high-fat, high-cholesterol diets can be correlated with increased Aβ deposition in the brains of mice, guinea pigs and rabbits (Sparks, et al., 1994; Howland, et al., 1998; Refolo, et al., 2000; Sparks et al., 2000). Finally, further support for an association between the regulation of cholesterol homeostasis and risk for LOAD is derived from epidemiological studies reporting that the use of statins, a family cholesterol-lowering used in the treatment of hypercholesterolemia, may decrease the prevalence of AD in some (Jick, et al., 2000; Wolozin, et al., 2000; Rockwood, et al., 2002), but not all (Wagstaff, et al., 2003; Zhou, Teramukai, & Fukushima, 2007), populations.

Although there is growing evidence to suggest that vascular disease contributes to the generation and progression of the AD pathology, the mechanisms regulating this association remain poorly understood. It remains to be determined if the presence of vascular disease contributes either directly or indirectly to the LOAD pathophysiology or if the diseases are independently influenced by common risk factors, such as apoε4 genotype which, in addition to its association with AD risk previously discussed, has been identified to contribute to atherosclerosis and coronary disease (Ilveskoski, et al., 1999; Peng, Zhao, & Wang, 1999; Fabrizio Rodella, et al., 2007).

1.1.4. CONTRIBUTION OF CHOLESTEROL DYSREGULATION TO AD

Considering the association of apoE and vascular diseases to the risk of AD, the contribution of cholesterol dysregulation to the disease pathophysiology has been a subject of growing interest. Cholesterol is an essential component of eukaryotic cell physiology, supporting both structural and signaling integrity. Accounting for an estimated twenty to twenty-five percent of cellular membrane lipid mass, free, unesterfied cholesterol (FC) contributes to the regulation of membrane fluidity through the extension of the phase-transition temperature range of the lipid bilayer (Horton, et al., 2002). In comparison, cholesterol may be esterfied (CE) by the activity of acyl-coenzymeA:cholesterol acyltransferase (ACAT) (Dobiásová & Frohlich, 1999) and stored intracellularly in the cytoplasm, providing a cholesterol reserve that may be converted back to free cholesterol and fatty acids by the enzyme neutral cholesteryl ester hydrolase (NCEH).(Zhao, et al., 2005).

Changes in membrane cholesterol content have been proposed to occur during aging (Schroeder, 1984) and may contribute to AD pathogenesis through the influencing of APP metabolism. Enriched cholesterol microdomains have been demonstrated to interact with, and influence the function of many membrane-bound proteins, including those involved in the processing of APP. How cholesterol mediates Aβ generation remains unclear. The results of studies conducted in primary hippocampal cell culture have studies have suggested that the β-secretase BACE is sequestered away from APP by cholesterol-enriched lipid rafts (Abad-Rodriguez, 2004). Depletion of membrane cholesterol has been proposed to enable the co-localisation of BACE with APP and thereby promoting the amyloidogenic processing of APP and Aβ generation (Abad-Rodriguez, 2004). In line with these results, analysis of AD brain cholesterol levels have demonstrated significant reductions in membrane cholesterol content, especially in areas of high amyloid plaque density (Ledesma, et al., 2003; Crameri, et al., 2006). As cholesterol has been demonstrated to support neurite outgrowth and synaptogenesis *in vitro* (Mauch, et al., 2001), deficits in cholesterol may also contribute to reduced plasticity and impair compensatory sprouting responses.

As evidence mounts in support of a role for the dysregulation of cholesterol homeostasis in the pathogenesis of AD, much research is being conducted in an attempt to understand the mechanisms regulating cholesterol homeostasis in the CNS.

1.2 CHOLESTEROL HOMEOSTASIS IN THE CNS

The brain is recognised as being the most cholesterol-enriched organ in the body, accounting for an estimated 2 percent of the total mass, but containing nearly 25 percent of the body's unesterfied cholesterol (Dietschy & Turley, 2004). This cholesterol pool—present primarily in neural cell membranes and specialised myelin sheaves—functions to influence membrane fluidity and decrease permeability with repercussions for cell physiology (Chauhan, 2003). Additionally, cholesterol serves as the essential precursor in the synthesis of isoprenoids and neurosteroids vital for the integrity of cell signalling cascades (Guarneri, et al., 1994; Matthies, et al., 1997). This duality of structural and signalling functions held by cholesterol homeostasis within the netting of cellular metabolism attests to the importance of this bio-molecule in nervous system physiology. Within the CNS, cholesterol homeostasis is maintained through the coordination of pathways regulating the synthesis, transport or recycling, and excretion of cholesterol.

1.2.1. REGULATION OF CHOLESTEROL SYNTHESIS

In the presence of an intact blood-brain-barrier, regulation of cholesterol homeostasis in the CNS is maintained independently from the periphery and negligible amounts of circulating cholesterol are incorporated into CNS cholesterol pools (Edmond, et al., 1991; Jurevics & Morell, 1995). Essentially all of the cholesterol demands of the CNS are met through *de novo* anabolism from acetyl-CoA, (as illustrated in figure 1) of

which, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA-R) functions as the rate-limiting enzyme. The conversion of HMGCoA to mevalonate catalysed by HMGCoA-R represents the first committed step in the biosynthesis of cholesterol. Located within the endoplasmic reticulum, HMGCoA-R is regulated by three independent mechanisms. As an interconvertible enzyme, HMGCoA-R may be inactivated by phosphorylation, providing short-term control of cholesterol synthesis. In addition, cholesterol and its derivatives have been proposed to provide feedback repression of HMGCoA-R gene transcription and promote enzyme degradation (Golstein & Brown, 1990) thereby lowering HMGCoA-R levels and providing longer term inhibition of cholesterol synthesis.

While both neurons and glial cells retain the ability to synthesise cholesterol in the mature, steady state (Vance, Hayashi, & Karten, 2005), during remodelling or repair, damaged neurons appear to require additional glia-supplied cholesterol to support the high-lipid demands imposed by reactive sprouting and synaptogenesis (de Chaves, et al., 2000; Mauch et al., 2001; Hayashi, et al., 2004). Studies conducted in sympathetic neurons by de Chaves and colleagues have demonstrated that following inhibition of cholesterol synthesis by statin treatment, axon regeneration could be supported by the addition of lipoprotein-associated cholesterol (de Chaves, et al., 1997; de Chaves, et al., 2000). Similarly, the results of studies conducted in retinal ganglion cell cultures suggest the formation of efficient mature synapses is promoted by the co-incubation of glial cells. Furthermore, apoE-associated cholesterol derived from these glia was sufficient to promote axon extension and synaptogenesis (Mauch, et al., 2001; Hayashi, et al., 2004).

1.2.2. REGULATION OF CHOLESTEROL TRANSPORT

Cholesterol is weakly polar by nature and cholesterol esters are hydrophobic, and therefore they cannot be readily transported between cells as free molecules. Instead, cholesterol and other lipids (including triacylglycerol and phospholipids) are complexed with the lipid-binding amphipathic apolipoproteins to form lipoparticles, which facilitate the transport of these lipids through aqueous media. With the notable exception that they tend to contain more unesterfied cholesterol, CNS lipoparticles are of comparable size and composition to the high-density lipoparticles (HDL) found in circulating plasma. (Illingworth & Glover, 1971; Pitas, et al., 1987; Gong, et al., 2002). These HDL-like lipoparticles are believed to facilitate the intercellular transport of cholesterol from glia towards neurons especially during dendritic sprouting or synaptogenesis (Mauch, et al., 2001)

1.2.2.1. A central role for apolipoprotein E in CNS cholesterol transport

As previously discussed, apolipoproteins mediate the transportation of cholesterol and other lipids between cells. Second only to the liver, the brain is a predominant site of apoE mRNA expression (Elshourbagy, et al., 1985). While low levels of other apolipoproteins, have been detected in the CNS, apoE is believed to function as the primary apolipoprotein in the CNS (Elshourbagy, et al., 1985). While present throughout the brain, apoE mRNA appears to be primarily expressed in astrocytes and microglial (Diedrich, et al., 1991; Poirier, et al., 1991; Stone, et al., 1997) and is believed to support the formation of glial-derived lipoparticles which, in turn, help support neuronal cholesterol requirements.

The importance of apoE in the maintenance of neural integrity has been investigated in the context of transgenic apoE knockout ($apoE^{-/-}$) mice. These studies have demonstrated that deletion of apoE can be associated with an age-dependent reduction in functional synapses (Masliah, et al., 1995). In agreement for a pivotal role of apoE in neural function, behavioural studies have demonstrated that spatial learning and memory deficits observed in $apoE^{-/-}$ mice may be significantly reversed with the ventricular injection of recombinant apoE (Masliah, et al., 1997).

Although there is evidence to suggest that glial-derived apoE-containing lipoparticles are essential in the maintenance of neural integrity and regeneration observed following trauma, the mechanisms through which the assembly of HDL in the CNS is regulated are not clearly understood. Much of our understanding of cholesterol efflux from cells has been gleaned from cardiovascular research and is only beginning to be applied in the context of CNS plasticity. These studies have identified a crucial role for members of the ATP-binding cassette (ABC) family of transporters in the regulation of cholesterol transport from cells and in the formation of HDL.

1.2.2.2. ATP-binding cassette transporters mediate cholesterol efflux

Divided into subfamilies A through G based on gene homology and substrate specificity, the more than 50 identified ABC transporters have been shown to mediate the translocation of a diverse range of macromolecules across biological membranes (Schmitz, Langmann, & Heimerl, 2001; Borst & Elfernik, 2002; Brewer & Santamarina-Fojo, 2003). Regulation of cholesterol efflux across the plasma cell membrane, and the formation of HDL, has been suggested to depend in part on the synergistic activities of ABCA1 and ABCG1 (Vaughan & Oram, 2006).

ABCA1 has been shown to regulate the efflux of cholesterol and phospholipids from diverse cell types to extracellular lipid-poor apolipoproteins, resulting in the formation of nascent discoidal HDL (Remaley, et al., 2001; Favari, et al., 2004). In humans, mutations in ABCA1 have been linked to Tangier disease and have been shown to result in afflictive deficits in plasma HDL, the intracellular accumulation of cholesterol, and, severe atherosclerosis (Bodzioch, et al., 1999; Brooks-Wilson, et al., 1999; Rust, et al., 1999). Although studies have not investigated the effects of ABCA1 deletion on neurodegenerative diseases such as AD, studies in transgenic ABCA1 knockout mice ($ABCA1^{-/-}$) suggest ABCA1 also plays a key role in the regulation of cholesterol homeostasis behind the blood-brain-barrier (Ohtsuki, et al., 2004).

Expressed ubiquitously by microglia, astrocytes and neurons (Koldamova, et al., 2003), ABCA1 has been shown to influence APOE secretion and lipidation in the mouse brain. Deletion of ABCA1 may be associated with a significant reduction in APOE secretion from cultured glial cells and in the brains of $ABCA1^{-/-}$ mice (Hirsch-Reinshagen, 2004). In addition, deficits in cholesterol loading to existing APOE are observed in these animals (Wahrle, et al., 2004).

Epidemiological studies examining the associations between polymorphisms and/or mutations in the ABCA1 gene with the onset and progression of AD suggest ABCA1 function may contribute in the development of the AD pathology. The non-synonymous R219K polymorphism in ABCA1 gene, previously associated with the risk of coronary artery disease (CAD), was shown to influence both CSF cholesterol levels and the age of LOAD onset in European populations (Wollmer, et al., 2003; Katov, et al., 2004; Sundar, et al., 2007). In addition, this polymorphism has been associated with an increased risk for LOAD in women but not men (Sundar, et al., 2007).

Initially identified due to its high sequence homology with ABCA1, the half-transporter ABCG1 has since been shown to participate in the regulation of cholesterol efflux and to direct the assembly of mature-HDL (Klucken, et al., 1999; Vaughan & Oram 2005; Gelissen, et al., 2006). Proposed to function either as a homodimer or heterodimer in conjunction with other ABCG transporters (Klucken, et al., 1999), ABCG1 has been demonstrated to facilitate the efflux of cholesterol to partially lipidated but not lipid-free lipoproteins (Kennedy, et al., 2005). In this context it has been proposed that cholesterol efflux and the formation of HDL is regulated through the synergistic actions of ABCA1 and ABCG1. Studies in macrophage cultures have demonstrated that lipoparticles produced by ABCA1, but not nascent apolipoproteins may serve as ligands for ABCG1, which, through the transfer of additional cholesterol and lipids, mediates the formation of mature HDL (Vaughan & Oram 2005).

Interference of ABCG1 expression through RNA inhibition has been associated with a 32 percent reduction in cholesterol excretion from cultured macrophages and a concomitant decrease in APOE secretion, independent of ABCA1 expression, providing strong evidence for a role of ABCG1 in the maintenance of cholesterol homeostasis (Wang, et al., 2004). Studies in ABCG1 knockout mice have demonstrated increased intracellular cholesterol accumulation in a wide range of cell types in response to challenging by a high-fat, high-cholesterol diet (Kennedy, et al., 2005). The observation that ABCG1 expression is significantly up-regulated in ABCA1 knockout mice further supports an important role for ABCG1 in the regulation of cholesterol efflux, at least in the periphery (Lorkowski, et al., 2001).

Within the context of the CNS, ABCG1 function is poorly understood. *In situ* hybridization studies in the mouse suggest ABCG1 is widely expressed in the brain

throughout development and maturity, appearing to co-localise with ABCA1 expression (Tachikawa, et al., 2005). Recent results from cerebellar astrocyte cell cultures suggest ABCG1 expression is up-regulated in response to increases in intracellular cholesterol concentrations (Karten et al., 2006). While these conditions failed to influence ABCA1 expression, the increase in ABCG1 mRNA was correlated with an increase in cholesterol efflux to exogenous apolipoproteins (Nakamura, et al., 2004; Karten et al., 2006). Although ABCG1 is gaining recognition as a potential regulator of cholesterol efflux in the CNS, associations between polymorphisms in the ABCG1 gene and risk for LOAD have yet to be examined.

1.2.2.3. Receptor-mediated endocytosis of cholesterol

There is accumulating evidence to suggest that in addition to mediating the formation of lipoparticles, apoE functions to regulate the binding and internalization of glial-derived lipoparticles, thereby facilitating the redistribution of cholesterol among cells of the CNS. While neurons do not appear to synthesis and secrete apoE (Pitas, et al., 1987; Nakai, et al, 1996), they have been demonstrated to express receptors that bind to, and mediate the endocytosis of, apoE-associated lipoparticles (Beffert, et al., 1998; Xu, et al., 1998) presumably via the clatherin-coated pit pathway (Havel, 1998). Several different lipoprotein receptors have been identified in the CNS; including the low-density lipoprotein and very-low density lipoprotein receptors (apoE(LDL)R and VapoE(LDL)R respectively), the LDL-related protein (LRP), and apolipoprotein E receptor 2 (apoER2), which are believed to participate in the binding and endocytosis of apoE-associated lipoparticles (Pitas, et al., 1987; Willnow, et al., 1992; Williams, et al., 1994; Hayashi, et al., 2004; Petit-Turcotte, et al., 2005). Mutations in these receptors may result in the

extracellular accumulation of lipoparticles as they are unable to be bound and metabolised by cells (Brown & Goldstein, 1976).

1.2.3. REGULATION OF CHOLESTEROL EXCRETION

Cholesterol synthesis continues in the mature CNS and therefore mechanisms regulating the removal of cholesterol are necessary to maintain cholesterol homeostasis (Quan, et al., 2003; Xi, et al., 2003). Neural cells are unable to catabolism cholesterol and therefore excess cholesterol must be removed from the CNS into the peripheral circulation for disposal by the liver (Elferink & Groen, 1999). This regulation of cholesterol efflux from the CNS is achieved primarily through the conversion to the more soluble hyrdroxylated cholesterol derivative, 24(S)-hydroxycholesetrol by the activity of 24-hydroxylase (Lütjohann, et al., 1996; Lund, et al., 2003). 24(S)-hydroxycholesterol has been demonstrated to readily cross the blood brain barrier (Haines, 2001; Leoni, et al., 2003) and may serve as a precursor for the hepatic synthesis of bile acids (Björkhem, et al., 2001). In addition to serving as a mechanism through which cholesterol may be excreted from the CNS, hydroxyl sterol derivatives have been shown to function as important signaling molecules via the liver-x-receptor (LxR) pathway (Liang, et al., 2004; Abildayeva, et al., 2006) Analysis of mRNA expression suggest that the brain in the primary site of 24-hydroxylase expression and approximately ninety percent of plasma 24(S)-hydroxycholesterol is derived from the CNS and therefore circulating levels have been proposed to indicate cerebral cholesterol turnover (Björkhem, et al., 1998; Lund, Guileyardo, & Russel, 1999; Meaney, et al., 2001). In addition, secondary mechanisms, mediated by apoE-containing lipoparticles and ABC transporters, have been proposed to

contribute, in a lesser extent to the efflux of cholesterol from the CNS (Obtsuki, et al., 2004).

1.3. CHOLESTEROL REGULATION FOLLOWING CNS INJURY

Albeit limited in capacity, the mature CNS is capable of functional recovery following injury through the compensatory sprouting of surviving neurons and the reorganisation of synaptic connectivity. Perhaps the most widely employed paradigm for the investigation of neural plasticity in the adult CNS is the unilateral entorhinal cortex lesion (uECL). This model maintains relevance in the context of AD pathophysiology as it has been demonstrated that the earliest evidence of tangle formation and cell loss occurs in the entorhinal cortex several years prior to disease diagnosis (Braak & Braak, 1997). While uECL fails to induce the accumulation of neurofibrillary tangles and extracellular amyloid plaques so characteristic of the AD pathology, it does provide a useful model to investigate the mechanisms regulating cholesterol homeostasis in response to denervation and reactive sprouting responses.

1.3.1. THE ENTORHINAL CORTEX LESION PARADIGM

Functioning as a bidirectional cortical interface between multimodal association cortices and the hippocampus proper, the entorhinal cortex (EC) provides the primary source of hippocampal afferentation *via* the perforant path (Van Hoesen, et al., 1991). The popularity of the entorhinal cortex lesion (ECL) paradigm in the analysis of lesion-induced plasticity in the rodent CNS is due in part to the well-described topography and

laminated termination of the entorhino-dentate projections in addition to the physical distance from the lesion site (EC) to the location of reactive sprouting (dentate gyrus). More specifically, the cytoarchitecture of the dentate gyrus makes it possible to assess the relative contributions of homotypic and heterotypic sprouting in the observed lesion-induced reorganisation. Deafferentation of the hippocampal formation through uECL results in an 80 to 90 percent reduction in the synaptic input to the outer molecular layer of the ipsilateral dentate fascia (Matthews, Cotman, & Lynch, 1976a, b; Steward, et al., 1990; Phinney, et al., 2004). While reorganisation does occur in the contralateral dentate gyrus by virtue of compromises to transhippocampal circuitry, the resulting deafferentation is estimated to represent an approximate five percent loss of synapses (Steward & Scoville, 1995), therefore the contralateral hippocampus may be used as a negative control to which lesion-induced plasticity may be compared. In response, compensatory sprouting of surviving cells has been shown to replace as much as eighty percent of the lost synapses in the denervated region (Matthews, Cotman, & Lynch, 1976a, b; Steward, et al., 1990).

The heterotypic reactive sprouting responses of the cholinergic septohippocampal projections in response to ECL are well described (Matthews, Cotman, & Lynch, 1976a, b; Phinney, et al., 2004; Marrone, LeBoutillier, & Petit, 2005) and are commonly employed as a measure of reinnervation following deafferentation of the dentate. Histochemical analysis of the cholinergic synaptic marker AChE demonstrates marked laminar staining in the intact dentate gyrus, being confined to the inner one-third of the dentate fascia. Denervation of the outer-molecular layer through uECL results in a well-documented expansion of this inner molecular layer cholinergic lamination and may be used as an indication of lesion-induced compensatory sprouting. Importantly, this

deafferentation of the dentate also results in shrinkage of the outer molecular layer that must also be considered when estimated changes in synaptic density (Phinney, et al., 2004).

1.3.2. PROMOTION OF CHOLESTEROL RECYCLING

Previous studies have demonstrated that mechanisms regulating cholesterol homeostasis are dynamically regulated in the hippocampal formation in response to uECL. As evidence suggests APOE expression and secretion is up-regulated in response to diverse CNS traumata (Poirier, et al., 1994; Nathan, et al., 2001; White, Nicoll, & Horsburgh, 2001) a central role for apoE in the recycling of lipids released from degenerating terminals for use during remodelling has been proposed.

Activation of dentate astrocytes in response to terminal degeneration enables the active phagocytosis and clearing of debris. Essential for the permission of reactive sprouting, this degenerative phase of neural remodelling is rapid in its onset and, in the mouse, is estimated to continue for up to six days post-lesion (Matthews, Cotman, & Lynch, 1976; Steward, et al., 1990). Following this period, axon extension and synaptic replacement in the denervated dentate may continue for up to 30 days and is supported through glial-mediated lipid recycling (Phinney et al., 2004).

The accumulation of cholesterol and lipids within activated astrocytes is believed to provide a large and accessible pool of lipids that may be processed and secreted as apoE-containing lipoparticles and directed towards surviving neurons undergoing reactive sprouting and synaptogenesis (Goodrum, 1990; Poirier, et al., 1993; Goodrum et al., 1995; Beffert, et al., 1998). In support of this hypothesis, it has been observed that

following uECL astrocytes localised in the denervated region of the dentate gyrus display a significant increase in apoE expression during the early phase of regeneration (Poirier, et al., 1991). To further support for a central role for the recycling of lipid in the promotion of reactive sprouting, HMGCoA-R expression is observed to be down-regulated in response to neural injury (Goodrum, 1990). The reduction of cholesterol biosynthesis in addition to an up-regulation in apoE expression observed during remodelling suggest that the increased cholesterol demands imposed during this period are being met through apoE-mediated recycling. The importance of cholesterol recycling pathways in the support of reactive sprouting response and synaptogenesis are underscored by the observation that deletion of APOE results in cytoskeletal abnormalities and impaired synaptic remodelling in the CNS following deafferentation (Masliah, et al., 1995; Champagne, Rochford, & Poirier, 2002).

There is now accumulating evidence to suggest that apoE-mediated recycling of lipids is essential for the synaptic remodelling observed following denervation in the CNS. In light of these results, there is much interest in understanding the mechanisms regulating apoE activity and the mobilisation of lipids. As previously mentioned the ABC transporters ABCA1 and ABCG1 have been implicated in the regulation of lipid efflux from astrocytes and the generation of lipoparticles (Brewer & Santamarina-Fojo, 2003; Vaughan & Oram, 2006). In this study we will quantify changes in the mRNA transcript and protein expression of ABCA1, ABCG1 and APOE during recovery from uECL in order to determine whether these transporters are involved in the regulation of glial-mediated cholesterol recycling observed in response to trauma.

1.4. PHARMACOLOGICAL MANIPULATION OF CHOLESTEROL

Increasing evidence suggesting that the dysregulation of cholesterol homeostasis may compromise neural function has fuelled interest into the effects that the pharmacological manipulation of cholesterol regulation may have on neural integrity and recovery from trauma.

1.4.1. INHIBITION OF HMGCoA-R ACTIVITY

Statins represent the most commonly prescribed family of drugs for the treatment of hypercholesterolemia and function through the competitive inhibition of HMGCoA-R activity. In this action, statins reduce the synthesis of both cholesterol and its derivatives, thereby exerting pleiotropic influence on cell physiology. While not without controversy (Orsi, Sherman, & Woldeselassie, 2001; Wagstaff, et al., 2003), there is evidence from epidemiological, clinical and experimental studies to suggest that therapeutic statin treatment may have cognitive benefits, decreasing the prevalence of AD (Jick, et al., 2000; Wolozin, et al., 2000; Fassbender, et al, 2001) and increasing recovery from cerebral ischemia (Yrjanheikki, et al., 2005).

1.4.2. ARE STATINS NEUROPROTECTIVE?

The mechanisms through which statin therapy exerts its neuroprotection are not fully understood. Inhibition of cholesterol biosynthesis has been shown to influence regulation of cholesterol efflux, resulting in decreased ABCA1 and G1 mRNA expression

and APOE secretion proportional to deficits in cellular cholesterol status (Naidu, et al., 2002; Wong, et al., 2004; Zanotti, et al., 2006). Additionally it has been noted that the observed down-regulation of cholesterol export mechanisms can be correlated with an increase in LDL receptor expression, indicating cholesterol import mechanisms are up-regulated in response (Wong, et al., 2004). In addition to its cholesterol lowering properties, the potential for statin treatment to interfere with isoprenoid synthesis has been demonstrated to influence microglial inflammatory responses (Kiener, et al., 2001; Bi, et al., 2004). In this manner, the neuroprotective actions of cholesterol inhibition may function through promoting the clearing of lipid debris in response to trauma while impeding additional damage induced by inflammation.

The effect of systemic statin therapy on plasticity in the CNS is an area of increasing interest. Evidence suggests that the neuroprotective effect of statin therapy in reducing cell death following ischemia may be attributed to anti-inflammatory effects instigated through the reduction in isoprenoid synthesis. Studies examining the effects of statin therapies on the promotion of neurite extension are limited by the extremely high statin doses used and the culture systems employed (Sato-Suzuki, & Murota, 1996; Tanaka, et al., 2000). During the second phase of our study the effect of clinically relevant statin therapy on cholesterol efflux and reactive sprouting responses *in vivo* will be examined. Inhibition of cholesterol synthesis has been demonstrated to increase the endocytosis of exogenous cholesterol and lipoproteins while inhibiting cholesterol efflux. It is hypothesised therefore that statin treatment may influence the extent or time-course of reactive sprouting and synaptogenic responses observed in the deafferented rodent hippocampus, and influence mechanisms regulating cholesterol efflux.

Many studies indicating inhibition of HMGCoA-R activity may influence neural plasticity *in vivo* have investigated the neuroprotective effects of statin therapy in the context of cerebral ischemia (Yrjanheikki, et al., 2005; Nagaraja, et al., 2006) and studies examining the effects of statin treatment on reactive sprouting and synaptogenesis are lacking. And while cell culture studies suggest that the competitive inhibition of HMGCoA-R with statin treatment may influence ABCA1-mediated cholesterol efflux from macrophages (Wong, Quinn, & Brown, 2004), these effects have yet to be examined in the brain. In most cases, the clinical relevance of these findings is unclear due to the high doses of statins applied. Therefore, this study aims to determine whether therapeutic (low dose) statin therapy is sufficient to influence cholesterol regulation or reactive sprouting responses observed following uECL in the mouse.

1.5. RATIONALE

The derivation by neurons of cholesterol and other lipids from glial cells is necessary for the support of reactive sprouting and synaptogenesis observed during recovery of function following trauma. However, mechanisms regulating the efflux of these molecules and the generation of transporting lipoparticles in the CNS remain poorly understood. Thus, this proposed study aims to examine a potential role for the transporters ABCA1 and ABCG1 in the regulation of cholesterol efflux from glial cells in an *in vivo* model of reactive sprouting. In addition, the effects of systemic statin therapy on these mechanisms and the possible implications for cholinergic remodelling will be investigated. Previous studies suggest that ABCA1, ABCG1 and APOE may be co-regulated and function together in the facilitation of cholesterol efflux and the formation of HDL in the periphery (Hirsch-Reinshagen, et al., 2004; Wahrle, et al., 2004; Gelissen, et al., 2006). The functions of these transporters in the regulation of CNS cholesterol homeostasis are poorly understood and most studies examining a role for ABCA1 and ABCG1 in the regulation of neural cell cholesterol homeostasis have focused on cell culture experiments (Hirsch-Reinshagen, et al., 2004; Karten, et al., 2006). To date only a single study has examined the *in vivo* expression of these transporters in the mouse brain (Tachikawa, et al., 2005).

The results of *in situ* hybridisation experiments conducted by Tachikawa and colleagues demonstrate widespread expression of ABCA1 and ABCG1 mRNA throughout the murine brain thereby suggesting a function for these transporters in neural physiology (Tachikawa, et al., 2005). The results of culture experiments suggest that ABCA1 and

perhaps ABCG1 participate in the generation of lipoproteins by glia through the regulation of cholesterol and phospholipid efflux to extracellular APOE (Hirsch-Reinshagen, et al., 2004; Karten, et al., 2006). As glial-derived lipoproteins have been shown to mediate reactive sprouting and synaptogenesis in cell culture it is hypothesised that ABCA1 and ABCG1 expression will be up-regulated, paralleling the reported increase in APOE expression observed during the regenerative phase following lesion-induced injury, thereby facilitating the recycling of cholesterol from dead or dying cells towards neurons undergoing compensatory synaptogenesis and terminal proliferation.

Together these studies suggest ABCA1 and ABCG1 may be involved in regulating cholesterol efflux from neural cells however fail to demonstrate a role for these transporters in the dynamic regulation of cholesterol homeostasis observed during reactive sprouting and synaptogenesis. In order to address this, we examined the expression of ABCA1 and ABCG1 will be examined in relation to APOE expression during lesion-induced reorganisation. During the first phase of investigation a potential role for the ABC transporters A1 and G1 in the regulation of cholesterol recycling in response to hippocampal deafferentation was examined.

CHAPTER 2

EFFECT OF LOW-DOSE PRAVASTATIN TREATMENT ON MARKERS OF CHOLESTEROL HOMEOSTASIS AND REACTIVE SPROUTING FOLLOWING uECL IN THE MOUSE

2.1. MATERIALS AND METHODS

2.1.1. ANIMALS

The *in vivo* investigation of a potential function for ABCA1 and ABCG1 during reactive sprouting was conducted in 12-week-old male C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were housed individually in an enriched environment and fed a diet of standard laboratory chow *ad libium.* A 12-hour light-dark cycle was maintained with light onset at 07:00 and offset at 21:00, local time. Following one week of acclimatisation, mice were randomly assigned to receive daily subcutaneous injections of either a low, pharmacologically relevant dose (0.6mg/kg) pravastatin solution or, a 0.9% saline vehicle solution. Pharmacological treatment proceeded for two weeks prior to the electrolytic lesioning of the entorhinal cortex and continued throughout recovery until sacrifice. 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.

2.1.2. SURGICAL PROCEDURES

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). At the age of 15 weeks, following two-weeks of drug treatment (either pravastatin or saline vehicle), mice were anaesthetised to by the intramuscular injection of a ketamine/xylazine/acepromazine mix (dose: 1μg/g of body weight) and placed into a stereotaxic apparatus with mouse adapter in the flat skull position. Lambda 0 was

determined by aligning the electrode with suture lines. The skull was drilled to allow the electrode to pass, at an angle of 6°, 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], and a current of 1mA was applied for a 10s 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 lowered only 1mm and no current passed. Following surgery, mice were given a subcutaneous bolster (0.1ml) of physiological saline to prevent dehydration. Animals were nursed throughout their recovery; remaining under a heat lamp for 24h 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, 5 mice from each treatment group were administered a lethal anaesthetic dose and perfused transcardially with 30ml of ice cold 0.01M phosphate buffered solution (PBS) in order to minimise contamination of brain tissues through contact with blood. These time points have been selected in order to represent the dynamic regulation of lipid homeostasis during the critical phases of reactive sprouting previously mentioned. Following ECL, the hippocampus displays marked synaptic loss in the outer molecular layer of the dentate gyrus continuing up to 6 days post lesion. Starting between 8 to 10 days, new synapses are being formed by terminals originating in the hilar region and septal areas. The 14 days time point corresponds to the most active phase of reinnervation which lasts an estimated two weeks. By 40 and 60 days following injury, up to eighty-percent of the lost entorhinal cortex inputs are replaced by synaptic connections originating of the septal and hilar regions.

This temporal analysis enables the dissection of the degenerative period immediately following lesioning from the subsequent phase of active reinnervation, creating a dynamic picture of the recovery process *in vivo*.

For the analysis of region-specific mRNA, protein, and enzyme activity levels brains were dissected and the cerebellum, hippocampi, entorhinal cortices, frontal cortices, parieto-temporal cortices, striatum and brain stem structures isolated. Whole-brain tissue dissects used for histochemical analyses were removed, flash-frozen at -40°C in isopentane, and stored at -80°C until use.

2.1.3. EVALUATION OF CHOLINERGIC SPROUTING

2.1.3.1. Tissue/ Slide Preparation.

Fresh frozen whole-brain dissects from 20 pravastatin-treated and 20 saline-treated mice (sacrificed at 2, 6, 14, and 21 DPL; N=5 per time point per drug group) were used for the assessment of cholinergic sprouting in the outer-molecular layer (OML) of the dentate gyrus following uECL as a function of drug-treatment. 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 4°C, and stored at –80°C until use. Sections through the region of the dorsal hippocampal formation were used for histochemical analysis.

2.1.3.2. Acetylcholinesterase Histochemistry.

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 for five

minutes in dH_2O three times and then placed in the developer solution (0.38% sodium sulphide; pH 7.8) for 6 minutes. After a second series of dH_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 then rinsed again three times in dH_2O for five minutes. AChE histochemistry was post-fixed in a 4% solution of PFA in 0.01M PBS at a pH of 7.4 for two hours. Slides were then rinsed a final three times in 0.01M PBS, pH 7.4 before being dehydrated in a series of alcohol baths and cleared in xylene (2 minutes 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).

2.1.3.3. Assessment of Cholinergic Sprouting.

Digital microphotographs of brain sections were captured using a Zeiss Axiostar Plus Transmitted-Light Microscope and the Northern Eclipse Version 6.0 Image Analysis Software. In order to assess the time course and extent of cholinergic sprouting in the dentate gyrus following uECL the relative optical density of AChE staining in the dentate both ipsilateral and contralateral to the lesion was evaluated using the MCDI-II image analysis system.

Measurements of AChE reactivity were taken at six points along the dorsal blade of the outer molecular layer of the dentate facia in five sections per animal (N=5 per drug treatment group per time point). Staining density was be calculated as the relative optical density ratio of ipsilateral to contralateral measures, thereby providing a within-section control for variations in histochemical processing.

The lamina-specific denervation of the dentate gyrus following uECL has been shown to result in atrophy localised to the OML that may confound the histochemical

estimation of AChE-reactivity (Caceres and Steward, 1983; Phinney, et al., 2004). Therefore measures of OML width were assessed at six 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 (1994) outlined in equation 1 below:

$$\text{AChE Density Ratio (ipsi/conta)} = \frac{[(\text{AChE Density})_{\text{ipsilateral}}]\left[\frac{[(\text{OML Width})_{\text{ipsilateral}}]}{[(\text{OML Width})_{\text{contralateral}}]}\right]}{[(\text{AChE Density})_{\text{contralateral}}]} \quad [1]$$

Equation 1: Quantification of acetylcholinesterase staining density. This model, described by Fagan and Gage (1994) enables the estimation of AChE reactivity in the ipsilateral OML relative to the contralateral OML (which serves as the internal negative control) while taking into account the effect of lamina shrinkage in the ipsilateral OML on the appearance of AChE density.

2.1.4. EVALUATION OF MRNA EXPRESSION

2.1.4.1. RNA Source and Total RNA Extraction.

Tissue dissects of left and right frontal cortices and left and right hippocampi from pravastatin- and saline-treated sham-operated (N=5 per drug treatment) and lesioned mice at each time point (2, 14, 21, 40, 60 DPL; N=5 per drug treatment per time point) were used in the evaluation of the effect of pravastatin treatment on ABCA1, ABCG1, APOE, apoE(LDL)R and HMGCoA-R mRNA expression following uECL. Total RNA was extracted from these brain regions using the QIAGEN RNeasy Mini Kit (QUIAGEN Inc.,

Mississauga, ON) according to manufacture's guidelines for the isolation of total RNA from animal tissues.

2.1.4.2. Primer Design and Real-Time RT-PCR.

Following extraction, 2μg of total RNA from each sample was reverse-transcribed 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 100ul (all reagents were purchased from PE Biosystems, Foster City, CA). The reverse transcriptase programme included the following thermal cycle: 10min at 25°C, 30min at 48°C, followed by 5min at 95°C to stop the reaction.

Real-time PCR was conducted using the SYBR Green technique in the GeneAmp5700 sequence detection system in triplicate for each RT product sample. 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 for RT and PCR reactions were purchased from PE Biosystems (Perkin Elmer, Foster City, CA). The programme applied for real-time PCR cycling consisted of 2 min at 50°C, followed by 10 min of denaturation at 95°C. Amplification was then achieved through 40 cycles through a two-segment real-time PCR programme consisting of 15 sec at 95°C followed by 1 min at 60°C.

Primer pairs used for PCR amplification in this study were generated using the Primer Express PE Biosystems software, and are summarised in table A below. Primer specificity was confirmed through dissociation curve analysis which demonstrated single

product specific melting temperatures (T_m) as outlined in table A. No Primer-dimers were observed during the 40 PCR cycles.

Table A: Summary of primer sequences generated for the quantification of mRNA expression by real-time RT-PCR of target and candidate housekeeping genes. Melting temperature determined through dissociation analysis is denoted by T_m.

Summary of mRNA Primer Sequences

	Forward Primer	Reverse Primer	T_m
Target Gene mRNA			
ABCA1	5'-GACCGTACTCTCGCAGGG-3'	5'-GCGGCCTTGCCGGTAT-3'	75.7 °C
ABCG1	5'-CCGATGTGAACCCGTTTCTT-3'	5'-AGGCGGAGTCCTCTTCAGC-3'	81.4 °C
APOE	5'-TCCATTGCCTCCACCACAGT-3'	5'-GGGCGTAGTGAGGGATGA-3'	80.7 °C
HMGCoA-R	5'-GCCGGGACCTGACAGACTAC-3'	5'-CCTCTCAGCTGTGGTGAA-3'	76.3 °C
APOE(LDL)R	5'-TGGACCGCAGCGAGTACAC-3'	5'-CCACCACATTCTTCAGGTTGG -3'	81.5 °C
Housekeeping Gene mRNA			
β-ACTIN	5'-TGACCGAGCGTGGCTACA-3'	5'-TCTCTTTGATGTCACGCACGAT-3'	80.4 °C
PRDX2	5'-ATGACCTACCTGTGGGACGCT-3'	5'-CCTGGACTAGGCGTAGAGCCT-3'	77.1 °C
TPP2	5'-GTTGATGCTGCCAATGCTGTT-3'	5'-TGCAAGAGCGGTTTGATCG-3'	76.0 °C

2.1.4.3. Identification of Stable Housekeeping Gene

Identification of an appropriate housekeeping gene for this study was conducted through the comparison of expression stability of three candidate mRNA transcripts, β-actin (β-ACTIN), peroxiredoxin 2 (PDRX2), and tripeptidyl peptidase II (TPP2), using the BestKeeper© excel-based analysis tool (for pair-wise correlations) as previously described by Pfaffl and colleagues (Pfaffl et al., 2004). Real-time RT-PCR protocols applied were identical to those described earlier however the PCR reaction was carried out in RT product samples pooled by group. Selection of these candidate housekeeping

genes was made based upon previous studies reporting expression stability of these genes in the mouse hippocampus following ischemia-reperfusion injury (Nishida et al., 2006), and uECL (Blain *et al.,* 2004). Following this analysis, β-ACTIN was selected to serve as the housekeeping gene as its expression levels were determined to be the most stable of the three across all treatment groups and time points, as outlined in table B below.

Table B: Descriptive statistics generated by the BestKeeper© program for the three candidate housekeeping genes studied (β-ACTIN, PRDX2, TPP2) based upon observed crossing point values (CP).

Candidate Housekeeping Genes – BestKeeper© Descriptive Statistics			
	β-ACTIN	**PRDX2**	**TPP2**
N (Groups)	8	8	8
Geometric Mean of CP	18.92	23.59	23.60
Arithmetic Mean of CP	18.92	23.60	23.60
Minimum CP	18.30	22.63	22.91
Maximum CP	19.41	24.20	24.33
St. Dev. [± CP]	0.32	0.54	0.40
Variance (as % of CP)	1.68	2.30	1.68
Min [x-fold]	-1.57	-2.74	-1.70
Max [x-fold]	1.44	1.91	1.76
St. Dev. [± x-fold]	1.26	1.49	1.34

2.1.4.4. Quantification of Relative mRNA Expression

The quantification of mRNA transcript expression was determined relative to the selected stable housekeeping gene according to the Pfaffl model previously described (Pfaffl, 2001). This model (outlined in equation 2 below) enables the comparison of target mRNA expression (quantified relative to the expression of a selected reference gene) in isolated tissue dissects following experimental manipulation, with those observed in the control condition. This method considers differences in primer

efficiencies and deviations in CP values between the target and housekeeping gene and therefore boasts the advantage of increased reproducibility and accuracy while compensating for sources of error generated during the reverse-transcriptase reaction (Pfaffl, 2001).

$$\text{Relative Expression Ratio} = \frac{(E_{target})^{\Delta CP_{target}\,(\text{control - sample})}}{(E_{reference})^{\Delta CP_{reference}\,(\text{control - sample})}} \quad [2]$$

Equation 2: Quantification of relative mRNA expression ratios according to the Pfaffl method (Pfaffl, 2001). E_{target} denotes RT-PCR primer efficiency for target mRNA; $E_{refernce}$ denotes the primer efficiency for selected housekeeping gene; ΔCP_{target} denotes the difference in the observed crossing point (CP) for the control treatment (mean CP of saline-treated, sham-operated group) from that of the CP of each experimentally manipulated (by recovery time following uECL and/or drug-treatment) samples for each of the target mRNAs studied; $\Delta CP_{reference}$ denotes the difference in the observed CP for the control-treatment from the experimentally manipulated (by recovery time following lesion and drug-treatment) samples for the housekeeping gene.

The efficiencies of primer pairs during one cycle of the exponential phase were calculated according to Pfaffl (2001) from the slope of the standard curve as generated by the PE Biosystems GeneAmp 5700 SDS software according to equation 3. Crossing point values for each mRNA transcript studied were calculated as the cycle number marking the half-way point during the logarithmic phase of cDNA amplification.

$$E = 10^{-1/slope} \quad [3]$$

Equation 3: Calculation of real-time PCR primer pair amplification efficiencies. Where E denotes the primer efficiency as determined by the slope of the standard curve for each primer pair as previously described by Pfaffl (2001).

2.1.5. EVALUATION OF ABCA1 PROTEIN EXPRESSION

2.1.5.1. Protein Source and Total Protein Extraction.

Left and right hippocampal whole-tissue dissects were used for the analysis of ABCA1 protein expression following uECL (at 2, 14, 21, 40 or 60 DPL) or sham surgery as a function of pravastatin or saline treatment (N = 5 per group). Tissue isolates were sonicated on ice in a phosphate-buffered solution containing protease inhibitors (AEBSF, aprotinin, bestatin, E-64, and pepstatin A; purchased from Sigma Aldrich Canada Ltd., Oakville, ON). Total protein concentration was then assessed using the bicinchoninic acid (BCA) technique (Pierce Biotechnology Inc., Rockford, IL).

2.1.5.2. PAGE and Immunoblot Analysis.

In order to quantify ABCA1 protein expression was relative to β-tubulin, 12μg of total protein homogenate was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a NuPAGE Novex® 4-12% gradient Bis-Tris Midi Gel in an XCell SureLock Midi-Cell (Invitrogen, Burlington, ON) for 3h 20min at room temperature. Transfer to a nitrocellulose membrane was conducted using the iBlot™ Dry Blotting System according to manufacturers specifications for Novex ® 1mm thick Midi Gels (programme, P2; 23 Volts for 6 min). Coomassie Blue staining was used to confirm equal protein loading.

Following transfer, nitrocellulose membranes were blocked overnight in 5% dried-milk TBS-T (20mM Tris base, 137mM sodium choride, and 0.05% Tween-20) solution (pH = 7.5), and then rinsed three times (1 x 15min, 2 x 5 min) in TBS-T. Membranes were incubated with the primary antibody, mouse monoclonal (AB-H10) to ABCA1 (dil. 1/500; Abcam, ab18180) for two hours at room temperature with gentle agitation. After a

second washing (three rinses; 1 x 15min, 2 x 5 min), membranes were probed with the sheep-anti-mouse IgG, HRP-linked secondary antibody (dil. 1/5000; Amersham, Catalogue No. NA931) for one hour with gentle agitation. Following a final cycle of washing, detection of ABCA1 was conducted using the Western Lightning ™ Chemiluminescence Reagent Plus (Perkin-Elmer LAS Inc., Boston, MA) according to manufacturer's specifications on a Kodak Image Station 440CF (Eastman Kodak Co., Rochester, NY).

Membranes were then stripped of all antibodies prior to detection of β-tubulin. Briefly, following the detection of ABCA1, nitocellulose membranes were twice rinsed for 10 min in TBS-T prior to incubation in erasing buffer (62.5mM Tris-Cl (pH = 6.8), 2% SDS, 10mM 2-mercaptoethanol) at 70°C for 30 min. Following this, membranes were again washed twice (10 min each) in TBS-T and blocked overnight in a 5% dried-milk TBS-T solution as before. The protocol used for immunoblotting against β-tubulin was similar to that described above for ABCA1. After blocking, membranes were incubated with the primary antibody, mouse monoclonal (Dil. Ascites Clone IU-01) to the N-terminus of β-tubulin (dil.1/100; BioDesign, Catalogue No. M61409M), for one hour at room temperature, with gentle agitation. After washing, membranes were similarly probed with the secondary sheep-anti-mouse IgG, HRP-linked antibody (dil. 1/5000; Amersham, Catalogue No. NA931). Detection of β-tubulin reactivity was carried out as described above.

The densiometric analysis of immunoblot results was conducted using the Kodak 1D Image Analysis Software of the Image Station 440CF (Eastman Kodak Co., Rochester, NY). Lane analyses were used to determine the mean optical densities (OD) of ABCA1 and β-tubulin bands. Results were calculated as the mean OD of ABCA1

relative to β-tubulin in the hippocampus ipsilateral to, relative to the hippocampus contralateral to the uECL location for each mouse.

2.1.6. EVALUATION OF HMGCoA-R FUNCTIONAL ACTIVITY

2.1.6.1. Protein Source and Total Protein Extraction.

In order to determine whether the low-dose (0.6mg/kg/day) pravastatin regimen applied in this study was sufficient to inhibit cholesterol biosynthesis, the functional activity of HMGCoA-R was measured in the right (ipsilateral) parieto-temporal cortex and liver tissue dissects isolated from sham and lesioned animals treated with either saline or pravastatin as previously described and sacrificed at 2, 14, 21 and 40 DPL (N = 3 per group).

Homogenisation of 10 mg of frozen tissue dissects was conducted in 600µl of incubation buffer (30 mM EDTA, 250 mM NaCl, 1 mM DTT, 50 mM K_2HPO_4; pH = 7.2,) by sonication on ice (reagents purchased from Fisher Scientific, Ottawa, ON). Tissue homogenates were then centrifuged at 1000g for 20min at 4°C. The supernatant was collected and subsequently used for the determination of total protein concentration by the BCA technique (Pierce Biotechnology Inc. Rockford, IL), and HMGCoA-R functional activity.

2.1.6.2. HMGCoA- Reductase Activity.

Aliquots (10µl for liver, 25µl for parieto-temporal cortex) of tissue homogenates were completed a volume of 300µl by the addition of incubation buffer and pre-heated to a temperature of 37°C for 10min. Following this, 50µl of the NADPH regenerating system (1.5nmol $NADP^+$, 15nmol glucose 6-phosphate, 2U glucose 6-phosphate

dehydrogenase, and 150nmol HMGCoA in incubation buffer; reagents purchased from Sigma Chemical Co., St. Louis, MO) was added to the homogenate mixture and incubated for 60 min at 37°C. The reaction was stopped by the addition of 150 µl of a 100mM K_2HPO_4 (pH = 5.0, Americans Chemical Ltd, Saint-Laurent, QC) solution containing 60nmol of N-propionyl CoA which serves as an internal standard in subsequent analyses. A blank sample (without homogenate) was included to calculate the amount of HMGCoA remaining after incubation in the absence of HMGCoA-R activity. The final activity of HMGCoA-R in tissue isolates, expressed as the nmoles of HMGCoA transformed per minute per milligram of total protein (nmol HMGCoA/min/mg protein) was calculated as the decrease in the amount of HMGCoA in the sample homogenates in comparison with the blank sample as expressed in equation 4 below.

$$\text{HMGCoA-R Functional Activity} = \frac{\left[\frac{(\text{nmol HMGCoA})_{\text{Blank}} - (\text{nmol HMGCoA})_{\text{Sample}}}{60\text{min}}\right]}{(\text{mg Total Protein})_{\text{Sample}}} \quad [4]$$

Equation 4: Calculation of the functional activity (nmol HMGCoA/min/mg protein) of HMGCoA-R in parieto-temporal cortex and liver tissue isolates. Subtraction of nmol of HMGCoA present in each sample homogenate after controlled reaction conditions from that present in the blank control enables the determination of nmol HMGCoA transformed by HMGCoA-R present in the tissue homogenates during the reaction time allowed (60min). Milligrams of total protein present in each sample volume analysed was determined using the BCA technique.

2.1.6.3. High-Pressure Liquid Chromatographic Separation

Coenzyme A (CoA), HMGCoA and N-propinoyl CoA were separated in 30 µl of final reaction mixture injected into a High-Pressure Liquid Chromatography (HPLC) Polaris Varian System equipped with a 5u-C18-A Polaris column with a metaguard 4.6mm Polaris 5uC18 from Varian. The wavelength detector was set at 254nm for the analysis of resolved CoA molecular species. Separation was performed using a gradient system of 75mM K_2HPO_4 (Fisher Scientific, Ottawa, ON) and 100% acetonitrile (A&C American Chemicals Ltd, Saint-Laurent, QC) for a 43 min run at a flow rate of 1.0 ml/min according to previous studies conducted by Deutsch, Rapoport and Rosenberger (2002).

The standard curve used for HMGCoA quantification was generated by obtaining a linear relationship between the injected amount of HMGCoA and the area under the observed signal peak. The detection limit of this assay was determined to be 1nmol HMGCoA. Standard solutions were prepared by dissolving each standard (purchased from Sigma Chemical Co., Ottawa ON) in 75mM K_2HPO_4 buffer. The standard curve for CoA and HMGCoA ranged from 5 to 30 nmol with 10nmol of N–propionyl CoA as an internal control.

2.1.7. STATISTICAL ANALYSES

2.1.7.1. Analysis of Cholinergic Sprouting.

The corrected ipsilateral: contralateral AChE density ratios for both the pravastatin- and saline-treated groups at each of the time points (2, 6, 14, and 21 DPL, n = 5 per group) were averaged and analysed using the two-way between subjects (drug

treatment [2] x time [4]) ANOVA of the SPSS version 14.0 software. Significant interactions were decomposed by tests of simple main effects, and Tukey's HSD Test applied for all pair-wise comparisons. Results were considered significant when $p < 0.05$. All results are reported as mean ± standard error of the mean (SEM).

2.1.7.2. Analysis of Relative mRNA Expression

A two-way between subjects (drug treatment [2] x recovery time [6]) ANOVA was applied to investigate the effects of recovery time on the relative expression levels (expressed as ipsilateral-to-contralateral relative expression ratios) of each of the three different target mRNA sequences following uECL in the frontal cortex and hippocampus as a function of drug treatment (pravastatin or saline). Significant interactions were decomposed with simple main effects tests, and Tukey's HSD test was applied for all pair-wise comparisons. Results were considered significant when $p < 0.05$. All results are reported as mean relative expression ratio ± standard error of the mean (SEM).

2.1.7.3. Analysis of ABCA1 Protein Expression.

A two-way between subjects (drug treatment [2] x recovery time [6]) ANOVA was applied to investigate the effects of recovery time on the measured OD of ABCA1 protein relative to β-tubulin levels (expressed as ipsilateral-to-contralateral relative OD ratios) following uECL in the hippocampus as a function of drug treatment (pravastatin or saline). Significant interactions were decomposed with simple main effects tests, and Tukey's HSD test was applied for all pair-wise comparisons. Results were considered significant when $p < 0.05$. All results are reported as mean OD ± the standard error of the mean (SEM).

2.1.7.4. Analysis of HMGCoA-R Functional Activity.

A two-way between subjects (drug treatment [2] x recovery time [5]) ANOVA was applied to investigate the effects of recovery time on the calculated functional activity of HMGCoA-R (expressed as nmol HMGCoA/min/mg protein) following uECL in the ipsilateral parieto-temporal cortex and liver as a function of drug treatment (pravastatin or saline). Significant interactions were decomposed with simple main effects tests, and Tukey's HSD test was applied for all pair-wise comparisons. Results were considered significant when $p < 0.05$. All results are reported as mean functional activity (n=3 per group) ± the standard error of the mean (SEM).

2.2. RESULTS

2.2.1. TIME COURSE OF CHOLINERGIC SPROUTING

Denervation of the dentate gyrus by uECL results in the well-described reactive sprouting of cholinergic fibres in the outer one-third of the OML in the dentate ipsilateral to the lesion location (Phinney et al., 2004; Champagne, Rochford, & Poirier, 2005; Blain, Sullivan, & Poirier, 2006). In order to determine whether the low-dose pravastatin treatment applied in this study was sufficient to influence cholinergic sprouting responses, of AChE-staining densities in the OML of the ipsilateral DG were compared to those of the contralateral dentate at 2, 6 14 and 21 DPL. Figure 2 illustrates the location of AChE-staining density and OML width measures under high resolution. Representative photomicrographs demonstrating the patterns of AChE-staining observed in the pravastatin and saline-treated animals are illustrated in figure 3. Analysis of these results, illustrated in figure 4, yielded a significant increase in AChE-staining density over time in both the pravastatin and saline treated animals. Specifically, it was observed that in both treatment groups AChE-staining density was significantly increased at 14 DPL ($p's<0.05$) and remained so at 21 DPL ($p's<0.05$) when compared to the other time points studied.

2.2.2. EFFECT OF PRAVASTATIN TREATMENT ON CHOLINERGIC SPROUTING

As depicted in figures 2, 3, and 4, no significant differences were observed between the saline-treated and pravastatin-treated animals at any of the time points studied. These results suggest that the low-dose pravastatin regime applied in this study was insufficient to influence the measures of cholinergic sprouting applied in this study.

2.2.3. EXPRESSION OF ABCA1, ABCG1, APOE, apoE(LDL)R, AND HMGCoA-R mRNA FOLLOWING uECL

2.2.3.1. Frontal Cortex.

The assessment of expression of target mRNA transcripts relative to β-actin in the frontal cortex was conducted using real-time quantitative RT-PCR to ensure the specificity of changes in target mRNA levels to within the hippocampus following uECL. This region, selected for its distance from the lesion location, served as an internal control for the lesion technique as it also received the electrical stimulation applied, but does not suffer the extensive denervation observed in the hippocampal formation. As illustrated in figure 5, no significant differences were observed in the mRNA expression levels of ABCA1, ABCG1, APOE or HMGCoA-R, in the frontal cortex ipsilateral to lesion site relative to that contralateral, in lesioned mice at any of the time points studied (2, 14, 21, 40, and 60 DPL) when compared to sham-operated animals.

2.2.3.2. Hippocampus.

The levels of target mRNA transcripts (ABCA1, ABCG1, APOE and HMGCoA-R) in the hippocampus relative to β-actin, was examined in sham-operated and lesioned mice (sacrificed at 2, 14, 21, 40 and 60 DPL) to determine the temporal pattern of changes in mRNA expression induced by uECL.

As depicted in figure 6, a transient two-fold increase APOE mRNA expression was observed in the ipsilateral hippocampus of saline-treated animals at 14 DPL ($p<0.05$), returning to within normal levels by 21 days. These results are in accordance with those widely reported elsewhere, (Poirier et al., 1993; Blain et al., 2004) and suggest a marked up-regulation of APOE expression during the active phase of reinnervation and synaptic remodelling.

Similarly, a significant three-fold increase in ABCA1 mRNA expression in the hippocampus ipsilateral to the lesion site was observed at 14 DPL ($p<0.01$) with transcript levels returning to baseline by 21 days in saline-treated animals, as illustrated in figure 7. This temporal pattern of ABCA1 mRNA expression was noted to coincide with that observed for APOE transcripts and may indicate a role for ABCA1 during the most active period of regeneration in the hippocampus following uECL.

Interestingly, the expression of ABCG1 mRNA was unchanged in the ipsilateral hippocampus of saline-treated mice following uECL. As illustrated in figure 8, no significant changes in ABCG1 mRNA levels were observed at any of the time-points studied when compared to sham-operated animals.

In addition, apoE(LDL)R mRNA transcript expression was not observed to be significantly affected by uECL. As depicted in figure 9, no significant changes in apoE(LDL)R mRNA expression were observed in the deafferented hippocampus at any of the time-points studied, although there is a non-significant trend towards a transient increase in apoE(LDL)R mRNA levels at 14DPL.

Finally, the expression of HMGCoA-R following uECL was examined at each time-point in the hippocampus of saline-treated animals. As shown in figure 10, no significant changes in the levels of HMGCoA-R mRNA transcripts were observed in the ipsilateral hippocampus when compared to those of sham-operated animals.

2.2.4. EFFECT OF PRAVASTATIN TREATMENT ON EXPRESSION OF ABCA1, ABCG1, APOE, apoE(LDL)R AND HMGCoA-R mRNA FOLLOWING uECL

2.2.4.1. Frontal Cortex.

The effects of low-dose pravastatin treatment on the expression of target mRNAs (APOE, ABCA1, ABCG1, and HMGCoA-R) were examined in the frontal cortex of both lesioned and sham-operated mice and compared to those which received saline-treatments previously described. As predicted, the results of this analysis, illustrated in figure 5, uncovered no significant effect of pravastatin-treatment on the mRNA expression of target transcripts in the frontal cortex when compared to saline-treated animals at each of the time points studied. These results also suggest that the low-dose pravastatin treatment applied in this study does not influence the expression of these transcripts in the frontal cortex in either the sham-operated or lesioned animals.

2.2.4.2. Hippocampus.

The relative expression ratios of APOE, ABCA1, ABCG1, apoE(LDL)R, and HMGCoA-R mRNA transcripts in the hippocampus in sham and lesioned mice were investigated in pravastatin and saline-treated animals to determine whether the administration of therapeutically relevant doses of pravastatin (0.6mg/kg/day) influenced the temporal patterns of target mRNA expression observed following uECL after 2, 14 21, 40 or 60 days recovery.

The effects of pravastatin treatment on the relative expression of APOE mRNA transcripts in sham-operated and lesioned mice are depicted in figure 6. The significant up-regulation of APOE mRNA expression observed in saline-treated mice at 14DPL when compared to sham-operated animals was also observed in pravastatin-treated

animals ($p<0.05$). The temporal pattern of APOE mRNA expression observed in the ipsilateral hippocampus of saline-treated mice was not found to be significantly affected with pravastatin treatment as no significant differences in the levels of APOE mRNA transcripts were observed between saline and pravastatin treated mice at any of the time points studied. No significant differences in the relative expression of APOE mRNA were observed between pravastatin and saline-treatment in sham-operated animals.

As illustrated in figure 7, the low-dosage of pravastatin administered in this study was found to have a significant effect on the temporal pattern of ABCA1 mRNA expression in the ipsilateral hippocampus following uECL. The significant transient increase in ABCA1 expression observed in saline-treated animals at 14DPL, when compared to sham-operated animals, was paralleled in pravastatin-treated mice ($p<0.05$). Interestingly however, this up-regulation of ABCA1 mRNA expression was found to persist in the pravastatin-treated animals, with this group demonstrating significantly increased ABCA1 mRNA levels in the ipsilateral hippocampus at 21 DPL when compared to saline-treated animals ($p<0.05$). By 40 DPL, the ABCA1 mRNA levels measured in the pravastatin-treated animals were found to have returned to within control levels. Treatment with pravastatin was not found to affect the expression of ABCA1 mRNA transcripts in sham operated animals.

Pravastatin treatment was not found to have a significant effect on the relative expression of ABCG1 mRNA transcripts in the ipsilateral hippocampus following uECL. As depicted in figure 8, no significant differences were observed between pravastatin and saline-treatment in either the sham-operated or lesioned groups at any of the time points studied.

Interestingly, a significant interaction between time and drug-treatment was observed to influence apoE(LDL)R expression in the deafferented hippocampus. As illustrated in figure 9, a significant ($p<0.01$) increase of approximately 250% was observed in the levels of apoE(LDL)R mRNA expressed in sham-operated animals receiving pravastatin treatment when compared to saline-treated, sham-operated mice. While levels of apoE(LDL)R mRNA expression were similar in pravastatin and saline treated animals at 2 and 14 DPL, a significant difference was observed at 21DPL. To contrast the effect observed in sham-operated animals, apoE(LDL)R mRNA transcript expression was observed to be significantly ($p<0.05$) lower in pravastatin treated animals when compared to saline-treated animals sacrificed at the same time.

Finally, the effects of pravastatin treatment on the expression of HMGCoA-R mRNA transcripts were investigated. As shown in figure 10, no significant differences were observed in the relative expression of HMGCoA-R mRNA between the saline- or pravastatin-treated sham-operated animals. However, pravastatin treatment was found to significantly affect the temporal pattern of HMGCoA-R mRNA expression following uECL. Specifically, pravastatin-treated animals demonstrated a significant down-regulation in HMGCoA-R mRNA expression levels in the ipsilateral hippocampus at 21 DPL when compared to both to sham-operated and saline-treated animals sacrificed at the same time point. This down-regulation observed in the pravastatin-treated mice was transient, with HMGCoA-R mRNA levels returning to within control levels by 40 DPL.

2.2.5. EXPRESSION OF ABCA1 PROTEIN EXPRESSION FOLLOWING uECL

2.2.5.1. Hippocampus.

In order to examine whether the up-regulation of ABCA1 mRNA transcripts observed to occur in the ipsilateral hippocampus at 14 DPL translated into an increase in ABCA1 protein expression, the expression of ABCA1 protein present in hippocampal homogenates was determined relative to β-tubulin by immunoblot analysis in sham-operated and lesioned animals following 2, 14, 21, 40 and 60 days recovery. As illustrated in figure 11, a significant up-regulation (two-fold) in the levels of ABCA1 protein was observed at 14 DPL in the hippocampus ipsilateral to the lesion location in saline-treated animals ($p<0.05$). This increase in ABCA1 protein expression was noted to parallel the observed up-regulation of ABCA1 mRNA in this region, and demonstrated a transient pattern of induction, returning to near control levels by 21 DPL.

2.2.6. EFFECT OF PRAVASTATIN TREATMENT ON ABCA1 PROTEIN EXPRESSION FOLLOWING uECL

2.2.6.1. Hippocampus.

While pravastatin treatment was not observed to influence the expression of ABCA1 mRNA, the effects of drug administration on the levels of ABCA1 protein expression were examined in both sham and lesioned mice to determine whether pravastatin treatment influenced the temporal pattern of ABCA1 protein expression independent of its observed effects on mRNA transcript levels. As illustrated in figure 11, pravastatin treatment was not found to effect ABCA1 protein expression, relative to β-tubulin, in sham-operated animals. Interestingly, it was observed that animals receiving pravastatin failed to display the up-regulation of ABCA1 protein expression observed in

saline-treated mice at 14 DPL discussed above ($p<0.05$), and instead were not found to differ significantly from sham-operated animals at any of the time points studied. These results suggest that low-dose pravastatin treatment may attenuate the observed induction of ABCA1 protein expression observed during the active phase of regeneration (14 DPL) without influencing the relative expression of ABCA1 mRNA transcripts.

2.2.7. EFFECT OF PRAVASTATIN TREATMENT ON FUNCTIONAL ACTIVITY OF HMGCoA-R FOLLOWING uECL

While statins function to impair cholesterol biosynthesis through the competitive inhibition of HMGCoA-R, the extensive diversity of regulatory mechanisms controlling this enzyme's activity led us to investigate the effects of pravastatin treatment on the functional activity of this protein. The turnover rate of HMGCoA, the substrate of HMGCoA-R enzymatic activity, was investigated by HPLC techniques in the liver (the primary site of pravastatin bioactivity), and within the right parieto-temporal cortex (a region immediately superior to the lesion location and therefore potentially accessible to the administered drug through breaches in the blood-brain-barrier) in both sham operated and lesioned mice. The effects of pravastatin treatment on HMGCoA-R activity following uECL was investigated in these regions at 2, 14, 21, and 40 DPL (n=3 per group).

2.2.7.1. Liver.

As illustrated in figure 12, the functional activity of HMGCoA-R was observed to by dynamically regulated in the liver following uECL. Specifically, it was observed that HMGCoA-R activity was significantly down-regulated (approximately 80%) at 2 DPL in

liver tissue homogenates when compared to saline-treated, sham-operated animals ($p<0.01$). While a transient increase in HMGCoA-R activity from levels observed at 2 DPL was found to occur at 14 DPL, this increase remained significantly lower than the measures of activity observed in the sham-operated, saline-treated group ($p<0.05$). At 21 DPL, HMGCoA-R activity was observed to be depressed to levels similar to those observed at 2 DPL and remained at depressed at 40 DPL, the last time point studied.

The ability of the low-dose pravastatin treatment applied in this investigation to inhibit the functional activity of HMGCoA-R in the liver was also investigated. As illustrated in figure 12, treatment with pravastatin resulted in a significant 50% reduction ($p<0.05$) in measures of HMGCoA-R activity in the liver homogenates of sham-operated animals only. No differences in HMGCoA-R activity were observed following uECL between pravastatin and saline-treated animals at any of the post-operative time points studied.

2.2.7.2. Parieto-Temporal Cortex.

Analysis of HMGCoA-R functional activity in the right parieto-temporal cortex in sham-operated and lesioned mice demonstrated a dynamic regulation of HMGCoA-R activity in this region in response to uECL, although the temporal pattern of its activity was distinct from that observed in the liver. As demonstrated in figure 13, HMGCoA-R activity was significantly transiently up-regulated at 2 DPL when compared to the sham-operated, saline-treated group ($p<0.05$). Levels of HMGCoA-R activity were then observed to return to within control levels at 14 and 21DPL. A significant increase in the functional activity of HMGCoA-R, to levels similar to those observed at 2 DPL, was observed at 40 DPL in saline-treated animals.

To contrast the observed effects of pravastatin treatment on HMGCoA-R activity in liver homogenates, drug treatment was not found to influence the functional activity of HMGCoA-R in the cortex homogenates of either sham-operated or lesioned mice at any of the time points studied (as illustrated in figure 13). These results suggest that the low dose of pravastatin applied in this study was insufficient to significantly influence the enzymatic activity of HMGCoA-R in the parieto-temporal cortex.

FIGURES AND LEGENDS

Figure 1: The Mevalonate Pathway

Biochemical anabolism of cholesterol. Synthesis of cholesterol and its derivatives is primarily regulated through the function of HMGCoA Reductase, the rate-limiting enzyme in the mevalonate pathway. Statins inhibit cholesterol biosynthesis through the competitive inhibition of HMGCoA-R activity.

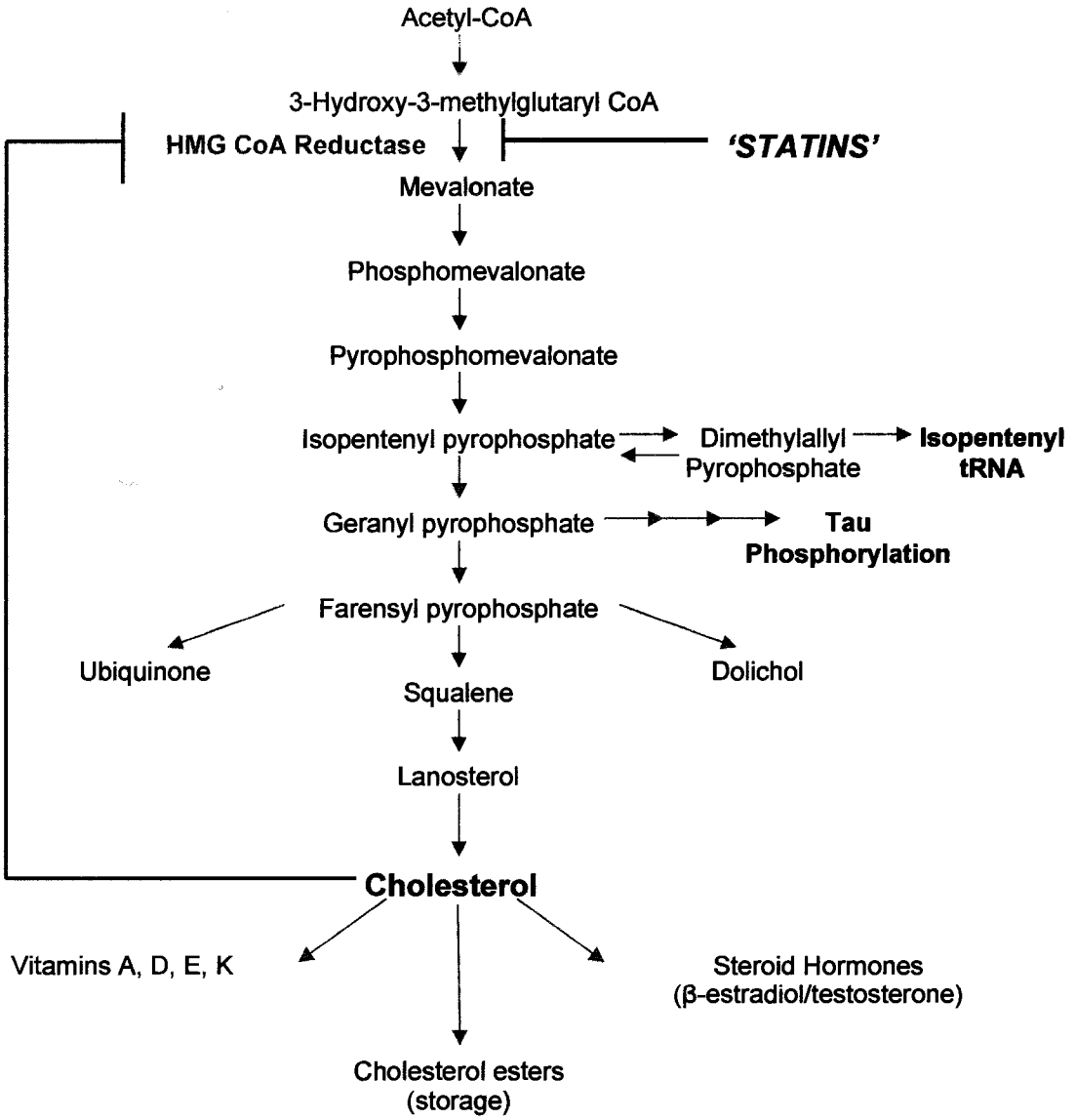

FIGURE 1

Figure 2: Measures of AChE staining density and lamina width.

To investigate the effects of pravastatin treatment on reactive sprouting responses in the mouse dentate gyrus (DG) following uECL, the density of AChE-staining was measured at six positions along the outer one-third of the outer molecular layer (OML) of the dorsal blade of the DG (as depicted by black boxes) in five sections per mouse. Changes in AChE density in the ipsilateral dentate were calculated relative to measures obtained from the contralateral dentate which served as an internal control in our analyses. Measures of OML width, taken at six-positions along the dorsal blade of the DG in five sections per mouse, were calculated as the absolute distance (perpendicular to the tangent of the arc of the DG OML) from the hippocampal fissure (indicated by asterisks) to the superior border of the inner molecular layer (IML). The location of the granule cell layer is indicated by GC. Measures of AChE-staining density were corrected for OML shrinkage as described in Materials and Methods. Magnification: 10 X.

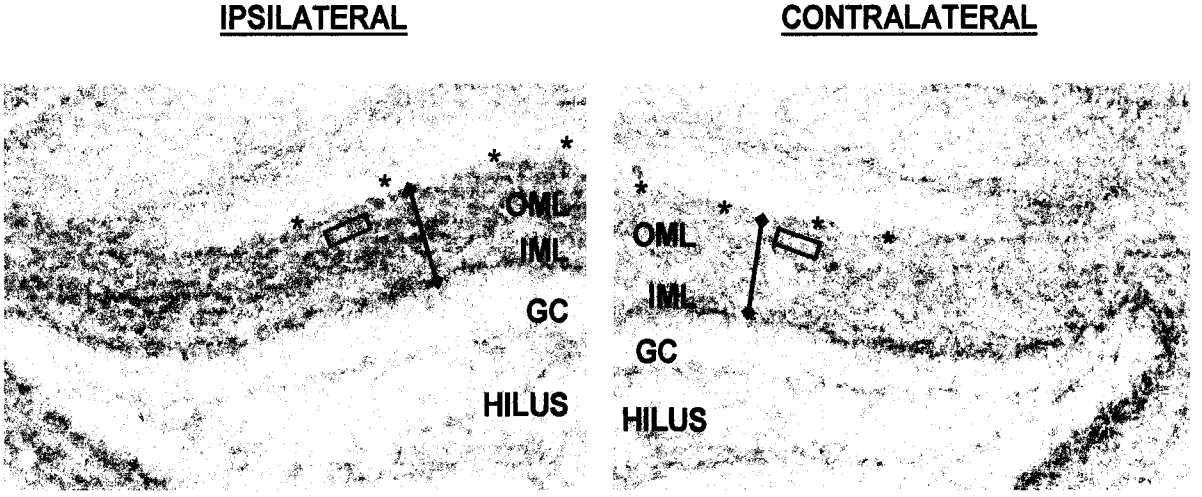

FIGURE 2

Figure 3: Patterns of AChE staining in the dentate gyrus of pravastatin and saline treated animals following uECL.

Representative photomicrographs of AChE staining densities in the dorsal region of the hippocampal formation, ipsilateral and contralateral to lesion site, at 2, 6, 14 and 21 days post-lesion (DPL) in pravastatin and saline-treated animals. No significant differences between the measures of AChE staining density were observed at 2 or 6 DPL in the ipsilateral DG. As indicated by arrows, a significant increase in the density of AChE staining in the OML of the DG ipsilateral to lesion (relative to contralateral measures) was observed at 14 and 21 DPL. Pravastatin treatment was not found to significantly affect measures of AChE staining density at any of the time points studied. Magnification: 2.5 X.

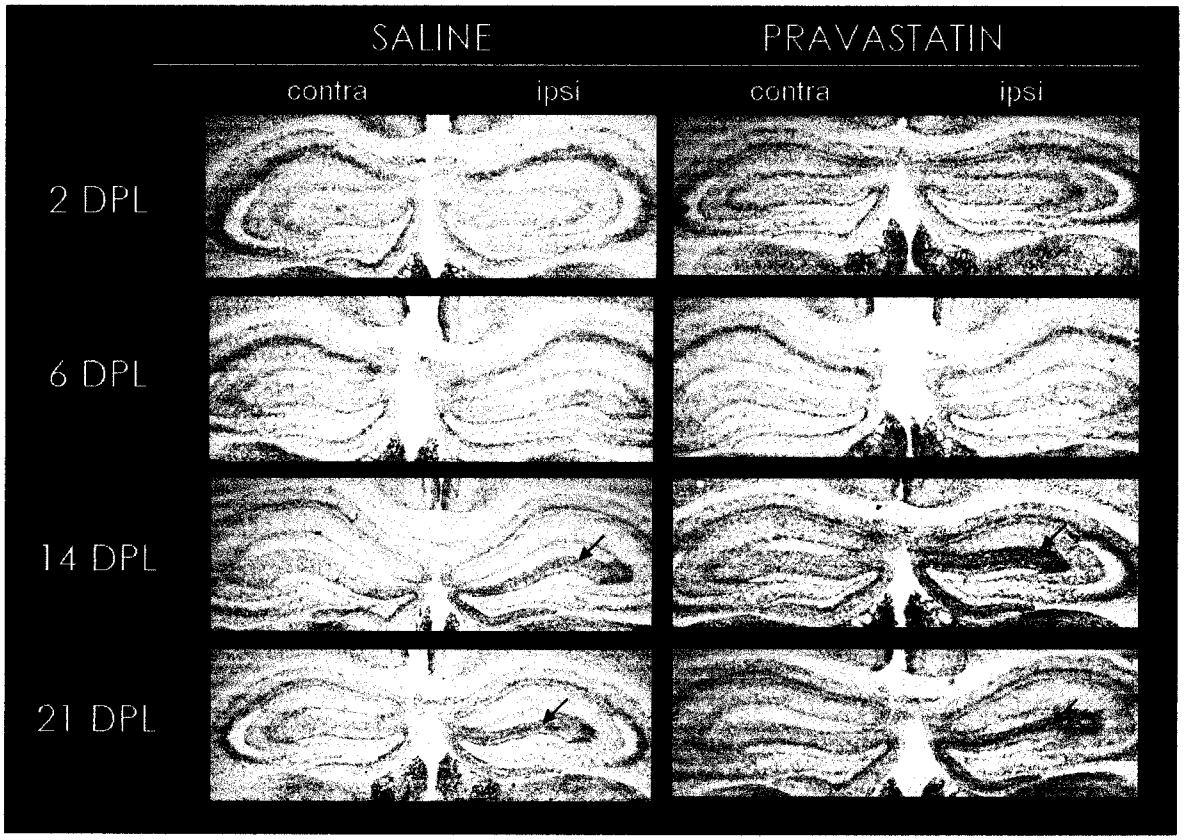

FIGURE 3

Figure 4: Quantification of AChE staining density in the outer-molecular layer of the dentate gyrus ipsilateral to lesion location in saline and pravastatin treated mice. Changes in AChE staining density in the OML of the ipsilateral DG were assessed relative to the contralateral, intact DG. The ipsilateral-to-contralateral ratios of relative optical density (ROD) measures of AChE staining, corrected for laminar shrinkage, were assessed in saline and pravastatin treated mice at 2, 6, 14 and 21 DPL. While no changes in AChE staining density were observed at 2 or 6 DPL, a significant increase in staining density was observed in the OML of the ipsilateral DG at 14 DPL and 21 DPL (*$p<0.05$). Treatment with pravastatin was not observed to significantly affect the density of AChE staining, when compared to saline-treated animals, at any of the time-points studied. Values are expressed as group mean of ROD ratios (ipsi/contra) corrected for OML shrinkage ± SEM (n=5).

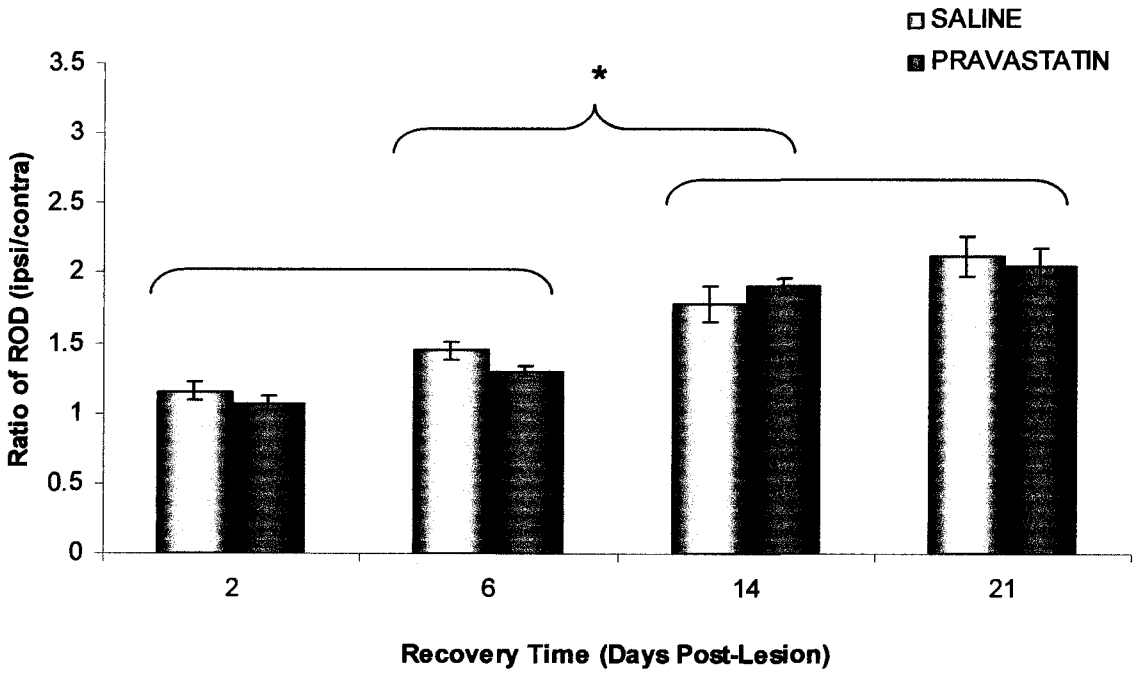

FIGURE 4

Figure 5: Quantification of target mRNA transcript expression following uECL in the frontal cortex of pravastatin and saline treated mice.

The ipsilateral-to contralateral expression ratios of APOE, ABCA1, ABCG1 and HMGCoA-R (illustrated in A, B, C, and D, respectively) mRNA transcripts (relative to β-actin) in pravastatin and saline treated mice at each post-lesion time point studied were compared to sham-operated animals. The relative expression of target mRNA transcripts in the ipsilateral frontal cortex was not observed to deviate significantly at any of the time points studied following uECL when compared to sham-operated expression levels. In addition, pravastatin treatment was not observed to significantly affect mRNA expression ratios in the frontal cortex at any of the time-points studied. Values are expressed as group mean relative (to β-actin) expression ratios (ipsi/contra) ± SEM (n=5).

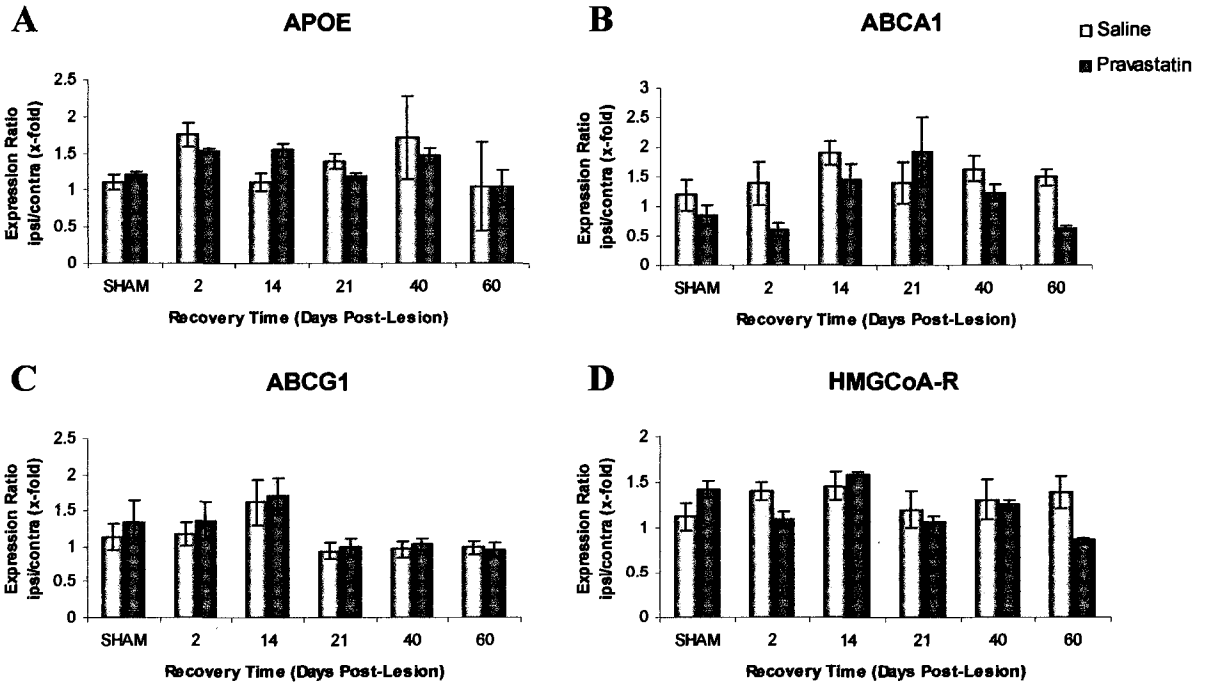

FIGURE 5

Figure 6: Quantification of APOE mRNA transcript expression in the hippocampus of sham-operated and lesioned mice following 2, 14, 21, 40 and 60 DPL.

(A) Fold-difference in APOE mRNA transcript levels (normalised relative to β-actin) measured in the ipsilateral hippocampus relative to contralateral values. (B, C) Expression of APOE mRNA transcripts, relative to β-actin, in the ipsilateral and contralateral hippocampus respectively. A significant (approximately two-fold), transient increase in the expression of APOE mRNA (normalised relative to β-actin) was observed in the hippocampus ipsilateral to uECL location at 14 DPL in both drug treatment groups relative to sham-operated (CTRL) mice (*$p<0.01$). Treatment with pravastatin failed to significantly influence the expression of APOE mRNA transcripts in sham-operated or leisoned mice at any of the time-points studied when compared to the relative saline treated animals. Values are expressed as group means ± SEM (n=5 per group).

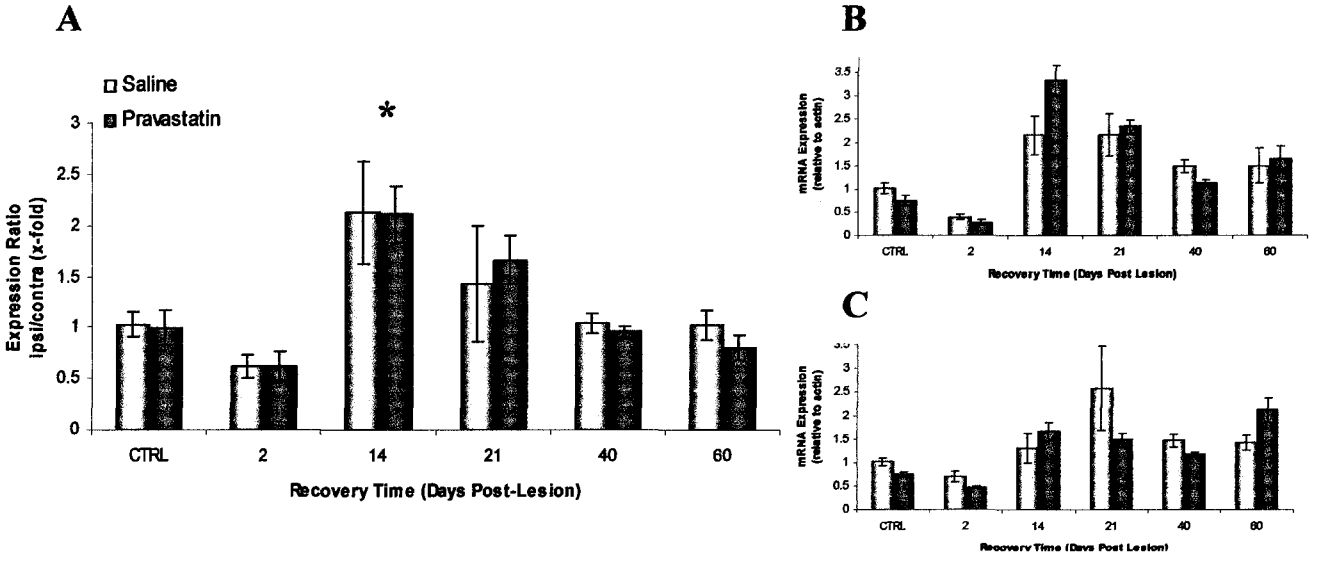

FIGURE 6

Figure 7: Quantification of ABCA1 mRNA transcript expression in the hippocampus of sham-operated and lesioned mice following 2, 14, 21, 40 and 60 DPL.

(A) Illustrates changes (x-fold) in ABCA1 mRNA transcript levels (normalised relative to β-actin) in the ipsilateral hippocampus expressed relative to contralateral values. (B, C) Measures of ABCA1 mRNA expression, relative to β-actin, in the hippocampus ipsilateral and contralateral-to lesion location respectively. A significant (approximately three-fold), increase in the expression of ABCA1 mRNA was observed in the hippocampus ipsilateral to uECL location at 14 DPL in both drug treatment groups relative to sham-operated (CTRL) mice (*$p<0.01$). While ABCA1 mRNA transcript levels were observed to return to baseline by 21 DPL, mice receiving pravastatin treatment maintained significantly increased ABCA1 mRNA expression at 21 DPL (approximately two-fold; †$p<0.05$), returning to baseline by 40 DPL.

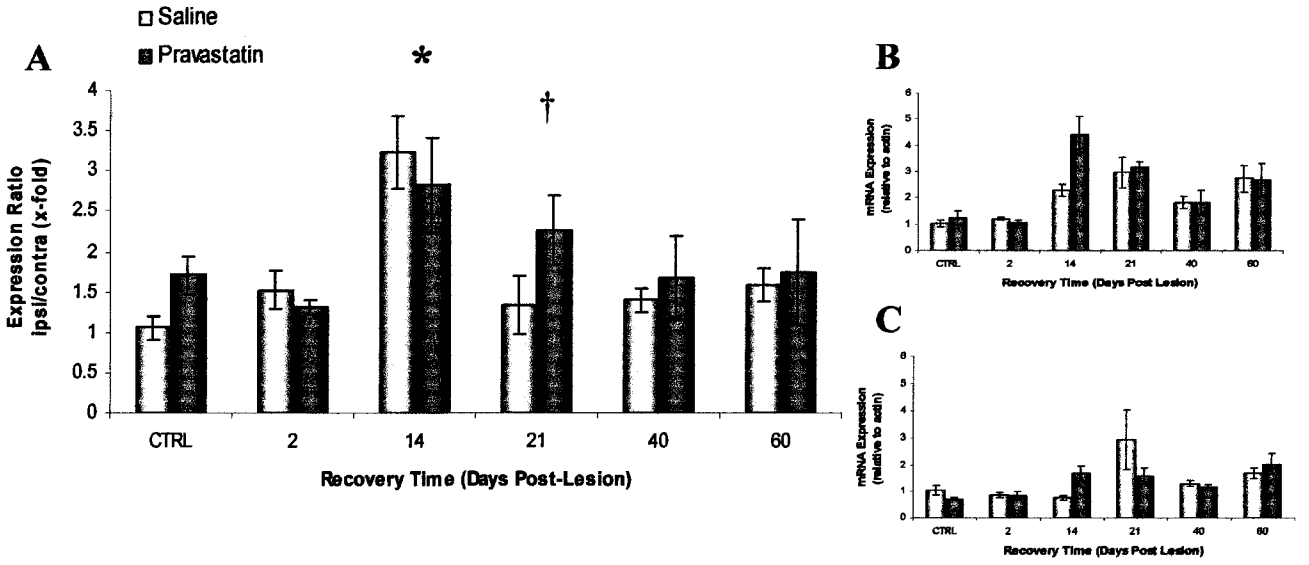

FIGURE 7

Figure 8: Quantification of ABCG1 mRNA transcript expression in the hippocampus of sham-operated and lesioned mice following 2, 14, 21, 40 and 60 DPL.

(A) Expression of ABCG1 mRNA transcript levels (normalised relative to β-actin) in the ipsilateral hippocampus relative to contralateral values. (B, C) Expression of ABCG1 mRNA transcripts as measured in the ipsilateral and contralateral hippocampus respectively. No significant changes were observed in the expression of ABCG1 mRNA in the ipsilateral hippocampus relative to the contralateral at any of the time points studied. In addition, pravastatin treatment failed to affect the expression of ABCG1 in either sham-operated or lesioned mice. All values are expressed as group means ± SEM (n=5 per group).

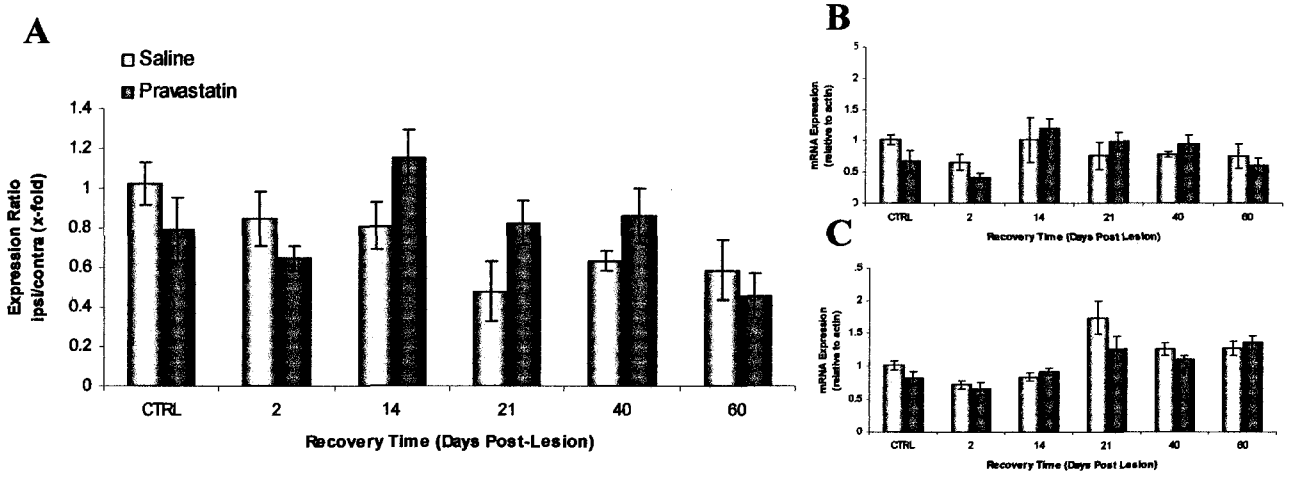

FIGURE 8

Figure 9: Quantification of apoE(LDL)R mRNA transcript expression in the hippocampus of sham-operated and lesioned mice following 2, 14, 21, 40 and 60 DPL. (A) Fold-change in apoE(LDL)R mRNA transcript levels (normalised relative to β-actin) in the ipsilateral hippocampus expressed relative to contralateral values. (B, C) Measures of apoE(LDL)R mRNA expression, relative to β-actin, in the hippocampus ipsilateral and contralateral to, lesion location respectively. No significant changes in the expression of apoE(LDL)R mRNA were observed in the hippocampus ipsilateral to uECL location at any of the time-points studied. Pravastatin treatment was observed to result in a transient but significant decrease in apoE(LDL)R mRNA expression in the ipsilateral hippocampus at 21 DPL (*$p<0.05$) when compared to both sham-operated and saline-treated mice sacrificed at the same time point. In addition, apoE(LDL)R mRNA expression was found to be significantly increased in sham-operated animals receiving pravastatin treatment when compared to saline treated sham-operated animals. Values represent group means ($n=5$) ± SEM.

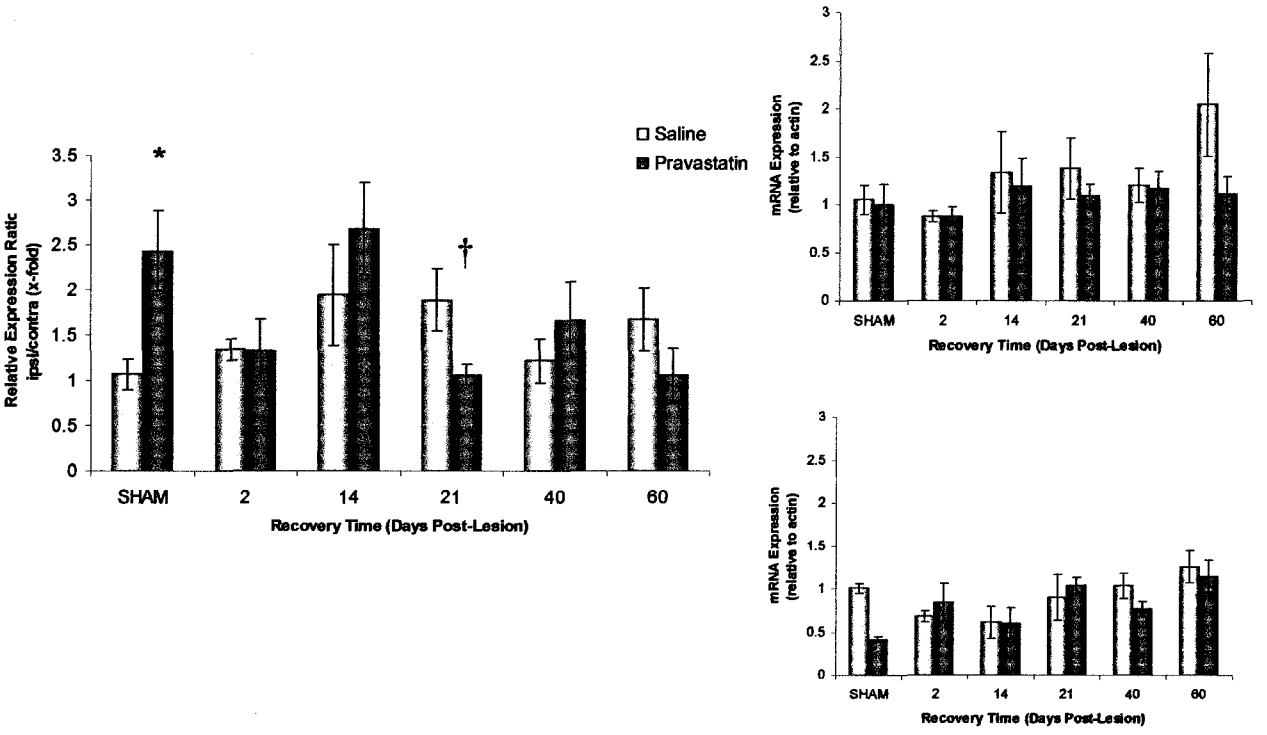

Figure 9: Quantification of apoE(LDL)R mRNA transcript expression in the hippocampus of sham-operated and lesioned mice following 2, 14, 21, 40 and 60 DPL.

Figure 10: Quantification of HMGCoA-R mRNA transcript expression in the hippocampus of sham-operated and lesioned mice following 2, 14, 21, 40 and 60 DPL. (A) Fold-change in HMGCoA-R mRNA transcript levels (normalised relative to β-actin) in the ipsilateral hippocampus expressed relative to contralateral values. (B, C) Measures of HMGCoA-R mRNA expression, relative to β-actin, in the hippocampus ipsilateral and contralateral to, lesion location respectively. No significant changes in the expression of HMGCoA-R mRNA were observed in the hippocampus ipsilateral to uECL location at any of the time-points studied. Pravastatin treatment was observed to result in a transient but significant decrease in HMGCoA-R mRNA expression in the ipsilateral hippocampus at 21 DPL (*$p<0.05$) when compared to both sham-operated and saline-treated mice sacrificed at the same time point. No significant differences were observed in HMGCoA-R mRNA expression between pravastatin and saline treatments in sham-operated animals. Values represent group means (n=5) ± SEM.

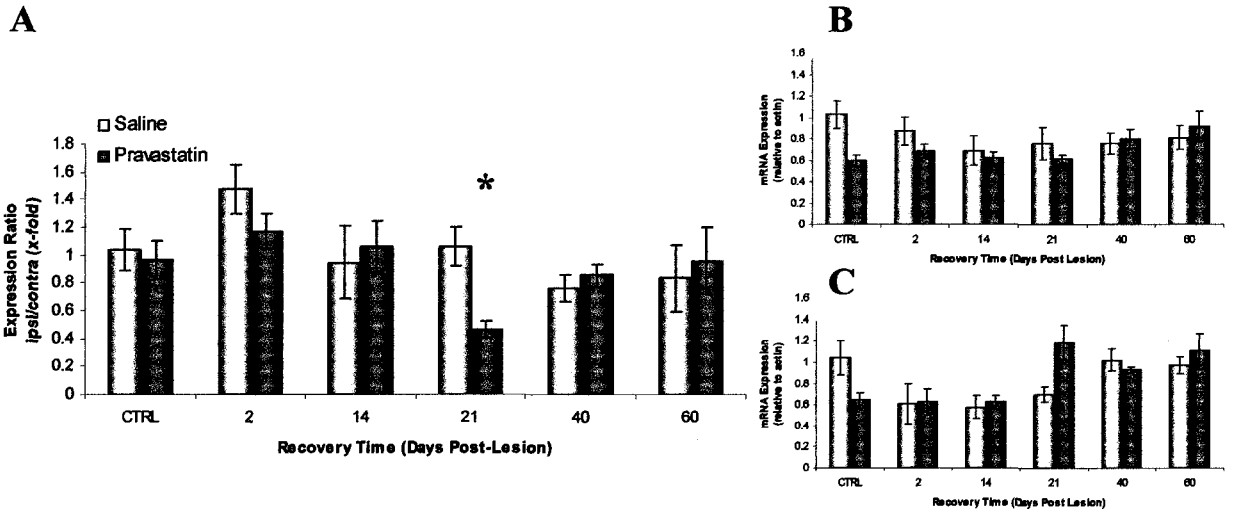

FIGURE 10

Figure 11: Quantification of ABCA1 protein expression in the hippocampus of sham-operated and lesioned mice after 2, 14, 21, 40 and 60 DPL.

Fold-changes in ABCA1 protein expression (normalised against β-tubulin) in the hippocampus ipsilateral to lesion site relative to the contralateral hippocampus as determined by immunoblot analysis are illustrated in (A). (B, C) Measures of ABCA1 protein expression, relative to β-tubulin, in the hippocampus ipsilateral and contralateral-to lesion location respectively. (D) Representative immunoblot results for sham and uECL mice illustrating ABCA1 and β-tubulin expression in the ispilateral (lanes 1-12) and contralateral (lanes 13-24) hippocampi of saline- (odd-numbered lanes) and pravastatin-(even-numbered lanes) treated mice. A significant (approximately two-fold), increase in the expression of *Abca1* protein was observed in the hippocampus ipsilateral to uECL location at 14 DPL in saline-treated animals relative to sham-operated mice (*$p<0.01$). Interestingly, treatment with pravastatin was observed to attenuate the up-regulation of ABCA1 protein expression observed at 14 DPL, with this group demonstrating significantly lower ABCA1 protein levels when compared to saline-treated animals sacrificed at the same time-point (†$p<0.05$). All values are expressed as the mean optical density ± SEM (n = 5 per group).

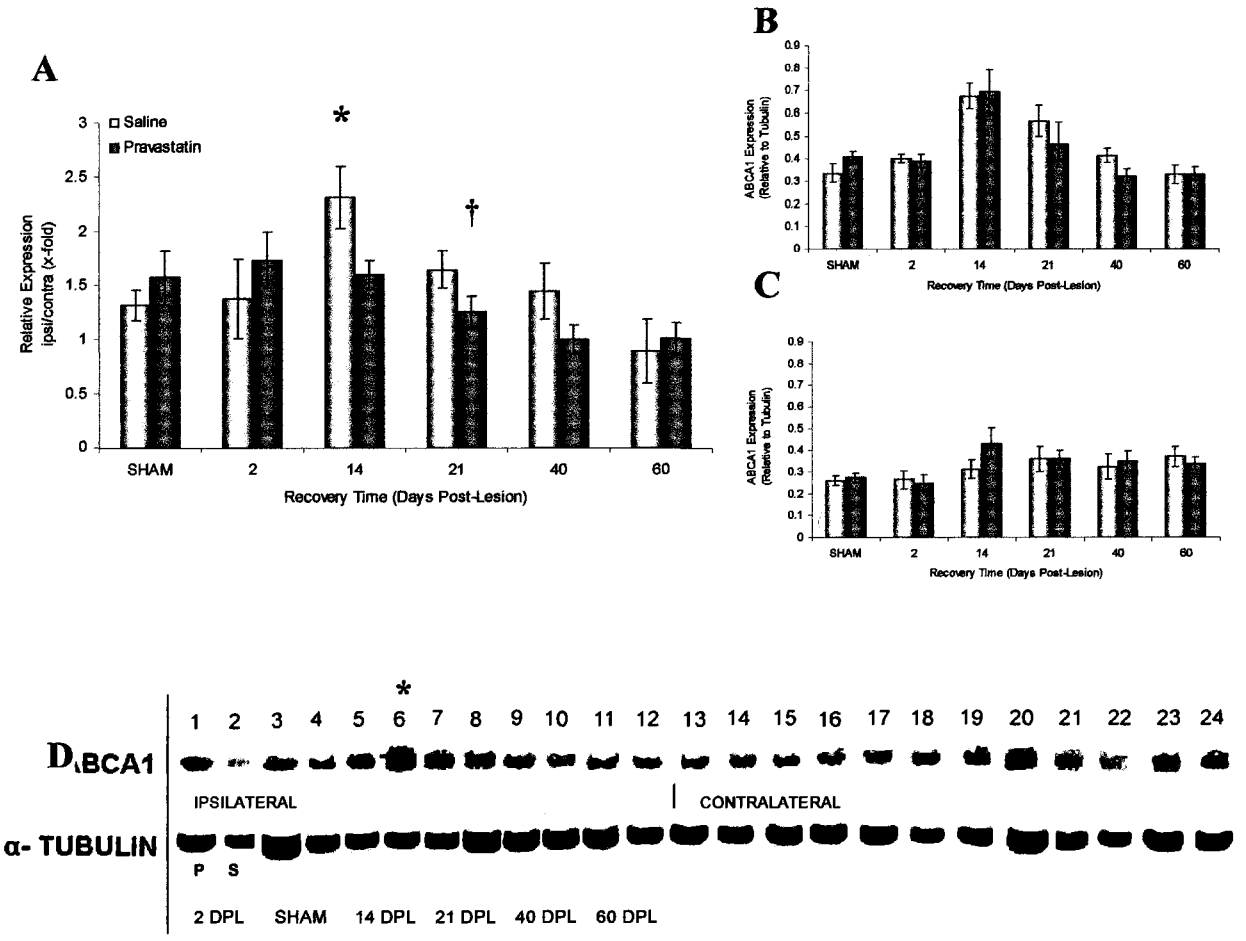

FIGURE 11: Quantification of ABCA1 protein expression in the hippocampus of sham-operated and lesioned mice after 2, 14, 21, 40 and 60 DPL.

Figure 12: Quantification of HMGCoA-R functional activity in the liver following uECL in pravastatin and saline treated mice.

Determination of HMGCoA-R enzymatic activity in the liver (expressed as nmole of HMGCoA transformed per minute per μg of total protein) was conducted by HPLC as described in Materials and Methods. The affects of pravastatin treatment on the functional activity of HMGCoA-R were found to be different in sham-operated and lesioned mice. Specifically, HMGCoA-R activity was significantly decreased (approximately 50%; †$p<0.01$) in sham-operated mice receiving pravastatin-treatment when compared to those receiving placebo. Differences in the HMGCoA-R activity between saline and pravastatin-treated animals were not observed in lesioned mice at any of the time-points studied. Both saline and pravastatin-treated mice demonstrated similar patterns of HMGCoA-R activity following uECL. A significant (*$p<0.01$) reduction in HMGCoA-R activity was observed in the liver of both drug treatment groups at 2, 21, 40 and 60 DPL when compared to sham-operated mice.

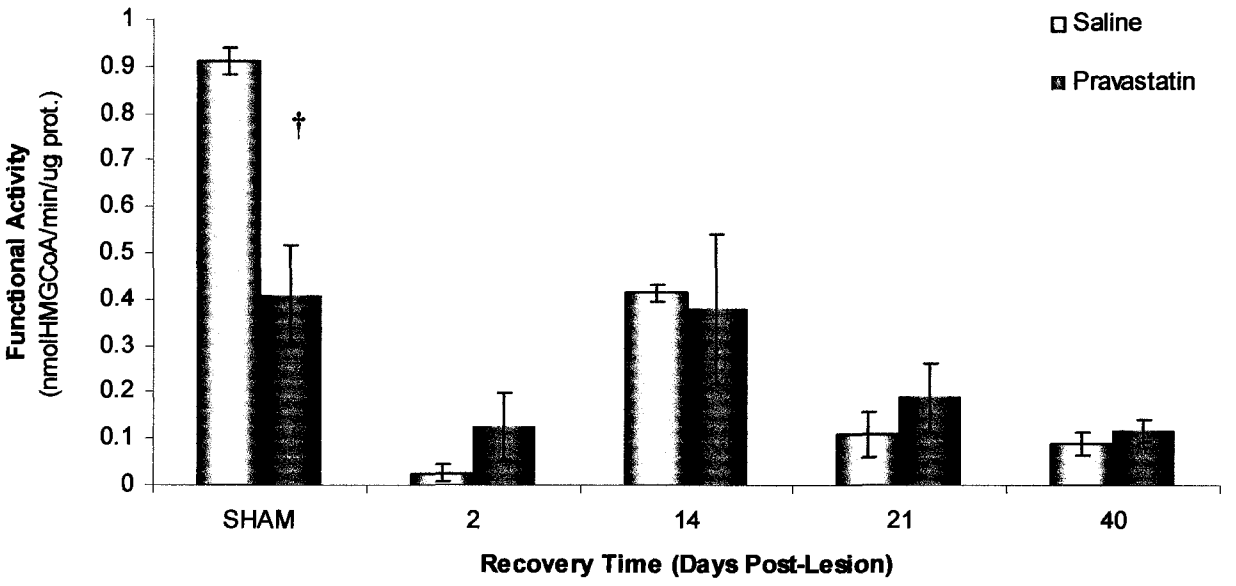

FIGURE 12: Quantification of HMGCoA-R functional activity in the liver following uECL in pravastatin and saline-treated mice.

Figure 13: Quantification of HMGCoA-R functional activity in the parieto-temporal cortex following uECL in pravastatin and saline-treated mice.

The functional activity (expressed as nmole of HMGCoA transformed per minute per μg of total protein) of HMGCoA-R in the parieto-temporal cortex (PT-Ctx) ipsilateral to lesion site was conducted by HPLC. It was observed that HMGCoA-R activity was dynamically regulated in the PT-Ctx following uECL. Specifically, a significant up-regulation in HMGCoA-R activity was observed at 2 and 40 DPL when compared to sham-operated animals (*$p<0.05$) with values remaining near control levels at 14 and 21 DPL. Pravastatin treatment was not found to affect the functional activity of HMGCoA-R in the ipsilateral PT-Ctx in either the sham-operated or lesioned mice at any of the time-points studied. All values are expressed as the mean ± SEM (n=3 per group).

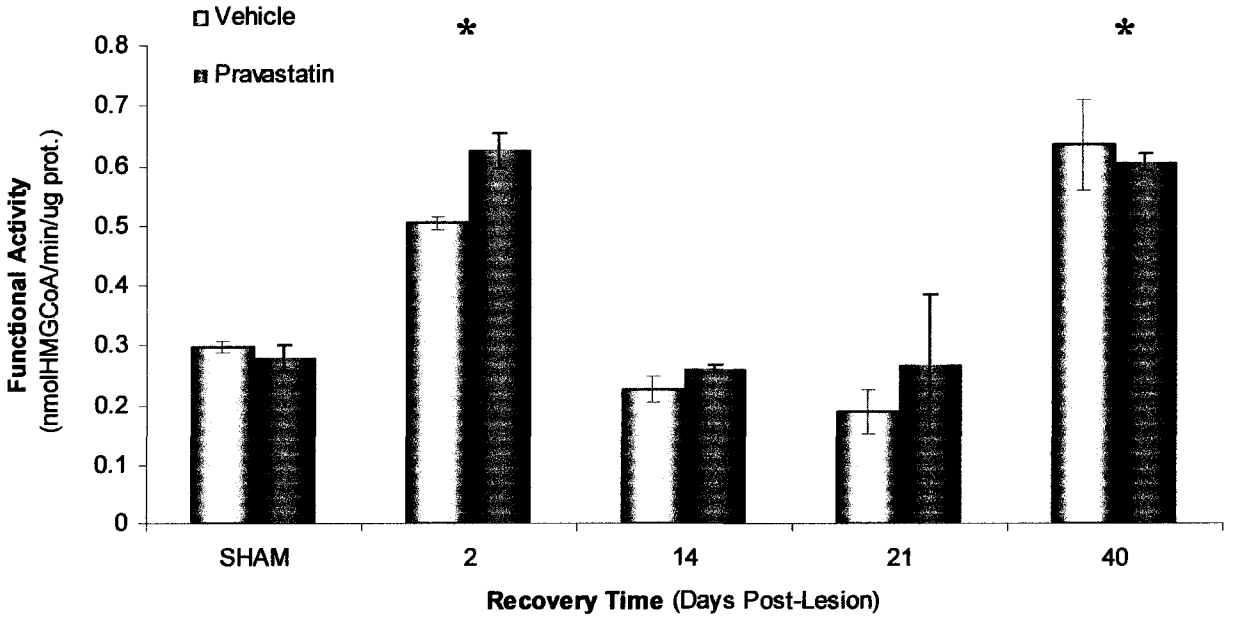

FIGURE 13

CHAPTER 3

DISCUSSION OF RESULTS

3. DISCUSSION

The accumulating evidence to suggest that apoE-containing glial-derived lipoparticles support reactive sprouting and synaptogenesis (Poirier, et al., 1991; Mauch, et al., 2001; Hayashi, et al., 2004) prompted this investigation into a potential role for the ABC transporters A1 and G1 in the regulation of cholesterol efflux during synaptic remodelling. These transporters have been demonstrated to be expressed throughout the CNS (Tachikawa, et al., 2005) where they have been proposed to mediate cholesterol efflux from neural cells (Hirsch-Reinshagen, et al., 2003; Wahrle, et al., 2004; Karten, et al., 2006) thereby influencing apoE lipidation. However their respective functions in the regulation of cerebral cholesterol homeostasis are not yet well described *in vivo*.

3.1. CHOLESTEROL HOMEOSTASIS IS DYNAMICALLY REGULATED DURING SYNAPTIC REMODELING

Here evidence is presented which demonstrates that following uECL, ABCA1 is up-regulated independently of ABCG1 in the deafferented hippocampus, suggesting a role for this transporter in the regulation of cholesterol efflux and formation of lipoparticles during reactive sprouting and synaptogenesis in the deafferented hippocampus.

3.1.1. Regulation of ABC transporters following uECL

The uECL paradigm is a well described model of reactive sprouting which enables the analysis of cholesterol regulation during synaptic sprouting responses distal to the lesion location. Previous studies have demonstrated that cholesterol homeostasis is

dynamically regulated in the deafferented hippocampus and mechanisms favouring the recycling of cholesterol and lipids from dead or dying neurons towards surviving cells are induced following injury and support neural remodelling (Poirier, et al., 1991; de Chaves, et al., 2001; White, Nicoll, & Horsburg, 2001; Goritz, Mauch, & Pfreiger, 2005). In order to investigate the time course and extent of synaptic remodelling following uECL, cholinergic sprouting was assessed in the OML of the dentate gyrus through the analysis of AChE staining density. A significant increase in the density of AChE staining was observed in the OML of the dentate gyrus ipsilateral to lesion location at 14 DPL and remained increased at 21DPL. These results therefore suggest that significant reactive sprouting and synaptogenesis of cholinergic fibres occurs at this time and represents a phase of active synaptic remodelling in response to denervation. These findings are in account with previous studies that suggest that in the mouse the active remodelling of dendrites and synapses occurs between six and thirty days following uECL (Blain et al., 2004; Champagne, Rochford, & Poirier, 2005; Blain et al., 2006)

In this study the mRNA expression of ABCA1, and not ABCG1, was observed to be significantly up-regulated at 14DPL, when compared to sham-operated animals. This increase was transient and was observed to return to baseline by 21 DPL. This increase was observed coincided with a significant, well-described up-regulation in APOE mRNA expression. Interestingly, ABCG1 mRNA expression in the hippocampus was not observed to be significantly affected by uECL. Taken together, these results demonstrate that ABCA1, and not ABCG1, mRNA expression is dynamically regulated in response to uECL along with APOE, and therefore suggests that ABCA1 and not ABCG1 may be important in the mediation of cholesterol efflux from glia cells and the lipidation of APOE in response to injury. In addition, ABCA1 protein expression was observed to be

similarly up-regulated at 14DPL, providing further supporting a role for this transporter in the regulation of cholesterol efflux during synaptic remodelling.

While ABCA1 has been demonstrated to mediate the initial lipidation of APOE, the affinity of this transporter to bind APOE is inversely proportional to the extent of APOE lipidation (Remaley, et al., 2001; Favari, et al., 2004) thus, the activity of ABCG1 is believed to mediate the formation of mature HDL from the nascent pre-β-HDL generated by ABCA1 (Vaughan & Oram, 2005; Gelissen, et al., 2006). As ABCG1 mRNA was not observed to be influenced during recovery from uECL, it may be proposed that the transport of lipids from glia toward neurons is facilitated through the formation of partially lipidated APOE and not through mature HDL-like lipoparticles. However, this study failed to examine the expression of the ABCG1 transporter protein and, while mRNA transcription was not significantly increased, it remains possible that ABCG1 protein levels may be increased similarly to ABCA1, perhaps through the inhibition of protein degradation. In addition, the relevance of ABCG1 potential to function as either a homo- or heterodimer may contribute to the differences in ABCA1 and ABCG1 expression observed in this study. Other members of the ABCG family of transporters such as ABCG4 have been demonstrated to dimerise with ABCG1 to form functional transporters (Cserepes, et al., 2004). Therefore the examination of the expression of other ABCG members during reactive sprouting may provide insights into this possibility. Regrettably, to date the investigation of ABCG1 protein expression has been impeded by the lack of commercially available specific antibodies, a limitation that will no doubt soon be rectified.

3.1.2. Regulation of cholesterol synthesis following uECL

In addition to investigating mechanisms regulating intercellular cholesterol transport, this study also considered the effect of uECL on the regulation of cholesterol biosynthesis. HMGCoA-R functions in the regulation of the first committed and rate-limiting step in the *de novo* synthesis of cholesterol and previous studies have demonstrated that during reactive sprouting responses, cholesterol synthesis is inhibited in favour of cholesterol recycling (Goodrum, 1990; Goodrum, et al., 1995). While no changes in HMGCoA-R mRNA expression levels were observed following uECL, significant changes in HMGCoA-R functional activity were observed. Specifically a significant increase in HMGCoA-R activity was observed in the PT-Ctx ipsilateral to the site of uECL. This increase in functional activity was observed early in recovery during the degenerative phase of synaptic remodelling (2DPL). This increase was transient, with values returning to within baseline at 14 and 21 DPL; however levels were again significantly increased at 40DPL. These results suggest that although HMGCoA-R activity is not regulated at the level of mRNA transcription in response to uECL in the brain, posttranscriptional regulatory mechanisms may significantly influence enzymatic activity during reactive sprouting.

Previous studies suggest that HMGCoA-R activity is down-regulated in during recovery from trauma (Goodrum, 1990), as cholesterol demands are being preferentially met through the apoE/LDL receptor uptake pathway involved in lipid recycling. It is important to note that measures of HMGCoA-R activity were taken in PT-Ctx homogenates. While the EC is believed to share reciprocal projections with the PT-Ctx via the perirhinal and postrhinal cortices (Johnston & Amaral, 1998), it does not provide the primary afferentation to this region. Indeed, the uECL may be considered to result in

the loss of a neural target for PT-Ctx neurons as opposed to the deafferentation experience by hippocampal neurons. This difference may underlie the discretions between the results presented here and those presented in previous studies (Goodrum, 1990). It is possible that the up-regulation in HMGCoA-R activity observed during the degenerative phase may represent an early up-regulation in isoprenoid synthesis or other inflammatory signalling cascades in regions upstream of the lesion location. However the generalisation of the results presented here regarding the functional activity of HMGCoA-R in the PT-Ctx are limited by the small sample sizes (n=3) and the unilateral measures employed. In order to determine if the synthesis of cholesterol is differentially regulated in the cortex and hippocampus following uECL further studies must be conducted with larger groups and include the bilateral examination of HMGCoA-R activity in cortex and hippocampus.

Interestingly, a significant change in hepatic HMGCoA-R functional activity was observed following uECL. Specifically it was noted that HMGCoA-R activity was significantly inhibited in the liver following uECL. While a transient increase in HMGCoA-R activity was observed at 14DPL when compared to measures taken at 2DPL, these values remained significantly lower that those of sham-operated animals and failed to return to baseline by the end of this study (60DPL). While limited by the small sample sizes employed, these results provide a compelling indication that CNS trauma may influence the regulation of somatic cholesterol homeostasis. Further studies are required to determine the mechanisms through which such influence may be exerted. It is conceivable that CNS trauma results in the promotion of cholesterol efflux from the CNS into periphery, either through the activity of ABC transporters or through the generation of 24(S)-hydroxycholesterol, thereby influencing hepatic cholesterol homeostasis.

3.1.3. Regulation of apoE(LDL)R expression following uECL

It has been proposed that in response to injury, cholesterol and lipids released from the dying and degenerating terminals are engulfed and processed by proximal astrocytes which then, once metabolised, complex this lipid pool with apoE to be directed towards regenerating terminals (Poirier, Baccichet, et al., 1993; Poirier, 1994). The uptake of these lipoparticles by neurons has been demonstrated to be facilitated through apoE(LDL)R-mediated endocytosis via the clatherin-coated pit pathway (Beffert, et al., 1998; Havel, 1998; Xu, et al., 1998). In order to assess the role of apoE(LDL)R in the recycling of cholesterol in the deafferented hippocampus in response to uECL the mRNA expression was quantified by real-time RT-PCR. While results presented here were not found to reach significance, a slight increase in apoE(LDL)R mRNA expression was noted in the denervated hippocampus at 14DPL when compared to sham operated animals. The results are consistent with observations made in uECL rats which exhibit marked I^{125}-LDL binding in the deafferented molecular layer of the dentate, ipsilateral to the lesion site at 14DPL, during the early phase of reinnervation (Poirier, et al., 1993). Again, this period corresponds to the early phase of reactive sprouting and synaptogenesis. Recent studies suggest that other members of the apoE(LDL)R family of proteins may be expressed by neurons and may participate in the mediation of endocytosis of glial-derived lipoparticles. Studies conducted by Petit-Turcotte and colleagues have suggested a role for the lipoparticle receptor apoER2 in the regulation cholesterol homeostasis and mediation of synaptic plasticity following uECL (Petit-Turcotte, et al., 2005). Deletion of apoER2 was observed to impair compensatory sprouting and synaptic remodelling in the deafferented hippocampus and therefore suggests that other receptors may participate with apoE(LDL)R in the mediation of

cholesterol uptake by neural cells during reactive sprouting responses (Petit-Turcotte, et al., 2005).

3.2. SUFFICIENCY OF LOW-DOSE PRAVASTATIN TREATMENT TO INFLUENCE CHOLESTEROL HOMEOSTASIS AND SYNAPTIC REMODELING

The increasing evidence supporting a central role for the regulation of cholesterol homeostasis in the maintenance of neural integrity has prompted investigations into the effects of pharmacological manipulation of cholesterol regulation of CNS function. While there is evidence to suggest that the inhibition of cholesterol biosynthesis through statin therapy may confer neuroprotection, the mechanisms through which statins exert such effects remain unclear. In addition, many studies are limited in their clinical-relevance by the supraphysiological concentrations of the drug applied (Sato-Suzuki, & Murota, 1996; Tanaka *et al.,* 2000). In order to address this, this thesis also examined the effects of a clinically relevant (0.6mg/kg/day) pravastatin regimen on the markers of cerebral cholesterol homeostasis and reactive sprouting responses following uECL previously discussed.

3.2.1. Pravastatin treatment influences cholesterol biosynthesis

Interestingly, the administration of clinical-dose pravastatin treatment was observed to significantly influence the mRNA expression of HMGCoA-R in response to uECL when compared to saline-treated animals. Specifically, a significant decrease in the expression HMGCoA-R mRNA relative to the housekeeping gene actin was observed in the hippocampus ipsilateral to the lesion location at 21 DPL. While pravastatin functions through the competitive inhibition of HMGCoA-R, these results suggest that

treatment with this statin may induce changes in the regulation of gene transcription, providing a second, potentially longer-term mechanism, through which statins may inhibit cholesterol biosynthesis.

As previously mentioned, HMGCoA-R is subject to extensive regulation and thus measures of mRNA transcript expression may not reflect the functional activity of this enzyme. Here we demonstrate that the low-dose pravastatin treatment applied in this study was sufficient to significantly inhibit the activity of hepatic HMGCoA-R by approximately fifty percent in drug-treated sham-operated animals when compared to saline-treated sham-operated mice. This effect is comparable to those observed in the clinical application of statins for the treatment of hypercholesterolemia (Jungnickel, Cantral, & Maloley, 1992). Interestingly, the difference in hepatic HMGCoA-R activity observed between saline and pravastatin treated animals was lost following uECL. At all time points studied, pravastatin-treated animals demonstrated levels of HMGCoA-R functional activity that were comparable to saline-treated animals sacrificed at the same time-point. These results suggest that in the absence of CNS trauma, low-dose pravastatin treatment is significant to inhibit the functional activity hepatic HMGCoA-R. However the mechanisms which underlie the dynamic regulation of hepatic HMGCoA-R in response to uECL are not influenced by the pravastatin therapy applied. In addition, the effects of low-dose pravastatin treatment on the functional activity of HMGCoA-R in the PT-Ctx were also investigated. No differences were observed between pravastatin and saline treated animal at any of the time points studied, suggesting that the statin therapy applied in this study was insufficient to influence the functional activity of HMGCoA-R in the brain, even in the presence of a breech to the blood brain barrier induced as a result of the lesion technique. Again, it is important to note that measures of HMGCoA-R

activity were made in the PT-Ctx and may therefore represent changes that are distinct from those observed in the deafferented hippocampus. Therefore further studies must be examined to determine if the change in HMGCoA-R mRNA expression observed in the deafferented hippocampus can be translated to a change in the functional activity of HMGCoA-R in this brain region.

3.2.2. Effects of statin treatment on expression of ABC transporters following uECL

Previous studies examining the effects of statin treatment of the regulation of cholesterol efflux have primarily focused on macrophage cell cultures with conflicting results (Wong, Quinn, & Brown, 2004; Argmann, et al., 2005; Wong, et al., 2007). As statins function in the inhibition of cholesterol biosynthesis it is reasonable to hypothesise that mechanisms regulating the efflux of cholesterol would be similarly inhibited as intracellular cholesterol stores cannot be readily accommodated by *de novo* synthesis. However, long-term treatment with statins may also result in the promotion of mechanisms regulating cholesterol recycling and, in response to injury the net result may be an increase in cholesterol efflux as glial intracellular cholesterol pools accumulate.

The results of this study demonstrate that low-dose pravastatin treatment was sufficient to significantly influence ABCA1 mRNA and protein expression but not the expression of ABCG1. Specifically it was observed that the up-regulation in ABCA1 protein expression observed in saline treated animals at 14DPL was attenuated in mice receiving pravastatin therapy. Thus pravastatin treated mice failed to demonstrate the dynamic regulation of ABCA1 protein expression observed in saline treated mice following deafferentation of the hippocampus through uECL. Interestingly, pravastatin treatment was observed to have a different effect on the expression of ABCA1 mRNA

transcripts following uECL. In particular, ABCA1 mRNA levels were observed to be significantly increased at 21DPL in following pravastatin therapy when compared to saline-treated animals. These results suggest that clinically relevant pravastatin therapy may differentially influence the expression of ABCA1 mRNA and protein with implications for the regulation of cerebral cholesterol homeostasis. Interestingly, APOE mRNA expression was unaffected by the application of statin therapy.

3.2.3. Effects of statin treatment on apoE(LDL)R expression following uECL

Although apoE(LDL)R mRNA expression was not observed to be significantly affected by uECL, treatment with pravastatin was observed to significantly influence apoE(LDL)R mRNA levels in the hippocampus. Specifically, apoE(LDL)R mRNA levels were observed to be significantly increased in statin-treated sham-operated animals when compared to saline-treated sham-operated mice. This up-regulation of apoE(LDL)R expression may indicate and increased emphasis on mechanisms regulating the endocytosis of exogenous cholesterol as biosynthesis is impaired. However to contrast, at 21 DPL pravastatin treatment was associated with a significant down-regulation of apoE(LDL)R expression. As other members of the LDLR family, have been demonstrated to participate in reactive sprouting responses (Petit-Turcotte, et al., 2005), further studies investigating the effects of low-dose statin therapy on the expression of other lipoparticle receptors must be conducted to examine this differential effect of pravastatin treatment during reactive sprouting when compared to sham-operated animals. Previous studies conducted in rat hippocampal slices by Poirier and colleagues demonstrated a significant increase in the binding of LDL in the outer molecular layer of the dentate during the reinnervation phase. This increase in binding has been proposed to

reflect an increased mobilisation of LDLRs to the cell surface (Poirier, et al., 1993). Further studies must be conducted to determine whether clinically-relevant pravastatin treatment influences the mechanisms regulating the mobilisation of LDLR to the surface of regenerating processes during synaptic regeneration.

3.2.4. Effect of pravastatin treatment on reactive sprouting responses following uECL

While the results presented thus far demonstrate that the administration of a clinically relevant pravastatin regimen is sufficient to influence mechanisms regulating cerebral cholesterol homeostasis, this study also investigated whether such treatments were sufficient to influence the compensatory reactive sprouting of cholinergic fibres in the deafferented hippocampus. No significant differences were observed between the density of AChE staining between saline and pravastatin treated animals, suggesting that the effects of such statin treatments on cholesterol homoeostasis were insufficient to influence the extent of synaptic remodeling. However, it is important to note that the analysis of heterotypic sprouting only represents one portion of synapses undergoing active remodeling. Additional histochemical analyses, including the measurement of total synaptic density assessed by synaptophysin immunoreactivity may uncover differences in the extent or time-course of synaptic remodeling that are not addressed in this study.

CONCLUSION

By facilitating the transportation of cholesterol and lipids, glial-derived lipoparticles have been demonstrated to support neuronal growth and synaptogenesis (Poirier, et al., 1991; Mauch, et al., 2001; Hayashi, et al., 2004). Perturbations in the regulation of cholesterol homeostasis have been demonstrated to compromise neural integrity and are observed in several neurodegenerative conditions including LOAD (Poirier, Davignon, et al., 1993; Strittmatter, et al., 1993; Ledesma, et al., 2003; Crameri, et al., 2006). The importance of mechanisms regulating the intercellular transport of cholesterol is underlined by the compelling association between polymorphisms in APOE and the risk for AD. Although there is evidence to suggest that glial-derived APOE-containing lipoparticles are essential in the neural regeneration observed following trauma, the mechanisms through which the assembly of HDL in the CNS is regulated are not clearly understood. To further the association between cholesterol regulation and nervous system integrity, there is growing evidence from epidemiological, clinical and experimental studies to suggest that statin treatment may confer cognitive benefits, decreasing the prevalence of AD (Wolozin *et al.,* 2000; Jick *et al.,* 2000; Fassbender *et al.,* 2001) and increasing recovery from cerebral ischemia (Daimon *et al.,* 2004; Yrjanheikki *et al.,* 2005; Nagaraja, *et al.,* 2006).

As a result, there is growing interest into the mechanisms regulating cerebral cholesterol homeostasis and the effects of pharmacological manipulation of cholesterol homeostasis on neural physiology. Here we present evidence to support an active role for the ABC transporter ABCA1 but not ABCG1 in the dynamic regulation of cholesterol

homeostasis observed following uECL. In addition we demonstrate that the administration of clinically-relevant (0.6mg/kg/day) pravastatin therapy is sufficient to influence mechanisms regulating cerebral cholesterol homeostasis, most notably the expression of ABCA1 and the apoE(LDL)R.

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APPENDIX