The effect of non-steroidal anti-inflammatory drugs on *in vitro* glial apolipoprotein E expression – implications for the mechanisms and treatment of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a neurological disorder characterized by plaque deposition and an elevated immune response. Epidemiological studies have shown that use of non-steroidal anti-inflammatory drugs (NSAIDs) by the elderly is associated with a decreased relative risk and a delayed onset of AD. Moreover, the apolipoprotein E (apoE) gene has been proven to be a risk factor for AD with apoE ε4 AD patients having been found to show lower levels of brain apoE. In the present study, treatment of primary rat mixed glial cell cultures with indomethacin, aspirin, and interleukin-1β resulted in significant increases in extracellular apoE protein. Furthermore, treatment of primary rat astrocyte cell cultures with aspirin, interleukin-6, and a cyclooxygenase-2 selective aspirin derivative was found to result in increased levels of apoE. Consequently, NSAID-induced increases in apoE protein may enhance apoE-mediated immunosuppression and compensatory synaptic plasticity, potentially resulting in decreased risk and delay of disease onset.

Résumé

La maladie d'Alzheimer (MA) est une pathologie neurologique caractérisée par la déposition de plaques séniles et une réponse immunitaire accrue. Des études épidémiologiques ont démontré que l'utilisation d'anti-inflammatoires non-stéroidiens réduit les risques de développer la maladie et retarde l'apparition des symptômes. De plus, le gène muté de l'apolipoprotéine E (apoE) appelé la forme e4, représente un facteur de risque pour la MA où les niveaux d'apoE cérébraux sont significativement réduits chez les porteurs de l'allèle anormale.

Des cultures primaires de cellules gliales mixtes ou pures de rat ont été utilisées pour examiner l'effet biologique de différents agents anti-inflammatoires sur la neurobiologie de l'apoE. Il en résulte une hausse significative des niveaux extracellulaires d'apoE. Cette hausse marquée de l'apoE cérébrale par les anti-inflammatoires non-stéroidiens pourrait, dans un second temps, stimuler l'immunosuppression médiée par l'apoE et ainsi promouvoir la plasticité synaptique compensatoire dans la maladie d'Alzheimer.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles, neuritic plaques composed of beta-amyloid (Aβ), dystrophic neurites, and cortical atrophy[1-3]. One key aspect of AD pathogenesis has been hypothesized to involve the apolipoprotein E (apoE) gene. Unlike rodents, three separate human apoE isoforms have been identified, namely apoE ε2, ε3, and ε4[4,5]. Individuals bearing two copies of the apoE ε4 allele have been found to be at significantly greater risk for AD[6]. In addition, apoE ε4 AD patients have been reported to exhibit lower brain apoE levels[7]. Consequently, reduced levels of apoE may significantly inhibit apoE-mediated lipid transport and homeostasis, synaptic plasticity, and Aβ clearance[8-11].

In recent years, many researchers have focused their efforts on the role of the immune system as an essential component of AD pathogenesis[12-15]. Studies have demonstrated high levels of microglial activation and clustering around AD plaques[16-18], as well as elevated levels of complement proteins, inflammatory cytokines, and acute phase proteins in human AD brains[13,15,19-22]. Thus, it has been hypothesized that a continuous host immune response may act in conjunction with environmental, hormonal, and genetic factors, thereby promoting inflammation, tissue damage, and clinical expression of AD symptomology[23-25]. Consistent with this hypothesis, epidemiological studies have demonstrated that there exists an inverse association between non-steroidal anti-inflammatory drug (NSAID) use and AD[26]. Specifically, use of NSAIDs by the elderly has been associated with a decrease in relative risk and a delay in AD onset[27-29].

Although the precise mechanisms underlying NSAID neuroprotection remain unclear, many have speculated that the benefits of NSAID use in AD may be the result of: (1) general suppression of a self-sustained AD immune response[30], (2) reductions in circulating Aβ derived from platelets[26], (3) reduced glutamatergic excitotoxicity, a process linked to cyclooxygenase (COX)-dependent events[29], and (4) free radical quenching[31]. Nevertheless, the majority of research has focused primarily on either direct inflammatory intervention or indirect cascade effects following immune modulation; non-immune mechanisms remain largely unexplored.

Although both the immune system and apoE have been extensively studied, the potential interaction between these two factors in the context of AD pathology remains unclear. Prior work has established a bi-directional relationship between apoE and the immune system with apoE showing potential immunosuppressive properties both *in vitro*[32-36] and *in vivo*[37,38] and inflammatory

mediators showing significant apoE regulatory effects[39-42]. In addition, epidemiological studies have revealed that the effect of NSAIDs is apoE genotype-dependent[29]. Specifically, it has been shown that the protective effect of NSAIDs is stronger in subjects lacking the apoE ε4 allele[29]

The question remains, however, as to whether the protective effects of NSAID treatment in AD are related to apoE. Based upon the bi-directional relationship between apoE and the immune system, the epidemiological link between NSAIDs and apoE, and the potential benefits of apoE in immune and lipid regulation, we hypothesize that the benefits of NSAIDs in AD may be due to an up-regulation of glial apoE production, thereby increasing the potential for compensatory synaptogenesis and immunosuppression. The objective of the current study was to examine the effects of inflammatory mediators such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), NSAIDs, and NSAID derivatives on apoE protein and mRNA regulation *in vitro*. In this paper, we demonstrate that treatment of primary rat mixed glial cell cultures with indomethacin, aspirin, and IL-1β resulted in significant increases in extracellular apoE protein. Furthermore, treatment of primary rat astrocyte cell cultures with aspirin, 17-β-estradiol, IL-6, and a COX-2 selective aspirin derivative was found to significantly increase levels of extracellular apoE. In contrast, significant decreases in apoE protein were detected following treatment of both astrocyte and mixed glial cell cultures with TNF-α and COX-2 selective indomethacin derivatives, as well as treatment of mixed glial cell cultures with an indomethacin derivative found to be inactive with regards to COX inhibition. The ability of NSAIDs, NSAID derivatives, and pro-inflammatory cytokines to significantly affect apoE regulation in glial cell cultures supports the hypothesis that apoE may play a role in NSAID neuroprotection in AD.

1.0 – Alzheimer's Disease

1.1 – Pathology

Alzheimer's disease (AD) is a neurodegenerative disorder whose pathology was first recognized by Dr. Alois Alzheimer in the early 1900's[43]. Epidemiological studies have established AD to be the most common form of dementia in adults with ~75% of all cases of dementia typically attributed to AD[44]. Age of AD onset may vary between the ages of 45 to 90 years; however, most patients demonstrate first onset between 60 to 80 years of age[45]. Clinically, AD has been characterized by memory loss, dementia, language impairments, and spatial disorientation[3,46]. Histologically, AD has been distinguished by the presence of neurofibrillary tangles, cortical atrophy, dystrophic neurites, and neuritic plaques composed of beta-amyloid (Aβ)[1-3].

Neurofibrillary tangles (NFT), key indicators of AD pathology, are composed of paired helical and straight filaments that typically occupy the cell body and dendrites[3]. Specifically, the paired filaments have been described as consisting of protofilaments arranged in a tubule formation with phosphorylated tau protein as a predominant component[3]. In addition to tau, tangles have been found to exhibit immunoreactivity for such proteins as kinase enzymes[47], ubiquitin[48], and Aβ[49]. In AD brain, NFT have been localized to the entorhinal cortex, hippocampus, locus ceruleus, nucleus basalis, and large pyramidal neurons of the neocortex[50-54]. Consequently, the prevalence of these abnormal neuronal structures has been hypothesized to promote neurodegeneration and AD symptomology[53]. As such, the functional implications of NFT have been demonstrated by studies establishing a correlation between the number of NFT present in AD brain and dementia severity[50,52,55-57].

In addition to the presence of NFT, significant selective cortical atrophy has been reported in AD. In fact, profound neuron loss has been observed in AD brain and particularly in regions such as the entorhinal cortex, hippocampus, and basal forebrain, thereby involving multiple neurotransmitter systems[58-60]. Numerous studies, however, have specifically demonstrated cortical cholinergic dysfunction in AD[60-65]. Specific cholinergic markers have been found to be significantly reduced in AD amygdala, hippocampus, and neocortex, as evidenced by significant decreases in choline acetyltransferase and acetylcholinesterase activity[61-65], as well as choline levels[66]. Furthermore, degeneration of cholinergic cells in the basal forebrain and of their innervation target cells in the amygdala, hippocampus, and neocortex has been observed[59,60,64]. Functionally, cholinergic deficits have been found to correlate with other pathological markers and dementia

severity[67,68].

Abnormal neuronal processes including axons, dendrites, and/or synaptic terminals, termed dystrophic neurites, have also been found to be widely distributed in AD brain, encroaching on regions such as the hippocampus, entorhinal cortex, superior temporal and frontal cortices, and inferior parietal cortex[50]. Dystrophic neurites have been classified primarily into neurofilament, tau, or chromogranin A-labeled forms[69]. In addition, labeling studies have established that dystrophic neurites are often associated with Aβ plaques and, in fact, may undergo a maturing process, whereby neurites convert from a neurofilament-abundant form to one primarily composed of tau[69,70]. Similar to results involving NFT load and cholinergic dysfunction, dementia severity has been correlated with dystrophic neurite density[50].

1.2 – Amyloid

Though the presence of NFT, cholinergic dysfunction, and cortical atrophy have all been identified as pathological hallmarks of AD, the presence of plaques composed of Aβ fibrils has been typically used as the defining characteristic of AD[71]. Such plaques have been isolated in numerous AD brain regions including the hippocampus, entorhinal cortex, superior frontal and temporal regions, as well as the inferior parietal region[50-52,72,73]. Aβ, a 4 kDa, soluble peptide, has been isolated as two main isoforms composed of 40 and 42 amino acid residues, respectively[71,74]. Following synthesis, Aβ may exist in monomeric, dimeric, oligomeric, or polymeric forms, the latter of which includes the fibrils isolated in AD plaques[3]. Researchers have postulated that Aβ may: (1) trigger apoptotic cascades[75], (2) promote microglial activation (i.e., IL-1 secretion)[76], and/or (3) corrupt potassium and calcium channels, thereby promoting neurodegeneration[77,78]. Consequently, the presence of pathological levels of Aβ in AD has led to the development of an Aβ hypothesis in which it has been proposed that gradual accumulation of Aβ monomers may lead to the production of oligomers and fibrils that develop into diffuse and, ultimately, mature plaques[79]. Accumulation of these plaques has been suggested to promote cellular dysfunction and trigger various pathways that contribute to neurodegeneration[79].

Cloning and complementary DNA (cDNA) sequencing of the Aβ peptide have shown that Aβ may be derived from a larger amyloid precursor protein (APP) that may function as: (1) a serine protease inhibitor[80-82], (2) a mediator of cell-cell and cell-substrate interactions[83], (3) a growth promoter[84], or (4) a type I, integral, cell-membrane receptor[74]. The human APP gene located on

chromosome 21, has been shown to code for five transcripts that are the products of alternative mRNA splicing[80-82,85,86]. Although five transcripts have been identified, the three common isoforms have been described to contain 695, 751, or 770 residues[87]. All three transcripts have been found to contain exons coding for three extracellular domains, a transmembrane domain, a cytoplasmic domain, and a region coding for the Aβ peptide[87].

Studies have suggested that, upon expression, APP may be processed such that a variety of peptide fragments, among them, a secreted APP form (sAPP), peptide p3, and Aβ[88,89], are produced. It has also been proposed that APP may be initially transported to the plasma membrane via secretory vesicles[87,90]. Subsequently, APP may undergo cleavage by an α-secretase enzyme, thereby producing a secreted APP fragment, as well as an approximately 10 kDa membrane-associated C-terminal fragment[89,91]. Thus, α-secretase-directed cleavage would preclude the synthesis of Aβ. The C-terminal fragment may then be cleaved by γ-secretase, producing an approximately 3 kDa peptide, p3[92]. In contrast, Aβ synthesis has been proposed to be the result of initial cleavage at amino acid residue 671, the N-terminus of Aβ, by β-secretase, thereby producing a large APP fragment that may be secreted, as well as a potentially amyloidogenic fragment containing the Aβ sequence[93]. Subsequent γ-secretase cleavage of the smaller fragment may then produce Aβ isoforms with 40 or 42 amino acid residues[87].

2.0 – Immune Aspects of Alzheimer's Disease

Recent evidence indicative of a protective effect of NSAIDs has led many researchers to focus on the role of the immune system in AD[27-29,94]. In human AD brain, studies have illustrated up-regulation of inflammatory mediators[20,22,95,96], complement[12,13,97,98], and microglial activation[99-102], thereby leading many to hypothesize that inflammation may contribute to neuronal damage[1,103]. Specifically, it has been proposed that inflammatory processes in the AD brain may reflect an initial scavenging system for the removal of pathological debris, the result of existing damage, as well as a later chronic, self-sustaining, auto-destructive force, whereby bystander neurons undergo immune and neurotoxic attack[1,103,104]. Consequently, continuing pathological damage, activated immune mediators, and inflammation-induced tissue damage may foster persistent inflammatory stimulation, producing a vicious cycle[1,103]. In addition to the role of inflammation in neuronal damage, it has been hypothesized that AD inflammatory mediators facilitate production of plaques, thereby perpetuating AD pathogenesis[1,103].

2.1 – Microglia

Microglia are bone marrow-derived cells that function as the macrophages of the central nervous system (CNS)[105]. Specifically, microglia are believed to be derived from systemic circulating monocytes that invade the CNS early during development, prior to the complete formation of the blood brain barrier[106]. Two subsets of microglia have been identified in adult brain: (1) a generally permanent population of resting, ramified microglia located throughout the brain parenchyma and (2) a dynamic population of perivascular, ameboid microglia, located in the basal lamina of brain capillaries and choroid plexus[107].

Functionally, microglia may present antigen to immune cells[108] and secrete a variety of pro-inflammatory cytokines and potentially neurotoxic mediators. As such, microglia have been found to produce: (1) cytokines such as IL-1β[109], IL-6[110], TNF-α[109,111], and interferon (IFN)-γ[112], (2) complement proteins[113], (3) reactive oxygen intermediates[114], (4) secreted proteases[115], and (5) nitric oxide (NO)[116]. In response to damage or infection of the CNS, microglia will activate, proliferate, and change their morphology[117-121]. Consequently, it has been suggested that microglia may participate in AD pathogenesis via microglial-mediated degradation of Aβ, perpetuation of chronic AD inflammation in response to neurodegeneration, and formation of senile plaques[102,122-125]. As such, immunohistochemical studies have identified an increase in the number of activated microglial cells and their processes clustered around and within compact Aβ deposits and senile plaques in both grey and white matter[16,18,98,100-102,126-129].

Activation of microglia has been hypothesized to be both a product of and contributing factor to AD pathogenesis. It has been suggested that microglia may play a reactionary role, in which pre-existing damage and Aβ deposition trigger microglial activation with subsequent microglial attempts to remove Aβ[100,122,130-132]. Cultured adult rat microglia have been shown to be capable of removing Aβ from serum free medium, with subsequent sequestering in phagosome-like vesicles[131]. Mouse mixed glial cell culture experiments have also confirmed the ability of microglia to internalize large quantities of Aβ microaggregates, as well as fibrillar and soluble Aβ[122,123,133]. Microglial-associated removal of Aβ has been proposed to utilize, in part, microglial class A scavenger receptors which mediate the adhesion of rodent microglia and human monocytes to Aβ fibril-coated surfaces[123,130]. In spite of the large quantities of Aβ internalized by microglia, limited Aβ degradation has been detected following uptake with only ~20% degradation

in the initial 3 days and little thereafter[122,133]. In fact, large proportions of intact Aβ have been found to be released from microglia with no further processing[133]. Although little is known as to how Aβ, both soluble and fibrillar, might avoid degradation, it has been speculated that resistance to degradation may be the result of: (1) a protease-resistant core within Aβ fibrils, (2) small Aβ fibril aggregation in late endosomes and lysosomes, or (3) intracellular Aβ fibril growth within pH friendly endosomes and lysosomes[133]. Nevertheless, in light of the sheer volume of Aβ deposition observed in AD, microglial phagocytosis and degradation would, most likely, prove ineffective[100,122].

APP- and Aβ-mediated microglial activation have also been hypothesized to significantly contribute to AD pathogenesis by potentially leading to increased expression of pro-inflammatory and neurotoxic mediators[112,124,134-136]. Specifically, *in vitro* treatment with Aβ has been found to induce macrophage and microglial activation[112,137,138] and subsequent production of IL-1[135,138-140], IL-6[135,140], TNF-α[112,139-141], chemokines[140], superoxide anions[134,141], and NO[135,137]. *In vitro* treatment of microglia with secreted APP components (sAPPα, sAPPβ) has also been shown to up-regulate microglial activation markers and expression of IL-1β and inducible nitric oxide synthase (iNOS), both potential toxic mediators[136]. Functionally, up-regulation of such inflammatory and toxic agents in culture has been associated with neuron killing, thereby confirming the capacity of microglial activation to contribute to AD cell loss and pathogenesis[112]. In addition, it has been suggested that synthesized IL-1 may play a role in senile plaque formation by up-regulating synthesis of APP mRNA, perhaps leading to increased deposition[142-144].

Although the precise mechanisms of Aβ-induced microglial activation remain largely unexplored, studies have established that Aβ may act in conjunction with other factors in order to facilitate AD inflammation[112,135]. In fact, a synergistic effect between Aβ and IFN-γ has been reported in the triggering of microglial production of reactive nitrogen intermediates and TNF-α, as well as subsequent neurite loss[112]. In addition, macrophage-colony stimulating factor (M-CSF) has been implicated in the production of IL-1, IL-6, and NO by microglial cells[135]. Simultaneous treatment of an immortalized microglial cell line with Aβ and M-CSF has been found to significantly amplify increases in IL-1 and IL-6, relative to treatment with Aβ alone[135]. Increased levels of M-CSF and M-CSF receptors have also been detected in AD brain[145,146].

In addition to the identified accessory factors, studies have established that Aβ-mediated microglial effects may be the result of complex signal transduction cascades[134,141,147]. Experiments

have revealed that Aβ treatment of cultured microglia induces expression of CD40, a receptor involved in cellular signaling and microglial activation[147]. Increased TNF-α production and induction of neuronal injury have also been detected following treatment of microglia with the CD40 ligand (CD40L)[147]. Consequently, it has been proposed that interactions between CD40 and CD40L may play a role in the activation of microglia through unresolved transduction pathways.

A variety of studies, however, have indicated that various kinase enzymes may be involved in microglial activation and the subsequent production of cytokines and superoxide anions[134,141]. Specifically, microglial exposure to Aβ fibrils has been associated with increased phosphorylation of the tyrosine kinases, Lyn, Syk, and FAK, with subsequent superoxide radical production[134]. *In vitro* treatment of human monocyte-derived macrophages with Aβ has also resulted in heightened activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) superfamily members, ERK1/2 and p38 MAPK[141]. Activation of these protein kinase pathways, through hardly fully understood, may also facilitate increased phosphorylation of transcription factors including nuclear factor-κB (NF-κB) and increased transcription of inflammatory genes[141,148].

Therefore, it has been generally hypothesized that high levels of sAPP and Aβ, as seen in AD, stimulate microglial activation and, ultimately, production of inflammatory cytokines, acute phase proteins, and toxic modulators, all of which may be responsible for neuronal damage and further Aβ deposition; however, microglial-produced inflammatory mediators may also create a positive feedback loop, whereby cytokines and subsequent neuronal damage produce further microglial activation[18,101].

2.2 - Astrocytes

Astrocytes are a subdivision of macroglia and have been subdivided into fibrous astrocytes located in white matter and protoplasmic astrocytes located in grey matter[149]. Functionally, astrocytes have been implicated in the modulation of inflammatory and immune responses in the CNS (i.e., secrete IL-1, present antigen to T lymphocytes), induction and maintenance of the blood brain barrier, and ion buffering[149]. Moreover, studies have shown that astrocytes are capable of synthesizing and releasing neurotrophic factors and such molecules as prostaglandins and lymphokines, some of which are inflammatory mediators[149].

In AD brain, an increase in the number of astrocytes and elevated astrocyte activation have suggested that these cells are involved in pathogenesis via production of cytokines and

chemokines[149-151]. Generally, this increase in the number of reactive astrocytes has not been found within Aβ deposits but rather around their periphery or near degenerating neurons[100]. As with microglia, studies have indicated that astrocytes may be activated by Aβ[150,152]. Specifically, rat astrocyte cell cultures, when treated with $A\beta_{40}$ and $A\beta_{42}$, have been found to undergo reactive morphological changes, up-regulate IL-1β and NOS mRNA, and increase NO, IL-1β, and TNF-α release[138,150,151]. Similarly, experiments with astrocyte cell lines have revealed significantly elevated IL-6 secretion and cell associated TNF-α[140]. Consequently, up-regulation of these cytokines and neurotoxins may mediate neuronal damage, enhance further Aβ-induced astrocyte activation, as well as perpetuate a positive feedback inflammatory cycle via IL-1-induced Aβ production and chronic microglial activation[108,143,153,154].

In addition to cytokines, release of chemokines by Aβ-activated astrocytes has been observed[151]. Treatment of astrocyte cell cultures with $A\beta_{40}$ has been found to increase MCP-1 and RANTES mRNA[140,151]. These chemokines are believed to be potent microglial and macrophage chemoattractants, thereby indicating that MCP-1 and RANTES may promote AD neuronal damage by recruiting microglia and macrophages with subsequent release of neurotoxins[151].

2.3 – Complement

The complement system refers to a series of plasma proteins that attack extracellular forms of pathogens[155]. In the human CNS, complement proteins are believed to be produced by neurons, astrocytes, and microglia[12,19,97,109,156,157]. In AD, the classical complement system has been implicated in pathogenesis since significant increases in C1q, C2, C3, C4, C5, C6, C7, C8, and C9 mRNAs have all been detected in post-mortem AD brain, particularly in pyramidal neurons[12,13,97]. Furthermore, up-regulation of C1q and C9 mRNA has been found to be greatest in areas of high pathology, including the entorhinal cortex, hippocampus, and mid-temporal gyrus[12].

In addition, the complement proteins, C1q, C1r, C1s, C2, C3, C4, C5, C6, C7, C8, and C9, have all been found to be more prominent in AD brains, compared to control brains[12,19]. Specifically, immunohistochemical and Western blot studies have demonstrated the presence of each complement protein in neuronal structures in AD hippocampus and temporal cortex, particularly in pyramidal neurons with intracellular tangles and senile plaques[12,19]. Immunopositive staining for the activation fragments, C3d and C4d, has also been observed in AD senile plaques, dystrophic neurites, and neurofibrillary tangles[12,98]. Furthermore, the membrane attack complex

(MAC) has been localized to dystrophic neurites and intracellular tangles in AD trans-entorhinal cortex[12].

Although much of the emphasis has been placed on the role of the classical complement pathway, recent evidence suggests that the alternative complement pathway may also contribute to chronic AD inflammation[158]. In fact, mRNA of a key pathway component, factor B, has been detected in AD frontal cortex while its cleaved derivatives, factors Bb and Ba, have been found to be significantly elevated[158]. In contrast, inhibitory regulatory factors H and I have both been found to show no accompanying increase in mRNA or protein[158].

The functional significance of complement up-regulation lies in its capacity to generate bystander cell lysis[12,19] and facilitate microglial activation[159,160]. Specifically, complement may promote inflammation through scavenger activation and lytic attack via the MAC[12,19,103]. Although these mechanisms typically target foreign pathogens, host tissue may also be vulnerable to auto-attack[19]. In addition to the direct threat that complement poses against cells, studies have established that complement proteins may indirectly perpetuate chronic inflammation. Classical complement components have been shown to activate microglia, facilitating release of pro-inflammatory mediators and promoting a destructive inflammatory loop[159]. Recent work has also implicated the complement activation peptide, C5a, in Aβ-mediated cytokine secretion from THP-1 cells[160]. Co-incubation of THP-1 cells with C5a and Aβ has been found to induce IL-1β and IL-6 secretion while also activating the transcription factor, NF-κB, a factor involved in inflammatory gene regulation[160].

Although the precise mechanisms of complement activation in AD remain unclear, many researchers have proposed that Aβ may be capable of initiating the complement cascade[161,162]. *In vitro* evidence supporting this hypothesis has established co-localization of C1q immunoreactivity with Aβ-containing senile plaques, as well as Aβ-C1q binding with subsequent complement activation[161].

Although many have focused upon the direct effects of up-regulated complement proteins, recent studies have detailed failures in compensatory complement inhibition by endogenous complement inhibitory regulators such as C1 inhibitor and CD59[163,164]. Experiments have revealed minimal increases in C1 inhibitor and CD59 in AD brain and, even then, only in regions of heavy AD pathology such as the entorhinal cortex, hippocampus, and mid-temporal gyrus[163]. Moreover, examination of AD hippocampal and frontal cortical samples has illustrated significantly reduced

levels of CD59 relative to non-demented elderly samples, indicating: (1) an inability of the AD brain to counter increases in complement activation endogenously and (2) an increased vulnerability to lytic attack[164].

2.4 – Cytokines

As with complement, up-regulation of cytokine expression in AD has been proposed to be a key mechanism of immune-associated AD pathogenesis. Among the cytokines elevated in AD brain are IL-1[20,125], S100β[20], IL-6[22,95,110], and TNF-α[96].

2.4.1 – Interleukin-1

Interleukin-1 (IL-1) is a pro-inflammatory cytokine with both local and systemic effects that include activation of lymphocytes and astrogliosis, vascular endothelium activation, local tissue destruction, and induction of cytokines[20,155,165]. In the human brain, biologically active IL-1 has been reported to be produced by astrocytes[165,166] and microglia[109,165,167,168], while IL-1 immunoreactivity has been isolated in neurons and axons[169]. In AD, however, significantly elevated levels of IL-1 and IL-1 immunoreactivity have been observed in cerebrospinal fluid (CSF)[170], plasma[171], and temporal homogenates[20]. Specifically, clusters of IL-1-positive reactive astrocytes have been detected around plaques in AD grey matter while IL-1-positive microglia have been located beyond the plaque corona[20]. The pattern of microglial IL-1α immunoreactivity has also been found to mirror the regional distribution of APP-positive neuritic plaques[172].

Elevated IL-1 expression may be the result of increased levels of microglial and astrocyte activation, both of which have been detected in AD. As previously discussed, secretory APP fragments and Aβ have been shown to induce microglial and astrocyte activation with associated expression of IL-1[135,138-140,150]. The functional role of IL-1 in AD, however, has been proposed to involve its pro-inflammatory characteristics, reciprocal influence on astrocytes and microglia, and impact on APP metabolism and plaque formation.

As previously described, IL-1 is a pro-inflammatory cytokine that activates macrophages and lymphocytes, thereby perpetuating sustained inflammation and consequently, host cellular damage[173]. In addition, IL-1 has been found to activate both astrocytes and microglia, thereby stimulating further production of IL-1[165], IL-6[165,167], TNF-α[165,174,175], complement proteins[176], chemokines[177], NO[175,178], and glutamate[178], all of which may perpetuate a chronic inflammatory

process, contribute to neuronal damage, and facilitate production of dystrophic neurites[125,179,180]. One potential mechanism underlying these induction effects has been proposed to involve IL-1-mediated activation of transcription factors[175,177]. Treatment of primary human fetal astrocytes has been shown to potently activate the transcription factors, NF-κB and activator protein-1 (AP-1), both of which have been reported to induce expression of multiple inflammatory genes including those coding for chemokines and cytokines[175].

IL-1α-induced astrocyte secretion of S100β has also been described *in vitro* and *in vivo*[14], consistent with evidence of elevated levels of S100β in AD[20]. Immunohistochemical studies have detected increased levels of S100-immunoreactive product in AD reactive astrocytes[20], as well as an increase in the number of S100-immunoreactive glia in AD brain[20]. Functionally, S100β has been implicated in dystrophic neurite and Aβ plaque formation, and further cytokine induction. As a neurite extension factor, S100β has been hypothesized to contribute indirectly to dystrophic neurite formation via its neurotrophic and neurite-extension properties[14,125,181,182]. Moreover, transgenic mice overexpressing a human APP minigene have shown progressive age-related increases in S100β and APP expression, prior to Aβ deposition, suggestive of a S100β role in Aβ pathology[180]. Finally, S100β has been reported to induce IL-6 expression in neurons[183].

In addition to the inflammatory consequences of elevated IL-1 in AD, experiments have revealed that IL-1 may play a significant role in Aβ metabolism and, ultimately, Aβ deposition[142-144]. Recent *in vivo* work has indicated that injection of synthetic IL-1β into rat brain results in increased detectable levels of S100β-positive astrocytes and significantly increased levels of APP isoforms[14]. These observations are consistent with the aforementioned hypothesis involving S100β as an IL-1-induced phenomenon and with previous work showing that IL-1 up-regulates translation of APP via 5' untranslated regions[143]. In addition, *in vitro* studies have shown that IL-1β treatment of neurons results in substantially increased APP mRNA[144]. Thus, it has been proposed that elevated IL-1 in AD brains may permit increased production of Aβ[14,184], in turn, increasing microglial activation[112,136] and subsequent IL-1 release[135,138].

2.4.2 – Interleukin-6

Interleukin-6 (IL-6) is a cytokine with wide-ranging effects including induction of acute-phase proteins, fever, lymphocyte activation, and immunoglobulin synthesis, as well as mediation

of hematopoiesis and neuronal protection[155,185,186]. In the CNS, IL-6 expression has been isolated in astrocytes[165,187,188], neurons[189], and microglia[109,165,188]. As with IL-1, studies have detected increased levels of IL-6 protein in the plasma[171,190], serum[191], CSF[170], and temporal cortex[22] of AD patients. In particular, IL-6 protein has been localized to AD cortical senile plaques[95,110]. In addition, elevated IL-6 mRNA levels have been detected in AD entorhinal cortex and superior temporal gyrus[192].

Furthermore, recent studies have proposed that expression of IL-6 may be a function of AD plaque evolution[15,110]. Clinically, non-demented individuals with predominantly diffuse plaques have exhibited minimal IL-6 immunoreactivity, compared to demented AD patients[110]. In fact, IL-6 immunoreactivity has been found to be significantly higher in early stage, diffuse plaques, compared to all other plaque types[110]. Thus, it has been hypothesized that IL-6 expression precedes neuritic changes and may, therefore, play a role in the evolution of primitive plaques from diffuse plaques[15].

Increased IL-6 expression in AD has been proposed to be the result of complex interactions between numerous factors involved in chronic AD inflammation, among them increased glial cell activation and cytokine production. As such, heightened levels of IL-6 may be the result of the increased number of astrocytes detected in AD and the increased activation of these astrocytes, as sustained by elevated levels of IL-1[20,140]. In addition, it has been suggested that increased activation of microglia by Aβ fibrils may, subsequently, result in IL-6 induction[135,140]. Specifically, the cytokine by-products of elevated microglial and astrocyte activation, in particular IL-1β, TNF-α, and S100β, have all demonstrated significant IL-6-inducing properties[165,175,183,193,194].

The potential functional implications of increased expression may be best illustrated by transgenic mice overexpressing IL-6[195]. Severe CNS abnormalities have been observed including neurodegeneration, astrocytosis, angiogenesis, and induction of acute-phase protein production[195]. In concert with these results, *in vitro* studies have established the ability of IL-6 to subtly induce complement proteins[176]. Consequently, IL-6 may prove to have both a direct and indirect pathogenic role in AD by virtue of its own pro-inflammatory characteristics, ability to facilitate chronic inflammation, and affect Aβ plaque formation.

2.4.3 – Tumour Necrosis Factor-α

Tumour necrosis factor-α (TNF-α) is a member of the TNF protein family that includes such proteins as the Fas ligand and CD40 ligand[155]. Though varied in its functions, TNF-α has been found to participate in lymphocyte activation, cytotoxicity, acute phase protein synthesis, vascular endothelium activation, increased vascular permeability, and septic shock[155,196]. In the CNS, TNF-α has been localized to microglia[109,165,188], astrocytes[165,188], and neurons[197]. Moreover, reports have indicated significantly elevated levels of TNF-α in AD sera[96] and TNF-α immunopositive ramified microglia in AD grey and white matter[124].

TNF-α induction, like that of IL-1 and IL-6, may be the result of increased astrocyte and microglial activation with subsequent cytokine production[112,139,140], as well as the result of positive feedback mechanisms in which existing cytokines trigger subsequent TNF-α synthesis[165,174,175]. In fact, IL-1β has been demonstrated to induce TNF-α production in both primary human fetal astrocytes and microglia[165,175].

Elevated TNF-α levels in AD brain may facilitate continuation of chronic inflammatory processes and Aβ metabolic effects. Studies have determined that TNF-α may be responsible, in part, for increased complement activation as TNF-α has been found to stimulate C1r, C1s, and C3 production[176]. Chronic AD inflammation may also be exacerbated by TNF-α-mediated activation of the transcription factors, NF-κB and AP-1, in a dose-dependent manner, thereby increasing subsequent expression of multiple inflammatory genes[175].

To date, the role of TNF-α in AD cell viability remains controversial. Though the potential detrimental inflammatory effects of TNF-α have been described above, numerous studies have suggested that TNF-α may, in part, mediate Aβ toxicity[198,199]. Recent work has determined that co-treatment of neuroblastoma cells with TNF-α and IFN-γ induces production of Aβ, thereby increasing the potential for Aβ deposition[198]. In contrast, pre-treatment of hippocampal neuron cell cultures with TNF-α has been found to significantly attenuate Aβ-mediated toxicity via anti-oxidant pathways[200]. Recent evidence, however, has demonstrated that the seemingly conflicting roles of TNF-α in Aβ toxicity may actually be a function of cell age and TNF-α concentration[199]. For example, high levels of TNF-α, in conjunction with Aβ, have proven to be toxic in neurons derived from old rats, compared to those derived from middle-aged or embryonic rats[199]. Low concentrations of TNF-α, however, have been found to be neuroprotective against Aβ toxicity[199].

Consequently, one might surmise that the effects of TNF-α are not entirely destructive or protective but rather a compromise of effects dependent upon age, TNF-α concentration, and Aβ load.

2.5 – Summary of Alzheimer's Disease Inflammation

From the available evidence, it is clear that inflammation in AD is a complex, integrative cycle characterized by positive feedback loops designed to promote a chronic and sustained immune response[1,173]. Initial activation of complement, microglia, and astrocytes has been hypothesized to be facilitated by APP and Aβ deposits and existing damage[112,150,151,161]. However, astrocyte and microglial up-regulation of cytokines and neurotoxic factors[124,150,151] are believed to continue this cycle by, in turn, facilitating further production of Aβ and APP[143], pro-inflammatory cytokines[165,174,188], chemokines[151], and neuronal damage[108,179,180,195] (Figure 1).

3.0 – Apolipoprotein E – Gene, Synthesis, and Processing

3.1 – Gene and Localization

Apolipoprotein E, a 299 amino acid single polypeptide, was first recognized in 1973 as a component of several human lipoproteins[201] and has since been linked to a gene on chromosome 19, composed of four exons and three introns with a relative sequence length of 3597 nucleotides[4,202]. Genetic studies have revealed that the apoE protein may be found as three major isoforms, the result of genetic variation with three alleles having been identified[4,203,204]. These alleles, ε2, ε3, and ε4, have been determined to code for apoE protein products with polymorphic sites at amino acids 112, 145, and 158, with corresponding single base substitutions in the DNA sequence[4,5].

ApoE mRNA and protein have been detected in a wide variety of species, as well as tissue and cell types. Specifically, apoE mRNA has been localized to the liver, adrenal glands, brain, kidney, and testes of the rat[205]. Furthermore, human apoE mRNA has been primarily isolated from the liver, intestine, and brain[206,207]. Circulating apoE has been identified in plasma and CSF, the latter of which has been hypothesized to be the result of nervous system synthesis[208].

Brain apoE expression has been described in mouse[209-211], rat[212,213], and human[214-216]. In transgenic mice expressing the human apoE gene, in situ hybridization has revealed apoE mRNA in glial cells of the cerebellum, striatum, and cerebral cortex, as well as in neurons of the cerebral

cortex[214]. Similarly, apoE has been determined to be predominantly synthesized by rat astrocytes, microglia, and oligodendrocytes[212,213,216-219]. Within the human CNS, apoE mRNA has been observed in Bergman glial cells and scattered astrocytes of the cerebellar cortex, selected cerebral cortical and hippocampal CA1-CA4 neurons, the granule cell layer of the dentate gyrus, and selected large neurons in the frontal lobe[214]. In spite of low-level neuronal localization of apoE mRNA, it has been typically suggested that neuronal apoE is the result of apoE uptake via available apoE receptors[220]. Nevertheless, it is generally accepted that brain apoE expression occurs primarily in astrocytes and microglia[212,221,222].

3.2 – Transcriptional Regulation

Although a great deal has been learned about hepatic apoE expression, very few investigations have been conducted into the regulation of apoE in brain cell types. Nevertheless, from the available literature, it is clear that regulation of apoE expression is a tissue-specific process involving complex interactions among positive and negative transcriptional control regions and transcription factors[223-227]. Examination of the 5' flanking region and the first intron of the human apoE gene in transfected HepG2 and Chinese hamster ovary (CHO) cells, has revealed multiple cis-acting regulatory elements responsible for modulation of apoE transcription[223,224]. Deletion analysis has identified at least three transcriptional regulatory domains within a 651 base pair region upstream of the apoE gene[223]. Within this region, a GC box transcriptional control element and three enhancer-like elements, URE1, URE2 and IRE1, have been established[223]. Furthermore, an additional enhancer element has been localized within the first intron[223]. DNase I footprinting assays have also demonstrated binding of nuclear extract proteins from HepG2 and CHO cells to specific sequences within transcriptional regulatory elements such as URE1 and binding of Sp1 or Sp1-like proteins to the GC box[223,224]. Further analysis of the URE1 enhancer element has revealed the presence of a specific protein-binding sequence, termed the positive element for transcription (PET)[228]. PET, a dominant regulatory element in the apoE promoter, has been found to bind to at least two protein factors including Sp1[228].

Finer mapping analysis within the 5' flanking region and first apoE intron has exposed nine positive control and three negative control regions that modulate apoE expression in both HepG2 and HeLa cells[224]. DNase I-protected footprints within these positive control regions have been ascertained to bear some homology to a serum-responsive element and an estrogen-responsive

element, as well as contain a GC box consensus sequence with the potential for Sp1 binding[224]. Similarly, the negative control regions have been found to contain repeated motifs with sequence similarities to the sterol responsive element found within the low density lipoprotein (LDL) receptor promoter[224] (Figure 2).

Further transcriptional complexity has been revealed to be the result of tissue and cell specificity with regards to regulatory activity[223,224,227]. Enhancer-like activity has been found to be dependent upon the cell line and promoter used[223]. Within the 5' flanking region and first intron, one positive and three negative control regions have been shown to have tissue-specific effects on HepG2 and HeLa cells, respectively[224]. In addition, site-directed mutagenesis studies have established that the pattern of positive and negative regulatory elements in the apoE gene differ between astrocytoma and hepatoma cells, thereby underscoring the tissue-specific nature of apoE gene regulation[229].

Several tissue-specific regulatory elements have also been identified downstream of the apoE gene, between the apoE and apoC-I genes, and downstream of the apoC-I gene[225-227]. High level liver expression of apoE has been shown to depend on a hepatic control region (HCR), located approximately 15 kilobases (kb) downstream of the apoE gene, that contains at least three nuclear protein binding sequences[225,226]. Expression of apoE in liver, however, has been found to require an additional non-specific proximal enhancer element in the apoE promoter, previously referred to as PET[226]. Transcriptional modulation may also be achieved via polymorphisms within the apoE promoter, thereby facilitating differential binding of nuclear proteins[230].

Similar complexity has been proven to exist in brain apoE expression. Elements found within the intergenic region between the apoE and apoC-I genes have been found to be responsible for stimulating apoE expression in the skin and brain while also inhibiting expression in the kidney[227]. Moreover, two astrocyte-specific distal enhancers, ME.1 and ME.2, have been recently identified via various transgenic mice constructs[231]. These distal sequences showing 95% sequence homology have been localized 3.3 kb and 15 kb downstream of the apoE gene[231]. Experiments have revealed that these cell specific enhancer regions are necessary for apoE promoter direction of *in vivo* gene expression in the brain[231]. Analysis of the enhancer sequences has exposed binding motifs for several common transcription factors such as the CAAT/enhancer-binding protein β (C/EBPβ) and steroid hormone receptors[231]. However, nucleotide differences between the two astrocyte-specific enhancers have been hypothesized to account for the

differential effects of the two regions on apoE gene expression in subsets of astrocytes[231]. Regional restrictions may also be the result of differential interactions of each enhancer region with other flanking regulatory sequences[231] (Figure 3).

In addition to regulatory elements found within and surrounding the apoE gene, transcriptional regulation may be modulated by such factors as cellular differentiation[232-234], cholesterol[235], diet[236,237], cyclic adenosine monophosphate (cAMP)[238], hormones[221,239,240], and various transcription factors[229,241,242]. Cholesterol loading of mouse macrophages has been found to increase apoE synthesis and secretion[243,244] while a high sucrose diet has also been found to stimulate increased apoE gene transcription in liver cells[237]. Moreover, cAMP has been shown to exert both a negative and positive effect on apoE transcription in HepG2 cells via specific sequences in the apoE proximal promoter[238]. Regulation of apoE by thyroid hormone[240] and 17-β-estradiol has been described in HepG2 cells and brain glia, respectively[221]. Elevated apoE mRNA has been observed at proestrus in cells of the CA1 hippocampus and hypothalamic arcuate nucleus, whereby both astrocytes and microglia appear to contribute to the estrogen-mediated increases in apoE mRNA[221].

In both liver and brain cells, transcription and other factors have also been reported to modulate apoE expression[229,241]. In human liver HepG2 cells, the transcriptional repressor factor, BEF-1, has been shown to reduce apoE mRNA via phosphorylation and a protein kinase C-mediated pathway[241,245]. In contrast, the transcription factors, Zic1 and Zic2, have been recently identified as activation factors in glial expression of apoE[242]. Specifically, three promoter-region binding sites for the Zic proteins have been isolated close to or overlapping binding sites for other described transcription factors such as Sp1 and AP2, emphasizing the importance of the promoter region, as well as the potential interactions between separate transcription factors in apoE regulation[242].

3.3 – Translational Regulation and Degradation

Experiments have revealed the apoE translation product in HepG2 cells and macrophages to be composed of one major and one minor isoprotein, collectively identified as pre-apoE[246,247]. Pre-apoE has been determined to contain an 18-amino acid NH_2 terminal extension signal peptide composed of predominantly hydrophobic residues[247]. Consequently, pre-apoE is composed of: (1) a 5' untranslated region of 60 base pairs, (2) a 3' untranslated region of 142 base pairs, (3) a

poly(A) tail derived from the polyadenylation signal, and (4) a signal peptide region[247]. Following removal of the signal peptide, synthesized apoE has been hypothesized to pool at the cell surface so as to be reinternalized and (1) recycled back to the Golgi for modification and eventual secretion or (2) degraded[248,249]. Specifically, it has been suggested that carbohydrate chains containing sialic acid are added to the apoE protein via O-glycosidic linkages, enabling subsequent secretion[247,249,250]. Extracellular desialation of the nascent sialo isoprotein has been proposed to then produce plasma asialo, mono, and disialo apoE forms[247,250].

Although regulation of apoE expression has been extensively explored, the precise process of apoE degradation remains unclear. It has been hypothesized that newly synthesized apoE may be directly targeted from the trans-Golgi network to lysosomes for degradation prior to secretion, without involving the plasma membrane and endocytosis[251,252]. Degradation of newly synthesized apoE has also been suggested to involve an intermediate density nonlysosomal cellular compartment, which is sensitive to proteosomal inhibitors[253]. In addition, apoE, upon internalization by receptors, may be degraded via a lysosomal pathway or elude degradation by lysosomes via retroendocytotic processes[248,254,255].

4.0 – Apolipoprotein E – Function

4.1 – Lipid Metabolism

Evidence suggests that the primary role of apoE involves the transport and metabolism of lipoprotein particles and cholesterol homeostasis, consequently affecting synaptic plasticity[213,222,256-258]. Various studies have demonstrated that apoE is a component of lipoproteins such as very low density lipoprotein (VLDL) and high density-lipoprotein (HDL), the result of specific domain interactions[259-261]. Moreover, in the periphery, apoE isoforms have illustrated preferential lipoprotein binding with apoE2 and apoE3 preferring HDL and apoE4 preferring VLDL[259-261].

Subsequently, apoE may facilitate internalization of these lipid complexes via interactions with cell surface LDL-receptors (LDL-R), LDL receptor-related proteins (LRP), very low density lipoprotein receptors (VLDL-R), apoE-receptor 2 (apoE-R2), and heparan sulfate proteoglycans (HSPG)[262-270]. Upon internalization via the LDL-R, degradation of cholesterol-rich lipoprotein particles within lysosomes is believed to occur, with subsequent release of cholesterol[271]. ApoE may then be degraded or resecreted for repeated action[248,254,255]. This apoE-mediated

internalization process, however, has been reported to be allelic and cell-type specific in nature[256]. In fact, LDL binding to iodinated apoE2-liposomes has been found to be significantly lower than that of apoE3- or E4-liposomes in cultured astrocytes or neurons[256]. Moreover, iodinated apoE4-liposome binding has been observed to be similar to that of apoE3-liposomes in astrocytes but lower in neurons[256].

In addition to internalization and uptake processes, it has been speculated that apoE may play a role in the cellular removal of lipoproteins from macrophages, neurons, and astrocytes, thereby promoting redistribution of cholesterol, reverse transport to the liver for removal, and general maintenance of lipid homeostasis[9,272,273]. The fact that cholesterol loading of mouse macrophages has been found to result in increased apoE synthesis and secretion may be indicative of a role for apoE in reverse cholesterol transport[243,244]. Although the precise mechanisms of such apoE action remain unclear, it has been suggested that apoE may act as an extracellular cholesterol acceptor rather than an intracellular transporter of cholesterol with subsequent secretion[273]. In addition, it has been hypothesized that cAMP plays a key role in apoE-mediated cholesterol efflux via induction of an apoE receptor[273]. As with apoE-mediated internalization, allelic differences in apoE-mediated efflux have been detected[272]. Exogenously added apoE has been found to promote cholesterol efflux with apoE2 being the most potent and apoE4 the least potent acceptor in neurons and apoE3 and apoE4 showing equal lower potency in astrocytes[272].

The importance of apoE-mediated lipid transport has been illustrated by studies utilizing models of CNS injury[222,258,274]. Lesions have been shown to induce apoE protein[275] as well as apoE mRNA[219] in the brain, particularly in hippocampal astrocytes[222]. In addition, bilateral carotid occlusion-induced forebrain ischemia and rat sciatic nerve crush injuries have been found to induce significant increases in apoE mRNA[276] and apoE protein[8], respectively.

In vitro studies have established that apoE mediates an allelic-dependent neurite extension process[258,277-279]. In fact, neuronal cell culture and hippocampal slice studies have reported that apoE3 and apoE4 mediate increases and decreases in neurite branching and extension, respectively[277,279]. This apoE3 extension effect has been shown to be an LRP dependent process supporting the hypothesis that apoE-mediated lipid homeostasis plays a role in neuritic remodeling[278]. Moreover, addition of apoE3 to apoE-knockout cell cultures has been found to fully restore previously defective mossy fiber sprouting while apoE4 facilitates recovery to only

58% of apoE3 levels[258]. Consequently, it has been suggested that increased levels of apoE may mediate increased availability of cholesterol for membrane and synapse formation, as well as nerve regeneration and remyelination, not only following injury but also during development[8,271,274,275].

The functional implications of apoE action in synaptic plasticity and neuronal remodeling have been affirmed by the observed deficits in apoE-deficient mice, deficits that are ameliorated by infusion of recombinant apoE[280-282]. Specifically, aged apoE-deficient mice have been found to suffer from synaptic and dendritic damage in the neocortex and limbic system[281], disruption of the microtubular cytoskeleton, as well as cholinergic and memory deficits[280,283,284]. In concert with the demonstrated differential recovery-inducing abilities of apoE3 versus apoE4 *in vitro*, expression of human apoE3 or apoE4 in neurons of transgenic mice lacking endogenous mouse apoE has been shown to result in allelic-dependent cognitive effects[285]. In particular, apoE4 mice exhibited impairments in learning a water maze task and in vertical exploratory behaviour, compared to apoE3 or control mice[285].

4.2 – Immune Regulation

To date, little has been published exploring the interaction between apoE and the immune system, especially in the CNS. Nevertheless, in the periphery, studies have established an interaction between inflammatory modulators such as endotoxins and cytokines and apoE expression[41,42,286]. In fact, it has been shown that lipopolysaccharide (LPS) endotoxin treatment of mouse macrophages, both in culture and *in vivo*, results in transient suppression of apoE secretion[286,287]. It has also been speculated that this inhibitory effect may be mediated by TNF as antibodies against TNF have been shown to neutralize the LPS-mediated reduction in apoE secretion[287]. In contrast, recent *in vivo* experiments involving intravenous injection of LPS into rodents have demonstrated significant increases in serum apoE[37].

Cytokines, such as IL-1β, TNF-α, IFN-γ, and transforming growth factor (TGF)-β, have also been shown to modulate apoE gene expression in macrophages[41,42,288] and HepG2 cells[39]. Addition of TNF-α to freshly isolated human monocytes has been found to result in a dose- and time-dependent 4-5 fold increase in apoE mRNA via stimulation of apoE promoter-dependent gene transcription[41]. Treatment of HepG2 cells with TNF-α and IL-1β has been found to result in significant increases in intracellular apoE and decreases in extracellular apoE, with no

accompanying changes in apoE mRNA, all of which may be indicative of a block in the apoE secretory pathway[39]. Pulse chase experiments have also illustrated that IFN-γ may significantly inhibit the accumulation of apoE in the supernatant of human monocytic THP-1 cells during and after differentiation via post-translational mechanisms[42].

Little evidence exists as to the influence of the immune system on CNS production of apoE. It has been shown, however, that IFN-γ treatment may increase the synthesis and intracellular expression of apoE in mouse astrocytes while decreasing secretion of apoE[40]. More recent work has demonstrated reduced apoE secretion by cultured human astrocytes upon treatment with IL-1β and IFN-γ[289].

In contrast, apoE has been proven to show immunoregulatory properties[32,34-36,290-293]. Specifically, it has been determined that apoE suppresses IL-2-dependent T lymphocyte proliferation[34,292], IL-4-stimulated lymphocyte proliferation[35], and neutrophil function[36]. Furthermore, apoE has been found to suppress glial secretion of TNF-α upon LPS stimulation with a trend towards increased suppression by apoE3 versus apoE4[33]. Although the mechanism of apoE-induced TNF-α suppression remains uncertain, it has been hypothesized that apoE may directly interact with microglia to suppress their responsiveness to inflammatory stimuli, thereby reducing TNF-α secretion[33]. Mixed glial cell cultures derived from apoE-deficient mice have also been proven to show a more robust immune response following LPS stimulation with an earlier and greater up-regulation of TNF-α and IL-6 mRNA when compared to apoE-containing cell cultures[32].

The *in vivo* immunological importance of apoE has been best described by studies investigating abnormalities in apoE-deficient mice[291,294,295]. Specifically, these mice have been found to exhibit significant elevations in antigen-specific IgM levels and a significantly decreased antigen-specific delayed-type hypersensitivity response when inoculated with tetanus toxoid plus adjuvant[294]. Similarly, apoE-deficient mice, when challenged with *Listeria monocytogenes*[291] and *Klebsiella pneumoniae*[38], have been found to demonstrate impaired immune responses with elevated TNF-α levels, increased bacterial outgrowth, and increased mortality. Intravenous LPS challenge of apoE-deficient mice has also been shown to induce significantly greater levels of TNF-α, IL-1β, and IL-6 mRNA, compared to those induced in wild-type control mice[32].

ApoE has been demonstrated to function not only in immune-challenged contexts but also under resting conditions. The sera of apoE-deficient mice have been reported to contain elevated levels of autoantibodies against neuron components[295]. These elevated levels are consistent with

a role for apoE in immunosuppression, indicating its potential ability to suppress autoimmunity[295].

Consequently, the gathered evidence, both *in vitro* and *in vivo*, suggests that apoE generally functions in an immunosuppressive manner. *In vitro* evidence indicates that in spite of increased mRNA and intracellular levels, apoE secretion is generally reduced following exposure to endotoxins or cytokines[39,41,286,287]; thus, initiation of an immune response may be accompanied by an initial decrease in apoE in order to facilitate progression of the immune reaction. The immunosuppressive properties of apoE have also been confirmed by the ability of apoE to inhibit immune cell proliferation and cytokine production[33-35]. Nevertheless, *in vivo* results indicate an increase in apoE upon LPS challenge and a significantly elevated immune response in the absence of apoE, suggesting that apoE may act to temper a stimulated immune response and, in part, prevent an uncontrolled reaction[32,37]. Thus, in an immune context, apoE levels may reflect a fine balance between the perpetuation of a required defensive immune response and a potentially harmful excessive reaction.

5.0 – Apolipoprotein E and Alzheimer's Disease

5.1 – Lipid and Amyloid Metabolism

Clinically, it has been determined that individuals bearing the apoE ε4 allele are at greater risk for sporadic AD and that apoE ε4 AD patients develop the disease earlier in life, compared to apoE ε2 or apoE ε3 AD patients[6,296]. An apoE ε4 gene dose effect has also been described, whereby age of AD onset is increased with decreasing number of apoE ε4 alleles[6]. Pathologically, the importance of apoE in AD has been established by virtue of its localization to Aβ plaques and dystrophic neurites[297-299], its decreased levels in serum[300] and the hippocampus and cortex of apoE ε4 AD patients[7], and its ability to bind and interact with Aβ in an isoform-specific manner[301,302]. Although the role of apoE in AD susceptibility has not yet been fully elucidated, the mechanistic implications of increased apoE ε4 allele frequency and decreased apoE levels in AD may involve Aβ fibrillization and clearance[220,302-305], cholinergic dysfunction[306-308], and loss of synaptic plasticity[222,275,309].

To date, the precise relationship between Aβ and apoE remains controversial[298]. Studies have shown that apoE3 has a higher affinity for Aβ binding than apoE4 in a lipid-dependent manner[302,305,310]. Consequently, it has been proposed that isoform-specific binding of apoE to Aβ

may play a role in the clearance and degradation of Aβ[220,302,305,311,312]. In support of this hypothesis, it has been recently shown that the presence of Aβ increases the binding and internalization of apoE into hippocampal neurons[313]. Further evidence of potential apoE-mediated Aβ clearance has been provided by apoE knock-in mice models, in which transgenic mice expressing human apoE3 or apoE4 but not mouse apoE, show markedly suppressed early Aβ deposition[314]. Functionally, preferential apoE-Aβ binding may explain the isoform-specific ability of apoE to reduce extracellular Aβ in neuronal cell culture with apoE3 proving to be more effective than apoE4[220,305,311]. Thus, decreased apoE4-mediated Aβ internalization and degradation may be partially responsible for increased plaque formation in apoE ε4 carriers and their greater risk for AD[220,305,311,315,316].

Cholinergic dysfunction in AD has been clearly illustrated by decreased levels of choline in the frontal and parietal cortices[11,66] and loss of cholinergic neurons, with accompanying loss of choline acetyltransferase (ChAT) activity[61,62]. Recent work has demonstrated a relationship between apoE and cholinergic dysfunction, whereby the apoE ε4 allele copy number exhibits an inverse relationship with brain ChAT activity in the hippocampus and temporal cortex of AD subjects[306]. Cholinergic dysfunction has been hypothesized to involve apoE and its potential ability to transport phospholipids, such as phosphatidylcholine, into neurons via the LDL-R-apoE pathway[262]. Thus, apoE4-associated decreases in apoE-mediated phospholipid transport may potentially explain the resulting decreases in choline and ChAT activity in AD[271,306].

Moreover, it has been proposed that apoE may be involved in AD pathogenesis via its role in lipid transport, cholesterol homeostasis, and synaptic plasticity[222,271,275,309]. In AD, apoE4-mediated lipid transport and cholesterol homeostasis may be dysfunctional, resulting in a loss of compensatory synaptogenesis and synaptic plasticity[271,309]. In fact, analysis of AD brain tissue has revealed that apoE ε4 patients show more severe degeneration and less plastic dendritic changes[317]. Moreover, an individual's apoE ε4 allele copy number has been found to have a significant effect on the pattern of dendritic arborization[317]. Evidence supporting this hypothesis may also be derived from *in vitro* studies exploring the role of apoE in neurite sprouting[258,277,279,318]. ApoE4-secreting cells have been found to promote a decrease in neurite branching and extension, compared to apoE3-secreting cells[277,279]. Loss of plasticity may not only be attributable to apoE dysfunction but also to a decreased capacity since apoE ε4 AD patients exhibit reduced brain

levels of apoE[7].

5.2 – Immune Regulation

Based on the *in vitro* and *in vivo* evidence, it is clear that there exists a bi-directional relationship between the immune system and apoE. The existence of such a relationship lends itself to the question as to whether apoE plays a role in the inflammatory processes of AD. Studies have established that apoE may have an isoform-specific modulatory role in complement as well as microglial and astrocyte activation, thereby potentially explaining, in part, the increased risk associated with apoE4. Aβ has previously been found to activate complement[161,162,319]. Recent studies, however, have indicated that apoE4 but not apoE3 or apoE2 potentiates Aβ-induced activation of classical complement *in vitro*[320].

ApoE has also been found to have isoform-specific effects on the number of microglia present in AD brain and their degree of activation. ApoE4 has been shown to have a dose-dependent effect on the increase in scattered microglia in AD brain[17]. In addition, an apoE effect on microglial activation has been observed in studies utilizing microglial cell cultures incubated with AD sera from genotyped patients[105]. ApoE 3/4 and apoE 4/4 genotype sera, as opposed to apoE2 sera, have been found to induce the highest percentage of microglia with an activated morphology[105,138]. Furthermore, increased IL-1β production by microglia has been detected upon incubation with sera from apoE3/4 and apoE4/4 AD patients[105]. Thus, apoE has been proposed to have an allelic effect on microglial activation.

In fact, it has been suggested that apoE may affect Aβ-induced microglial activation[136]. It has been shown that sAPP-α-induced microglial activation may be blocked by prior incubation of the protein with apoE3 but not apoE4[136]. Furthermore, apoE3 has been found to inhibit the ability of sAPP-α to increase nitrite production and to evoke microglial-mediated neurotoxicity[136]. Similarly, pre-incubation of microglia with apoE2 and apoE3, more so than apoE4, has been shown to significantly reduce microglial activation and TNF-α secretion following LPS stimulation[321]. These results are particularly interesting in light of the fact that isoform-specific apoE-Aβ binding has been observed with apoE3 showing greater affinity for Aβ than apoE4[302,311].

In addition, it has been suggested that apoE modulates astrocyte activation and proliferation. Recent studies have indicated that greater increases in astrogliosis may be detected among patients with the apoE ε4 allele[322]. Moreover, it has been found that: (1) pre-treatment of

astrocytes with apoE blocks subsequent Aβ-induced astrocyte activation, (2) Aβ, aged in the presence of exogenous apoE, reduces Aβ-induced astrocyte activation, and (3) exogenous apoE transiently reverses the activated phenotype of Aβ-treated astrocytes[152]. Recent experiments have also demonstrated that Aβ-induced astrocyte activation is associated with increased levels of total endogenous apoE, thereby leading the authors to suggest that astrocytes increase apoE levels in order to limit inflammatory processes[323].

Thus, the available evidence suggests that apoE may play a beneficial role in the inflammatory processes of AD, specifically, in the suppression of glial cytokine secretion[33], as well as microglial[105] and astrocyte[152] activation. It has been hypothesized that apoE is a potential immunosuppressent that reduces immune system activation, cytokine and neurotoxin production, and, ultimately, neuronal damage and chronic inflammation. Of particular interest, apoE4 has been found to have little effect on limiting the immune response of microglia or astrocytes, compared to apoE3[105,136,321,322], a fact that is consistent with apoE4 as a risk factor for AD[6]. Thus, one might theorize that apoE3 plays a role in immunosuppression while apoE4 is less effective in modulating the immune response, thereby perpetuating inflammation and neuronal degeneration.

6.0 - Treatment of Alzheimer's Disease

6.1 – Cholinesterase Inhibitors

To date, there exists no definitive treatment for AD that counters every disease symptom. Current clinical treatments typically address only the severe cholinergic deficits observed in AD brain. Examination of AD brains has revealed: (1) a reduction in choline levels in frontal and parietal cortices[66], (2) a selective loss of cholinergic neurons in the basal forebrain[60], and (3) a loss of ChAT activity[61]. Functionally, the severity of cholinergic dysfunction has been found to correlate with dementia severity[65]. Consequently, current clinical treatment strategies have focused upon preventing the breakdown of acetylcholine, thereby potentially alleviating some AD symptoms.

Numerous clinical trials have utilized cholinesterase inhibitors such as tacrine[324-327], donepezil, and metrifonate[328]. It has been demonstrated that treatment with tacrine, the most commonly utilized cholinesterase inhibitor, may produce mild to moderate cognitive improvement among some AD patients[327,329-331]. There exists, however, a population of patients that fail to respond to tacrine and are, therefore, labeled "non-responders"[324,329]. The failure of certain subgroups of AD patients to respond to tacrine has been shown to be a function of patient gender

and apoE genotype[324]. Specifically, the beneficial tacrine effect has been found to be significantly larger in women with apoE ε2-3 genotypes, relative to those with an apoE ε4 genotype[324]. Nevertheless, AD clinical strategies have typically focused upon the treatment of specific existing symptoms as opposed to disease prevention. As an alternative strategy, clinicians have reviewed the potential benefits of anti-inflammatory drug treatment in AD, particularly in light of the consistent up-regulation of immune modulators present in AD brain.

6.2 – Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) refer to a variety of drugs whose main effect is to inhibit the enzymes, cyclooxygenase-1 (COX-1) and cyclooxgenase-2 (COX-2), suppressing subsequent production of prostaglandins (PG), thromboxanes A_2 (TXA_2), prostacyclins (PGI_2), and cytokines[332,333 334,335]. During inflammation, PG have been shown to induce vasodilation, vascular permeability, sensitization of nerve receptors, and fever[336]. Although inhibition of prostaglandin production has been generally accepted as a key mechanism of NSAID effect, studies have also reported that NSAIDs may modulate the activity of other enzymes including lipoxygenase, phospholipase C, and iNOS[337-339].

In recent years, it has been discovered that COX exists in two main isoforms, COX-1 and COX-2[340-342]. COX has been found to exhibit cyclooxygenase and hydroperoxidase activities so as to form prostaglandin G2[343]. COX-1 is a uniformly expressed, constitutive form that has been hypothesized to perform cellular "house-keeping" functions including production of prostacyclin, coordination of hormones, and regulation of vascular homeostasis[344-346]. In the human CNS, COX-1 expression has been illustrated in almost all structures, with high levels having been detected in the forebrain[347]. Regulatory analysis of the COX-1 gene has revealed numerous putative regulatory sites including Sp-1, PEA-3, AP2, NF-IL6, GATA-1, and a shear stress response element[348].

The more recently discovered isoform, COX-2, has been described as an inducible form that is encoded by a different gene[341,342,349] bearing ~60% sequence homology to COX-1[343]. Structural analysis of the COX-2 gene has established the presence of a number of putative regulatory sites such as the cAMP, IL-6, and glucocorticoid response elements[348]. COX-2 expression has been observed in the cortex, hypothalamus, and hippocampus[350,351]. Furthermore, COX-2 activity has been found to be: (1) induced by inflammatory cytokines, mitogens, and

endotoxins and (2) regulated by intracellular messengers and substrate availability[334,336,343]. By virtue of the differential expression and regulation of COX-1 versus COX-2, it has been hypothesized that the anti-inflammatory benefits of NSAIDs may be attributed to COX-2 inhibition while the side effects associated with NSAID treatment may be the result of COX-1 inhibition[343].

For the purposes of this review, three NSAIDs will be discussed in further detail: aspirin, ibuprofen, and indomethacin. Aspirin is believed to inhibit both COX-1 and COX-2 by acetylating serine-530 and serine-516, respectively, thereby blocking the interaction of arachidonic acid with the enzyme active site and irreversibly inhibiting COX activity[352-354]. In spite of its ability to acetylate both COX-1 and COX-2, aspirin has been noted to be 10-100 times more potent against COX-1, relative to COX-2[346]. In addition to its ability to block COX activity, *in vitro* and *in vivo* experiments have revealed that aspirin has the capacity to inhibit COX-2 expression, specifically COX-2 transcription following cytokine and LPS stimulation[355]. Other biological effects of aspirin include: (1) NO regulation via inhibition of iNOS gene expression and NO radical scavenging[339,356] and (2) inhibition of tumour growth[357].

Another classic non-selective NSAID, ibuprofen, has been found to inhibit both COX-1 and COX-2 via competitive inhibition, as demonstrated by kinetic studies[358]. Unlike aspirin, ibuprofen has been found to have a 2-fold preference for COX-2 versus COX-1[346]. Ibuprofen, like many other profen family members, exists as an enantiomeric pair and is typically marketed as a racemic mixture[359]. Closer examination of R- versus S-ibuprofen has revealed that R-ibuprofen is relatively ineffective as an anti-inflammatory agent while S-ibuprofen is effective both *in vitro* and *in vivo*[359,360]. Nevertheless, R-ibuprofen has been found to undergo chiral inversion to S-ibuprofen, thereby increasing its anti-inflammatory capacity[361]. As with aspirin, ibuprofen has demonstrated other significant biological effects beyond COX inhibition; for example, *in vitro* treatment of macrophage and cerebellar glial cells has resulted in reduced levels of NOS mRNA and iNOS activity, respectively[339,362].

Indomethacin, a time-dependent COX inhibitor capable of crossing the blood brain barrier and binding to albumin in CSF[363,364], has been shown to induce an enzymatic conformational change resulting in irreversible binding and inhibition[333,334]. Specifically, structural studies have established that indomethacin binds deeply within the COX active site, leading to inactivation[333,334]. As with aspirin, indomethacin has demonstrated a 10-30 fold preference for COX-1 inhibition[346]. In addition, indomethacin, like both aspirin and ibuprofen, has been reported to play a role in NO

production, whereby reduced NOS mRNA has been noted following *in vitro* treatment of macrophages[339]. Moreover, indomethacin has been established to be an effective NO scavenger *in vitro*[356].

In light of the hypothesis that the anti-inflammatory benefits and side effects of NSAIDs are the result of COX-2 and COX-1 inhibition, respectively, many researchers have attempted to produce novel COX-2 selective inhibitors in order to relieve some of the gastro-intestinal side effects associated with prolonged NSAID use[365-368]. Of particular note, attempts have been made to biochemically convert existing NSAIDs such as aspirin and indomethacin into COX-2 selective inhibitors[365-368]. Recently, an aspirin-like molecule, o-(acetoxyphenyl)hept-2-ynyl sulfide (APHS), has been described to show preferential acetylation and irreversible inactivation of COX-2[368]. APHS, with an IC_{50} value of 0.8 μM, has been reported to be approximately 60 times more potent against and 100 times more selective for COX-2, relative to aspirin[368]. Nevertheless, the functional applicability of APHS has been confirmed both *in vitro* and *in vivo*[368]. In macrophages stimulated with LPS and IFN-γ, APHS has been shown to effectively inhibit COX-2 activity[368]. Furthermore, using an *in vivo* rat air pouch model, significant reductions in prostaglandin synthesis have been observed following treatment with APHS[368]. Similarly, indomethacin amides have been reported to exhibit COX-2 selective inhibitory activity in stimulated macrophages[365]. Of particular interest, indomethacin amide treatment in a rat footpad edema model has proven not only to be anti-inflammatory but also non-ulcerogenic[365].

6.3 – NSAIDs and Alzheimer's Disease – Epidemiological Evidence

Epidemiological studies have established that NSAIDs may have protective value with regards to the risk and onset[27-29,369,370], course[31,371], and pathology of AD[372-375]. Specifically, use of NSAIDs by the elderly has been shown to be: (1) negatively associated with the AD diagnosis and (2) associated with a decreased relative risk for AD[26,27,94,94,369,370,376]. In fact, the relative risk for AD has been reported to decrease with increasing duration of NSAID use[369,370]. Additionally, a review of 17 epidemiological studies has revealed that there may be a negative relationship between rheumatoid arthritis and AD, a relationship proposed to be the result of prolonged NSAID use for the treatment of arthritis[94]. Of particular interest, a recent epidemiological study has reported that the inverse association between NSAID use and AD may be detected at both high and low drug dosages, indicating that NSAID neuroprotection may be present at drug levels

unlikely to be anti-inflammatory[26].

The onset of AD has also been examined in a co-twin control study among elderly twin pairs with onsets of AD separated by at least 3 years[28]. The results have indicated a trend towards an inverse association between disease onset and daily use of NSAIDs[28]. In support of this study, it has also been determined that among siblings at high risk of developing AD, sustained use of NSAIDs is associated with delayed onset and reduced risk[29].

In addition to their preventative value, NSAIDs have been examined as potential treatments for diagnosed AD patients. Patients taking NSAIDs, including aspirin and ibuprofen, have been found to show less decline after one year on measures of verbal fluency, spatial recognition, and orientation[31]. Consistent with these results, AD patients taking NSAIDs, when compared to non-users, have performed better on measures of attention, speed, and language[377]. Moreover, a six-month, double-blind, placebo-controlled clinical study of indomethacin treatment in mild to moderately impaired AD patients has revealed that treatment appears to protect patients from cognitive decline[371]. In fact, indomethacin-treated patients have been shown to improve by 1.3% on cognitive tests while placebo-treated patients decline by 8.4%[371].

Based upon the epidemiological links between AD and NSAID use and the earlier successes in treating AD patients with NSAIDs, recent studies have used both NSAIDs and glucocorticoids as anti-inflammatory treatments in AD[378,379]. Treatment of mild to moderate AD patients with the NSAID, diclofenac, in parallel with the gastro-protective agent, misoprostol, in a 25-week, randomized, double-blind, placebo-controlled trial has revealed no significant effect of NSAID treatment[378]. However a non-significant trend towards greater deterioration in the placebo group has been reported[378].

In light of the elevated immune activation in AD and the common hypothesis that NSAIDs may function strictly in an anti-inflammatory fashion, researchers have begun to explore other potential treatments with anti-inflammatory effects including the glucocorticoid, prednisone[379]. No significant differences in cognitive decline, however, have been observed between prednisone and placebo-treated AD patients following a one-year, randomized, placebo-controlled, multi-centre trial[379]. In fact, prednisone-treated patients have been reported to show greater behavioural decline than control patients with increased agitation and hostility[379].

NSAID treatment has also been hypothesized to modify the pathological course of AD[372,373]. Post-mortem analysis of brain tissue from elderly, non-demented arthritic patients with a

history of NSAID use has shown significantly less microglial activation[373]. In support of these studies, recent *in vivo* experiments have also found that treatment of rats with a novel COX inhibitor, nitroflurbiprofen, attenuates LPS-induced neuroinflammation and microglial activation[380]. In AD brains, however, no differences have been detected in the mean number of plaques or the degree of neurofibrillary pathology following NSAID use[372,373,377].

6.3.1 - NSAIDs and Alzheimer's Disease – Potential Mechanisms

Although the precise mechanisms of NSAID function in AD remain unresolved, it has been proposed that NSAIDs may modify inflammatory processes by: (1) inhibition of COX-1 and COX-2 with subsequent reduction of prostaglandin synthesis[369,381], (2) inhibition of cytokine production via NF-κB-mediated pathways[382,383], (3) modulation of cytokine expression via peroxisome proliferator-activated receptor (PPAR) activity[384-386], and (4) modulation of Aβ production and deposition[374,375,387].

Numerous studies have determined that expression of both COX-1 and COX-2 is significantly increased in AD, underscoring the potential benefits of NSAIDs[381,388-391]. Importantly, significantly increased levels of COX-1 protein have been measured in AD brain, compared to control tissue[381]. In fact, Aβ plaque-associated COX-1 immunoreactivity[388] and increased density of COX-1-positive immunoreactive microglia[391] have both been observed in AD. Similarly, COX-2 expression has been found to be significantly increased in AD brain, specifically in neurons of the hippocampal CA1-CA4 region and among neurofibrillary tangles and damaged axons[381,390,392]. Up-regulation of COX-2 expression has also been detected in AD frontal cortex[389]. COX-2 up-regulation, like that of COX-1, may be associated with Aβ pathology since synthetic Aβ peptides have been found to induce COX-2 expression in SH-SY5Y neuroblastoma cells *in vitro*[389]

Classically, the protective effects of NSAIDs have been attributed to the inhibition of increased COX activity in AD, with subsequent reduction of prostaglandin synthesis and immune activation[393]. Increased prostaglandin levels, as would be seen with increased COX activity in AD, could facilitate chronic inflammation via vasodilation and vascular permeability, and eventually stimulate production of cytokines and other inflammatory mediators[336].

Recently, an alternate hypothesis regarding NSAIDs and COX inhibition has been proposed[394]. During the synthesis of prostaglandins, free radicals are produced as by-products of COX peroxidase activity[395]. It has been suggested that increased COX expression and activity in

AD could, consequently, produce increased levels of free radicals, leading to tissue damage and potentiation of Aβ toxicity[340]. Thus, NSAID-mediated COX inhibition would limit production of these reactive agents and subsequent damage.

In addition to the proposed NSAID effects on COX activity, it has also been demonstrated that NSAIDs including aspirin and ibuprofen, but not indomethacin, inhibit NF-κB activation[382,383,396-402]. NF-κB refers to a family of cellular transcription factors that are involved in the expression of such inflammatory and toxic mediators as IL-1, IL-6, TNF-α, COX-2, acute phase proteins, iNOS, as well as APP[403-405]. NF-κB typically exists as a latent, inactive heterodimer comprised of p50 and RelA subunits complexed with the inhibitor IκB[404]. Upon cellular activation by various stimuli including IL-1, TNF, Aβ, sAPP, and oxidative stress, IκB undergoes phosphorylation, ubiquitination, and, ultimately, degradation by the proteasome[136,404,406,407]. Upon degradation of IκB, the NF-κB heterodimer may then translocate to the nucleus, bind to consensus sequences in target genes, and increase expression[404]. Numerous studies have demonstrated that various NSAIDs inhibit NF-κB activation via prevention of IκB phosphorylation and degradation[382,397,400,408].

Consequently, in AD, elevated levels of IL-1β, TNF-α, and Aβ may work in concert to activate NF-κB, thereby inducing pathological levels of IL-1, IL-6, TNF-α, COX-2, and APP, all of which may continue to stimulate a chronic pro-inflammatory loop and further Aβ deposition. Consistent with this hypothesis, immunostaining of AD brain has identified activated NF-κB in senile plaques and in pyramidal neurons and astroglia surrounding primitive plaques[409]. Furthermore, *in vitro* treatment of primary neurons and astrocytes with Aβ has resulted in NF-κB activation[406,409]. Similarly, IL-1β stimulation of neuroblastoma cells has been found to induce NF-κB activation and production of COX-2 protein[407]. Thus, NSAID-mediated inhibition of NF-κB activation could limit reciprocal production of Aβ and cytokines, astrocyte and microglial activation, and neuronal damage.

Furthermore, it has been proposed that NSAIDs may function as peroxisome proliferator activated receptor (PPAR) agonists and may modulate various genes involved in inflammation and neurotoxicity[384,386,410]. PPAR-γ is a member of a nuclear receptor family of transcription factors that regulate ligand-dependent transcriptional activation and repression[411]. Like COX, significant increases in PPAR-γ levels have been reported in AD[381]. Indomethacin and ibuprofen have been

shown to bind and activate both PPAR-γ and PPAR-α at micromolar concentrations[384]. Recent experiments have illustrated that these PPAR-γ agonists inhibit production of cytokine synthesis from human monocyte cell cultures and downregulate iNOS and subsequent NO production[386,410].

With regards to the brain, treatment of Aβ-stimulated microglia with PPAR-γ agonists including indomethacin and ibuprofen, has been shown to suppress production and secretion of TNF-α and IL-6[387]. Furthermore, indomethacin has been found to inhibit LPS-induced COX-2 protein in glial cells[381] and iNOS expression in cerebellar granule cells[410]. Similarly, ibuprofen has been reported to reduce LPS/IFN-γ-stimulated iNOS expression *in vivo*[412]. Thus, it has been postulated that the anti-inflammatory effects of indomethacin and ibuprofen in AD may be due, in part, to PPAR activation and inhibition, at a transcriptional level, of cytokine, COX, and neurotoxin expression[386,410].

Although the focus of PPAR research in AD has been from an inflammatory perspective, recent studies have explored the role of PPAR in lipid homeostasis, an area in which apoE plays a vital role. Two recent studies have implicated PPAR-γ and PPAR-α in reverse cholesterol transport and cholesterol synthesis, respectively[413,414]. Specifically, increased expression of the ATP-binding cassette A1 protein transporter and cholesterol efflux have been observed following PPAR-γ agonist treatment of macrophages, fibroblasts, and intestinal cells[413]. Overexpression of PPAR-α has also been associated with significant increases in mitochondrial β-hydroxy-β-methyl-glutaryl-CoA (HMG-CoA) synthase mRNA[414]. Consistent with this finding, gene analysis has revealed a PPAR response element in the proximal promoter of the human HMG-CoA synthase gene[414].

Although very little information exists as to the relationship between apoE and PPAR, given the fact that NSAIDs may activate PPAR and that activation of PPAR is associated with increased cholesterol efflux, it begs the question as to whether an elevation in apoE might not be the next logical step in order to facilitate cholesterol transport and removal. In support of this hypothesis, recent evidence has demonstrated the presence of a peroxisome proliferator response element (PPRE) located in the apoE/apoCI intergenic region previously found to be involved in apoE regulation[415]. Treatment with a PPAR-γ agonist has been reported to induce apoE mRNA two-fold in THP-1 cells, an induction dependent upon a DR-1 sequence within the PPRE region[415]. Thus, the fact that PPAR-γ agonists have been proven capable of up-regulating apoE mRNA via PPRE interactions in an immune cell line, lends credence to the hypothesis that NSAIDs, many of

which have been shown to act as PPAR-γ agonists, have the potential to affect apoE homeostasis.

Finally, *in vitro* and *in vivo* experiments have illustrated NSAID-mediated effects on Aβ pathology[374,387]. Oral administration of ibuprofen to transgenic mice overexpressing APP has been found to significantly reduce IL-1 levels, the number and area of Aβ deposits, the number of dystrophic neurites, and the percentage area of microglia in plaques[374]. In addition to plaque pathology, Aβ-mediated toxicity in monocytes and PC12 cells and matrix metalloproteinase-9 activity in microglia have been found to be significantly reduced with indomethacin treatment[375,416,417].

Rationale

Based upon the accumulated evidence, it is likely that AD inflammation is a complex, interactive process, characterized by feedback loops that act to perpetuate chronic, self-sustained inflammation and neuronal damage. The importance of the inflammatory process is emphasized by: (1) the numerous immune irregularities in AD[18,20,22,151] and (2) the benefits of NSAID treatment in AD[29,369,371]. In terms of AD inflammation, apoE may act as an immunosuppressive modulator as well as a facilitator of immune activation. It has been determined that apoE has the capacity to inhibit microglial[136] and astrocyte[152] activation as well as glial secretion of cytokines[33], thereby limiting the production of further pro-inflammatory mediators and neuronal damage. Isoform-specific differences in apoE immunological function have also proven to be consistent with apoE ε4 as a major risk factor for AD. ApoE4 has been confirmed as being not only less effective in reducing the effects of chronic inflammation[136] but also more pathological by potentiating the activation of complement[320].

The question remains, however, as to whether the protective effects of NSAID treatment in AD are at all related to apoE. Epidemiological studies have revealed that the effect of NSAIDs is apoE genotype-dependent[29]. Specifically, it has been shown that the effect of NSAIDs is stronger in subjects lacking an apoE ε4 allele[29]. In addition, prior *in vitro* studies have determined that ibuprofen and aspirin reduced apoE mRNA and protein in organotypic hippocampal slice cultures derived from aged mice[418] while only aspirin reduced apoE mRNA in an NMDA-excitotoxicity model[419]. In spite of these initial results much remains unknown regarding the effect of other clinically viable NSAIDs and the mechanisms underlying such effects.

Global Working Hypothesis:

Based upon the bi-directional relationship between apoE and the immune system, the epidemiological link between NSAIDs and apoE, and the potential benefits of apoE in immune and lipid regulation, we hypothesize that the protective effect of NSAIDs in AD may be due to an up-regulation of astrocyte and microglial apoE production, thereby increasing the potential for compensatory synaptogenesis and immunosuppression.

Materials and Methods

1.0 - Experimental Objectives

The ultimate goal of the described experiments was to investigate the relationship between immune system mediators and brain apoE production. Specifically, experiments were used to assess the impact of NSAIDs, cytokines, and cyclooxygenase specific inhibitors on glial production of apoE, as well as the mechanisms underlying any effect. As an experimental model, primary rat astrocyte and mixed glial cell cultures were prepared and treated with common NSAIDs and other inflammatory mediators. Subsequently, extracellular apoE protein and cellular apoE mRNA levels were quantified. In order to fully characterize any potential effect, cell culture viability and purity were also assessed with acridine orange and immunostaining protocols, respectively.

2.0 – Tissue Cell Cultures

2.1 – Primary Rat Astrocyte Cell Cultures

Cell culture solutions and supplies were purchased from GIBCO (Grand Island, NY). Primary astrocyte cell cultures were obtained from the cortices of one day-old Sprague-Dawley rat pups (Charles River Laboratories Inc., St. Constant, Quebec). Upon decapitation, the rat brains were stored in cold 70% ethanol until removal of the hippocampi and meninges in cold dissection medium [Dulbecco's modified Eagle's medium (DMEM) with 20 mM HEPES]. Isolated cortical tissue was minced, centrifuged, and triturated following the addition of dispase (10 mg/mL phosphate buffer solution (PBS)) (Boehringer Mannheim Corp., Indianapolis, IN). The cell suspension was then shaken and passaged manually until homogenous following the addition of DNase I (1 mg/mL) (Boehringer Mannheim Corp., Indianapolis, IN). Subsequently, the suspension was filtered through a 70 μ nylon mesh (Becton Dickinson, Franklin Lakes, NJ). The filtered suspension was then centrifuged in fresh dissection medium twice and the cell pellet was ultimately resuspended in growth medium (DMEM-F12 supplemented with 10% fetal bovine serum (FBS) (Immunocorp, Montreal, QC), 1% penicillin/streptomycin, and 0.1% amphotericin B (fungazone), pH=7.6). The suspension was centrifuged a final time, resuspended in fresh growth medium, and plated in 75 cm^2 flasks (Sarstedt, Newton, NC), previously coated with poly-D-lysine (Sigma, St. Louis, MO). All cell cultures were incubated at 37°C and 5% CO_2. On the following day, the cell culture medium was replaced with fresh medium. Subsequently, the cell culture medium was replenished every 3-4 days until the astrocytes reached ~70% confluence and microglia were

visible.

The cell culture medium was then removed and PBS added. Cell cultures were shaken vigourously until detachment of the overlying microglia was visually confirmed. The microglia, suspended in PBS, was discarded and fresh medium added to the astrocyte cell cultures. Three to four days later, each flask was shaken again in order to purify the astrocyte cell cultures further. Astrocyte cell cultures were sustained with supplemented cell culture medium containing 10% FBS, 1% penicillin/streptomycin, and 0.1% fungazone. Plating of the astrocyte cultures in 24 well cell culture plates (Sarstedt, Newton, NC) was completed upon reaching ~85% confluence. Fluorescent microscopy established that astrocyte cell cultures were composed of ~95% astrocytes and 5% microglia.

2.2 – Primary Rat Mixed Glial Cell Cultures

The available literature suggests that there is a clear relationship between astrocytes and microglia in AD inflammation. Hence, by studying multi-typic cell cultures, we were able to better assess the dynamic between these cell types and any changes in apoE secretion. Mixed glial cell cultures were derived from the initial shaking of astrocyte cell cultures (Refer to 2.1). The mixed glial suspension was centrifuged and resuspended in fresh supplemented growth medium containing 10% FBS, 1% penicillin/streptomycin, and 0.1% fungazone. The cells were then plated in 75 cm^2 flasks, previously coated with poly-D-lysine, for 30 minutes, in order to ensure glial attachment. After 30 minutes, the cell culture medium was replaced so as to remove excess oligodendrocytes. Subsequently, the cell culture medium was replaced every 3-4 days until ~85% confluence was achieved. Antibody labeling revealed that cell cultures consisted of ~70% astrocytes, 25% microglia, and 5% oligodendrocytes.

2.3 – Plating of Primary Rat Astrocyte and Mixed Glial Cell Cultures

Upon reaching ~85% confluence, the cell cultures were gently rinsed with PBS, treated with 0.1% trypsin (0.025 g/mL), and warmed at 37°C for 10 minutes. The trypsin was then inactivated by adding supplemented cell culture medium containing 10% FBS, 1% penicillin/streptomycin, and 0.1% fungazone in equal volume. Upon shaking and detachment of the cells, the cell suspension was centrifuged and resuspended in fresh cell culture medium. All cell types were plated in 24 well cell culture plates, previously coated with poly-D-lysine, at a

density of ~50,000 cells/well. The cell culture medium was refreshed every 3-4 days until ~70% confluence was reached. All cells were utilized within three weeks of initial culturing.

2.4 – Primary Human Adult Microglial Cell Cultures

Primary adult human glial cell cultures were obtained from the laboratory of Dr. J. Antel (Dept. of Neurology and Neurosurgery, Montreal Neurological Institute, Montreal, Canada) following isolation from tissues removed during surgical resection. Cultures were derived from tissues distal from the primary epileptic foci, obtained during surgical treatment of non-tumour-related intractable epilepsy. Isolation of human microglial cells was based upon the differential adherence of glial cells. Briefly, 1-3 mm^3 tissue samples were dissociated enzymatically with trypsin (0.025%) and DNase (50 mg/mL) (both obtained from Boehringer Mannheim, Laval, QC) for 30 minutes at 37°C. Subsequently, cells underwent mechanical dissociation via passage through a 132 μm nylon mesh (Industrial Fabrics Corporation, Minneapolis, MN). Cells were then separated using a linear 30% Percoll gradient (Pharmacia, Baie d'Urfe, QC) produced by centrifugation at 15,000 rpm and 4°C for 30 minutes. Cells recovered from the interface were composed of ~65% oligodendrocytes, 30% microglia, and 5% astrocytes. In order to enrich the microglial fraction, mixed cells were suspended in Eagle's MEM, supplemented with 5% fetal calf serum (FCS), 2.5 U/mL penicillin, 2.5 mg/mL streptomycin, 2 mM glutamine, and 0.1% glucose (all from Life Technologies, Burlington, ON) and left overnight in 12.5 cm^2 tissue culture flasks (FALCON, Fisher Scientific, Montreal, QC) at 37°C and 5% CO_2. Less adherent oligodendrocytes were removed the following day by gentle pipetting while the remaining microglia and astrocytes were allowed to develop morphologically for 3 days. Subsequently, less adherent astrocytes were removed by rotary shaking for 2 hours at 200 rpm. Remaining microglia at a density of 1.2×10^5 cells/cm^2 were assessed by immunocytochemistry for CD68 expression (DAKO Diagnostics Canada Inc., Mississauga, ON) and were found to be 95% pure. Cells were maintained in 12.5 cm^2 tissue culture flasks at a confluence of ~85% until NSAID treatment. Fresh cell culture medium was provided every 3 days until treatment.

3.0 – Drug Treatment

3.1 – Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) were all purchased from Sigma (St. Louis,

MO). Primary rat astrocyte and mixed glial cell cultures were treated with the NSAIDs, indomethacin, ibuprofen, and acetylsalicylic acid (aspirin). In addition, primary adult human microglia were treated with indomethacin. Stock solutions of each drug were made in 100% ethanol. Cells were subsequently treated with each drug at various concentrations dissolved in fresh cell culture medium and incubated for a treatment period of 48, 72, or 96 hours. Each concentration was tested in triplicate. Upon completion of treatment, cells were visually assessed and the cell culture medium was collected and stored at -80°C.

3.2 – Cyclooxygenase Specific Inhibitors

Indomethacin, ibuprofen, and aspirin are all non-selective COX inhibitors whose ratios of COX-1/COX-2 inhibitory activity vary[346,352-354,358]. Consequently, in order to explore the individual role of each COX enzyme, COX-2 selective derivatives of indomethacin[365] and aspirin[366] (Gifts from Dr. L. Marnett, Dept. of Biochemistry and Chemistry, Vanderbilt University School of Medicine, Nashville, TN), were used to treat primary rat astrocyte and mixed glial cell cultures. Two indomethacin derivatives, indomethacin aromatic amide[365] and indomethacin phenethyl amide[365], that have been previously shown to mediate COX-2 selective inhibition, were utilized during a treatment regimen over a period of 24, 48, 72, or 96 hours. In addition, an inactive indomethacin derivative[365], characterized by a 4-bromobenzyl group on the indole ring, was included as a negative control. All three indomethacin derivatives were initially dissolved in dimethylsulfoxide (DMSO) (5mM) and subsequently in fresh supplemented cell culture medium for cell treatment. The remaining stock solution was stored at -20°C.

In addition, rat astrocyte and mixed glial cell cultures were treated with o-(acetoxyphenyl)hept-2-ynyl sulfide (APHS)[368], a COX-2 selective inhibitor, and APHS phenol[366], an inactive hydrolysis product of APHS. Both aspirin derivatives were provided in aqueous solution and subsequently dissolved in fresh supplemented cell culture medium. Stock solutions were stored at -20°C. Drug treatment was concluded following 24, 48, 72, or 96 hours. Following treatment with indomethacin and aspirin derivatives, the cell culture medium was collected from each well and frozen at -80°C for later analysis (Table 1).

3.3 – Cytokines

Previous work has shown that inflammatory mediators significantly affect apoE production

in mouse astrocytes[40]. Thus, the proposed experiments attempted to examine the potential effects of cytokine exposure on apoE production in primary rat cell cultures, as well as determine if pro-inflammatory mediator treatment resulted in opposite effects on apoE production, relative to those of NSAID treatment.

Primary rat astrocyte and mixed glial cell cultures were treated with pro-inflammatory mediators, specifically IL-1β (Geneka Biotechnology Inc., Montreal, QC) for 72 or 96 hours, as well as IL-6 (Amgen, Thousand Oaks, CA) and TNF-α (Geneka Biotechnology, Montreal, QC) for a period of 24, 48, 72, or 96 hours. As with the NSAIDs and cyclooxygenase specific inhibitors, each cytokine was dissolved directly in fresh supplemented cell culture medium for treatment. Cell culture medium was collected upon completion of treatment and stored at -80°C.

3.4 – Miscellaneous Drugs

17-β-estradiol (Sigma, St. Louis, MO), a drug previously having been shown to up-regulate apoE protein and mRNA expression in brain cells[221], was used as a positive control. A stock solution of 17-β-estradiol in 100% ethanol was created and dissolved in fresh supplemented cell culture medium. Subsequently, rat astrocyte and mixed glial cell cultures were treated for 48, 72, or 96 hours.

Rodent cell cultures were also treated with probucol (Sigma, St. Louis, MO), a hypolipidemic agent that has been proposed to play a role in AD via its ability to promote decreases in plasma cholesterol[420,421] and increases in apoE mRNA in animal models[422]. In addition, the effects of probucol have been shown to be apoE-genotype dependent[423]. Thus, probucol was utilized in order to explore the potential beneficial role of apoE in AD, as well as the impact of other clinically relevant drugs on the immune system. Probucol was initially dissolved in 100% ethanol, thereby forming a stock solution for further dissolution in fresh supplemented cell culture medium with ultimate treatment for a period of 24, 48, 72, or 96 hours.

4.0 – Primary Rat Cell Culture Immunolabeling

Fluorescent antibody labeling was used to assess rodent cell culture purity. Initially, the cell culture medium was removed from each well at room temperature. Cells were then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 15 minutes. Following fixing, the cells were rinsed with PBS and treated with a combination of 0.1%

bovine serum albumin (BSA) (Fischer Scientific, Fair Lawn, NJ) and horse serum (Vector Laboratories Inc., Burlingame, CA) in PBS for 90 minutes. The cells were then rinsed with PBS and permeabilized with 0.4% triton (Amersham, Arlington Heights, IL) for 30 minutes. Upon rinsing with PBS, the cells were incubated overnight at 4°C with primary antibodies designed to recognize an astrocyte-specific glial fibrillary acidic protein (GFAP), a perivascular and activated microglia/macrophage-specific cytoplasmic antigen in the adult CNS[424,425] (ED-1 antibody), or an oligodendrocyte-specific galactocerebroside (GALC) protein. The primary antibody testing conditions were as follows: (1a) single labeling with rabbit, anti-cow GFAP antibody (1:8000) (DAKO Diagnostics Canada Inc., Mississauga, ON), (1b) single labeling with mouse anti-rat ED-1 antibody (1:500) (Serotec, Raleigh, NC), (1c) single labeling with mouse anti-rat GALC antibody (1:500) (Chemicon International, Temecula, CA), (2) double labeling with rabbit, anti-cow GFAP antibody and mouse anti-rat ED-1 antibody, (3) double labeling with rabbit, anti-cow GFAP antibody and mouse anti-rat GALC antibody, and (4) PBS as a negative control. Each condition was tested in triplicate.

Following incubation, the primary antibodies were then removed, the cells incubated with PBS for 10 minutes, and fluorescent secondary antibodies added. The conditions were as follows: (1a) donkey anti-rabbit IgG antibody conjugated to Texas Red dye (1:1000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) (1b,c) donkey, anti-mouse IgG-Texas Red antibody (1:1000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) (2,3) donkey, anti-rabbit IgG-Texas Red antibody (1:1000) and donkey, anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC) (1:1000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and (4) negative controls of each secondary antibody without the accompanying primary antibody. The secondary antibodies were incubated for 1 hour in darkness at room temperature. Subsequently, the secondary antibodies were removed and the cells were rinsed with PBS and distilled water to remove any lingering salt. Finally, the coverslips were mounted on non-coated slides with Vectishield (Vector Laboratories Inc., Burlingame, CA) and stored in darkness at 4°C. Fluorescent microscopy analysis was achieved with a Nikon Eclipse E600 microscope (Nikon Inc., Melville, NY).

5.0 – Cell Viability Assay – Acridine Orange Staining

In order to eliminate the possibility of cellular and extracellular changes in apoE being due

to cell death, cell viability was assessed using an acridine orange labeling protocol following 96 hours of drug treatment. As a positive control, cells were treated with 0.01% and 0.001% hydrogen peroxide (Fischer Scientific, Fair Lawn, NJ) for 10 minutes prior to labeling. Initially, the cell culture medium and hydrogen peroxide were removed and 100 μL of fresh supplemented cell culture medium was added. Subsequently, cells were incubated with a stock buffer solution pH=3.5 (0.1% triton, 0.2 M sucrose, 10^{-4} M disodium EDTA, 0.02 M citrate phosphate buffer pH=3.0 based on phosphate) for 1 minute. Cells were then treated with an acridine orange (Eastman Kodak Comp., Rochester, NY) solution (2 mg/mL H_2O) diluted 1:100 with a second stock buffer solution (0.1 M NaCl, 0.01 M citrate phosphate buffer pH=3.8 based on phosphate) for a duration of 5 minutes. Upon completion of incubation, removal of the acridine orange solution was followed by a rapid rinse with PBS. Coverslips were immediately mounted on non-coated slides with Vectishield and observed using a fluorescent microscope.

6.0 – Apolipoprotein E Protein Quantification - Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of extracellular apoE levels in all collected cell culture medium samples was achieved using a protein-specific ELISA assay[426]. ELISA plates (Corning Costar E.I.A./R.I.A., Acton, MA) were coated with goat anti-human apoE capture antibody (International Immunology Corporation (IIC), Murrieta, CA, purified with a HiTrap Protein G Kit, Amersham Pharmacia Biotech, Baie d'Urfe, QC) in 10 mM sodium carbonate. Plates were sealed and stored overnight at 4°C. The capture antibody was subsequently blocked with 0.1% BSA in PBS and stored overnight at 4°C. The following day, each well was washed with 20 mM tris-base-salt-tween (TBS-T) between individual incubation periods of two hours. Defrosted cell culture medium samples and recombinant apoE4 (Panvera Quality Reagents, Madison, WI) standards (50-2000 ng/mL in PBS) were incubated in triplicate. Goat anti-human apoE antibody labeled with biotin (IIC, Murrieta, CA, purified with a HiTrap Protein G Kit, Amersham Pharmacia Biotech, Baie d'Urfe, QC, labeled with biotin, Boehringer Mannheim Corp., Indianapolis, IN) in 0.1% BSA in TBS-T was then added. Subsequently, wells were incubated at room temperature for one hour with alkaline phosphatase-streptavidin (Zymed Laboratories Inc., San Francisco, CA) diluted 1:1000 in 0.1% BSA in TBS-T. Following incubation, the plates were washed with TBS-T and once with distilled water. Attophos reagent (Promega Corporation, Madison, WI), warmed to room temperature, was then added. At

30 minutes and 60 minutes, measurements of emitted fluorescence were taken using a microplate fluorescence reader FL-600 (Bio-tek Instruments Inc., Winooski, VT), reading at a bandwidth of 450 nm/50 nm. Detectable levels of apoE protein were between 50-2000 ng/mL.

7.0 – Interleukin-1β (IL-1β) Protein Quantification - Enzyme-Linked Immunosorbent Assay (ELISA)

Extracellular IL-1β levels were quantified using a commercial IL-1β ELISA kit (Medicorp, Montreal, QC). Briefly, frozen cell culture medium samples were defrosted to room temperature. Recombinant rat IL-1β samples, diluted in provided standard diluent buffer (0-2000 pg/mL), were added in duplicate to wells on a 96 well plate pre-coated with anti-rat IL-1β antibody. Remaining wells were filled with a combination of 50 μL of each experimental sample and 50 μL of standard diluent buffer, all in triplicate. The sealed plate was shaken gently at room temperature for three hours. Subsequently, each well was washed thoroughly four times with diluted wash buffer. Following washing, 100 μL of biotinylated anti-IL-1β antibody solution was added to each well except those designated as chromogen blanks. The plate was then sealed and incubated at room temperature for one hour. Again, the plate was thoroughly washed and 100 μL of streptavidin-horse radish peroxidase working solution was added to each well except the chromogen blank wells. Provided stop solution was added to each well following incubation for 30 minutes at room temperature in darkness. Absorbency was then measured at 450 nm. Detectable levels of IL-1β protein were between 0-2000 ng/mL.

8.0 – Apolipoprotein E mRNA Quantification

In order to assess whether NSAID treatment had transcriptional effects on apoE expression, primary rat astrocyte cell cultures were prepared and plated in 75 cm^2 flasks. Rodent astrocyte cell cultures (~80% confluence) were then treated with indomethacin, ibuprofen, aspirin, and probucol diluted in supplemented cell culture medium. 17-β-estradiol and an inactive indomethacin derivative were used as positive and negative controls, respectively. Cells were incubated with the described compounds for 2, 4, 8, 16, 24, or 30 hours. The cell culture medium was collected and stored at -80°C. In addition, cells were rinsed with PBS and manually detached by scraping the flask surface. The cell suspension in PBS was centrifuged for 10 minutes upon

which the supernatant was removed. Samples were then stored at -80°C.

8.1 – RNA Extraction

Using an RNeasy Mini Kit (Qiagen, Mississauga, ON), frozen cells were disrupted by the addition of buffer RLT (10 μL β-mercaptoethanol/1 mL of buffer RLT) and subsequently homogenized using a QIAshredder column (Qiagen, Mississauga, ON). Samples were then centrifuged at 14,000 rpm for 2 minutes at 20°C. Following centrifugation, one volume of 70% ethanol was added to the homogenized lysate and mixed. The lysate was then applied to an RNeasy mini spin column and centrifuged at 10,000 rpm for 15 seconds. Subsequently, the mini spin column was washed with buffer RW1 and centrifuged for 15 seconds at 10,000 rpm. The spin column was then washed twice with buffer RPE and centrifuged at 10,000 rpm for 15 seconds following the first wash and at 14,000 rpm for 2 minutes following the second wash. Subsequently, the RNeasy column membrane was rinsed with RNase-free water, allowed to stand for approximately 1 minute, and centrifuged at 10,000 rpm for 1 minute. Extracted RNA samples were quantified using a spectrophotometer reading at 260 nm and then stored at -80°C.

8.2 – Real Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Extracted RNA samples were amplified using a two-step RT-PCR reaction[427,428]. In a total reaction volume of 50 μL containing 1X RT buffer, 5.5 mM $MgCl_2$, 500 μM of each deoxynucleotide triphosphate, 2.5 μM of random hexamers, 0.4 U/μL of RNase inhibitor, and 1.25 U/μL of Multiscribe Reverse Transcriptase, 1 μg of extracted RNA from each individual sample was amplified. The reaction volumes were sequentially held at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. Subsequently, the amplified cDNA templates were frozen at -20°C.

ApoE mRNA quantification was achieved using SYBR Green PCR Core Reagents (Molecular Probes Inc., Eugene, OR), designed for use with the GeneAmp 5700 Sequence Detector (PE Applied Biosystems, Foster City, CA). Specific rat apoE and β-actin oligonucleotide primers based on the rat apoE mRNA sequence[429] [Forward primer: nucleotides 921-940; Reverse primer: nucleotides 978-996] and rat β-actin cDNA sequence[430] [Forward primer: nucleotides 218-238; Reverse Primer: nucleotides 265-284], respectively, were designed using Primer Express

software (PE Biosystems, Foster City, CA). Each reaction utilized a total reaction volume of 35 μL containing 1X SYBR Green PCR Master Mix (Molecular Probes Inc., Eugene, OR), 1000 nM of each primer, and 3 μL of cDNA template, with completion to volume with RNase-free water. Each sample then underwent a thermal reaction cycle of 50°C for 2 minutes, 95°C for 10 minutes, and 40 repetitions of 95°C for 15 seconds and 60°C for 1 minute. Subsequently, the PCR product was stored at 4°C. All samples were amplified using both apoE and β-actin primers.

Quantification of mRNA was achieved by measuring changes in fluorescent signal emitted by SYBR Green Dye upon binding to double-stranded DNA[427,431]. Specifically, the threshold cycle (C_T) values of treated and non-treated samples were compared following normalization to the endogenous control, β-actin. The C_T value refers to the fractional PCR cycle number at which the amplified target reaches a fixed threshold, a condition indicated by the fluorescent signal emitted by the SYBR Green Dye.

9.0 – Statistics

9.1 – Extracellular Apolipoprotein E Protein

Statistical normality was initially assessed using Normcheck, version 1.0, J. Rochford, Montreal, QC. Statistical outliers were excluded at the 95% confidence level based upon the Dixon Test for Rejection of Outliers. Subsequently, statistical analyses were conducted utilizing Datasim, version 1.2, D.R. Bradley, Lewiston, ME. All individual data points were expressed relative to non-treated mean protein values within trial, drug, and time. Two-way independent measures analysis of variance (ANOVA) tests were then used to assess the effects of drug concentration and duration of treatment across trials on extracellular apoE protein levels. Post-hoc pairwise comparisons were completed as required using the Tukey's Honestly Significant Difference (HSD) test. Significant differences between non-treated and treated cells were then assessed using multi-sample 95% confidence intervals with the non-treated mean protein value arbitrarily set as a population mean of 1.0.

9.2 – Astrocyte Apolipoprotein E mRNA

In order to quantify sample apoE mRNA, a comparative C_T method[427,428] was utilized for each individual trial. C_T values derived from amplification with rat β-actin primers were subtracted from the C_T values derived from amplification of the same samples with rat apoE primers, thereby

producing a ΔC_T value. Consequently, the resulting data was normalized to an endogenous control. Subsequently, the ΔC_T values of treated samples were compared to those of non-treated samples within each time point. Ultimately, apoE mRNA quantities for each treatment condition were expressed relative to a non-treated value of 1.0^{428}.

Across trials, the data was collated such that an independent measures two-way ANOVA was conducted with duration of treatment and agent concentration as independent factors. Statistical differences between treated and non-treated cells were examined using multi-sample 95% confidence intervals with the non-treated mean apoE mRNA value set as a population mean of 1.0.

Results

Fluorescent antibody labeling of cell cultures

Rodent astrocyte and mixed glial cell cultures were double-labeled with fluorescent antibodies specific for glial fibrillary acidic protein (GFAP) in astrocytes, ED-1 antigen in microglia, and galactocerebroside protein (GALC) in oligodendrocytes. GFAP, a cytoskeletal protein and principle intermediate filament in mature astrocytes in the CNS, has been thought to be essential in providing structural stability to astrocytes while also modulating cellular motility and shape[432]. Moreover, it has been established that ED-1 antibodies recognize a CNS cytoplasmic antigen specifically found in perivascular and activated microglia/macrophages[424,425,433]. Galactocerebroside (GALC) is a galactolipid found in abundance in the myelin bilayer[434]. Functionally, galactolipids have been reported to transduce developmental signals, stabilize membranes, and facilitate protein trafficking[434]. Thus, oligodendrocytes, as an essential source of myelin proteins, have been identified through use of antibodies specific for membrane surface GALC protein[435].

Visual assessment of the fluorescent signal emitted from each individual cell type revealed that primary rat astrocyte cell cultures consisted of ~95% astrocytes and 5% microglia (Figure 4). In contrast, primary rat mixed glial cell cultures were composed of ~70% astrocytes, 25% microglia, and 5% oligodendrocytes (Figure 5). Primary human microglial cell cultures were provided at 95% purity as previously assessed by immunocytochemistry for CD68 expression (Data not shown).

Acridine orange assessment of cell viability

Acridine orange is a membrane-permeable, monovalent, cationic dye capable of intercalating into and interacting with double-stranded and single-stranded nucleic acids, respectively[436]. Consequently, cell viability was assessed by testing the integrity of the plasma membrane[437]. When viewed using fluorescent microscopy, viable cells fluoresce green upon inclusion of acridine orange and electrostatic interactions with cellular nucleic acid[436-438]. Numerous studies have successfully utilized acridine orange as a cell viability marker of CNS cells[439-441]. Acridine orange staining of drug-treated cells, when used in the reported concentrations, displayed significant green fluorescence. Rodent astrocyte and mixed glial cell

cultures, following 96 hours of drug treatment, displayed at least 95% cell viability consistently (Figure 6).

Non-steroidal anti-inflammatory drug (NSAID) treatment of primary rat astrocyte and mixed glial cell cultures significantly increased extracellular apoE protein levels

Indomethacin

ApoE secretion under control conditions (i.e., no exogenous drug treatment) remained constant with time over the course of all experiments. Two-way independent measures analysis of variance (ANOVA) testing of astrocyte data revealed a significant main effect of time, $F(2,259) = 4.38$, $p < 0.05$, with simple main effects testing demonstrating mean apoE protein levels to be significantly higher at 72 hours than at either 48 or 96 hours, $p_s < 0.01$. However, indomethacin treatment of astrocyte cell cultures resulted in no significant apoE protein differences between non-treated and treated cells (Data not shown).

Analysis of mixed glial data revealed a significant time-concentration interaction, $F(18,287) = 3.95$, $p < 0.0001$. Subsequent simple main effects analysis revealed significant differences in apoE protein levels between time points of 48, 72, and 96 hours, within specific concentrations, $F_s(2,287) \leq 21.76$, $p_s \leq 0.05$. As such, post-hoc pairwise analysis established a general trend of increased apoE protein with increased duration of incubation within wells treated with indomethacin at 10^{-17} M, 10^{-18} M, and 10^{-19} M, $p_s \leq 0.05$. Specifically, mean apoE protein values at 48 and 72 hours were significantly lower than those at 96 hours, $p_s < 0.05$. In contrast, a significant reduction in apoE over time was detected at a concentration of 10^{-14} M with mean protein values at 48 hours being greater than at 72 hours, $p \leq 0.01$. Confidence interval analysis demonstrated that indomethacin treatment of mixed glia induced significant increases in extracellular apoE protein levels after 96 hours, relative to non-treated cells, $p_s \leq 0.05$ (Figure 7).

Ibuprofen

Statistical analysis of astrocyte apoE protein data revealed a significant main effect of both time, $F(2,244) = 5.96$, $p < 0.01$, and ibuprofen concentration, $F(10,244) = 3.26$, $p < 0.001$. Pairwise comparisons between time points of 48, 72, and 96 hours found mean apoE protein levels at 48 hours to be significantly lower than those at either 72 or 96 hours, $p_s \leq 0.05$. In contrast, no

significant differences were detected between mean apoE levels of non-treated and ibuprofen-treated cells. Nevertheless, a subtle trend towards increased extracellular apoE was observed at concentrations of 10^{-11} M and 10^{-13} M (Figure 8A).

Similarly, ANOVA analysis of mixed glial data established a significant main effect of both time, $F(2,268) = 9.51$, $p < 0.0001$, and ibuprofen concentration, $F(10,268) = 2.73$, $p < 0.01$. Mean apoE protein levels were found to be significantly lower at 48 and 72 hours, relative to 96 hours, $p_s < 0.01$. Further analysis failed to show any significant differences between non-treated and treated cells. Trend analysis, however, revealed a significant quadratic dose-response curve, $F(1,263) = 13.74$, $p < 0.001$. In addition, as with astrocyte data, a subtle trend of increased apoE protein with ibuprofen exposure was observed (Figure 8B).

Aspirin

Significant main effects of both duration of treatment, $F(2,214) = 13.74$, $p < 0.0001$, and aspirin concentration, $F(9,214) = 4.35$, $p < 0.0001$, on astrocyte apoE levels were identified. In fact, apoE protein levels decreased over time, irrespective of concentration, as protein levels at 96 hours proved to be significantly lower than those at 48 or 72 hours, $p_s < 0.01$. Relative to non-treated cells, however, aspirin treatment was found to induce significant increases in astrocyte apoE protein at a concentration of 10^{-17} M, $p < 0.05$ (Figure 9A).

Following treatment with aspirin, mixed glia showed a significant main effect of drug concentration on extracellular apoE levels, $F(9,329) = 6.35$, $p < 0.0001$. However, no effect of duration of treatment was observed. Statistical analysis revealed a significant increase in apoE levels upon treatment with aspirin at a concentration of 10^{-11} M, $p < 0.05$ (Figure 9B).

Treatment of primary rat astrocyte cell cultures with cyclooxygenase-2 specific aspirin derivatives induced significant increases in extracellular apoE levels in a time and dose-dependent manner

o-(acetoxyphenyl)hept-2-ynyl sulfide (APHS)

The aspirin-like molecule, o-(acetoxyphenyl)hept-2-ynyl sulfide (APHS), has been recently described to show preferential acetylation and irreversible inactivation of COX-2[368]. APHS has been reported to be approximately 60 times more potent and 100 times more selective for COX-2

inhibition, relative to aspirin[368]. The functional applicability of APHS has also been confirmed both *in vitro* and *in vivo,* whereby COX-2 activity in stimulated macrophages has been inhibited by APHS treatment[368]. Furthermore, using an *in vivo* rat air pouch model, significant reductions in prostaglandin synthesis have been observed following treatment with APHS[368].

In the present study, a significant time-APHS concentration interaction effect was observed, $F(18,137) = 3.34$, $p < 0.0001$. Subsequent testing established a significant effect of time at an APHS concentration of 10^{-10} M, whereby mean apoE levels at 24 hours proved to be significantly greater than those at 48, 72, or 96 hours, $p_s < 0.01$. A significant increase in astrocyte apoE protein was also observed following 24 hours of treatment, relative to non-treated cells, $p < 0.05$ (Figure 10A).

o-(acetoxyphenyl)hept-2-ynyl sulfide phenol (APHS phenol)

In contrast, the phenol derivative of APHS has been shown to be inactive with no inhibitory activity against either COX-1 or COX-2[366]. ANOVA analysis substantiated a significant time-APHS phenol concentration interaction, $F(18,138) = 3.13$, $p < 0.0001$. Astrocyte apoE levels were generally found to increase with prolonged incubation at specific APHS phenol concentrations. Mean apoE levels at 24 hours proved to be significantly lower than at 72 hours at a concentration of 10^{-18} M, $p < 0.05$, and at 96 hours at concentrations of 10^{-12} M, 10^{-16} M, and 10^{-18} M, $p_s \leq 0.05$. In contrast, mean levels at 24 hours were significantly higher than at 96 hours at a concentration of 10^{-10} M, $p < 0.05$. Mean levels at 48 hours proved to be significantly lower than those at 72 hours at 10^{-16} M, $p < 0.05$, and at 96 hours at both 10^{-12} M and 10^{-16} M, $p_s \leq 0.05$. Finally, apoE protein levels at 72 hours were significantly lower than levels at 96 hours at 10^{-12} M, $p < 0.05$. In contrast to the results shown for APHS treatment of astrocyte cell cultures, cultures treated with APHS phenol showed no significant differences in mean apoE levels, compared to non-treated cells (Figure 10B). Nevertheless, analysis of the data revealed a trend towards increased apoE protein upon APHS phenol exposure at a concentration similar to that found with APHS.

Treatment of primary rat astrocyte and mixed glial cell cultures with indomethacin derivatives significantly reduced extracellular apoE protein levels in a dose-dependent manner

Three indomethacin derivatives, LM 4108, LM 4115, and LM 4192, were utilized in the current study. LM 4108, an indomethacin amide derivative, and LM 4115, an aromatic amide indomethacin derivative, have been characterized as COX-2 selective inhibitors[365]. Kinetic analysis has demonstrated that LM 4108 behaves as a slow, tight-binding inhibitor with a much slower time course of COX-2 inhibition compared to indomethacin[365]. Furthermore, both LM 4108 and LM 4115 have proven to be effective at inhibiting COX-2 activity in macrophage cell cultures, as well as *in vivo* in a rat footpad edema model[365]. In contrast, LM 4192, has been shown to be ineffective as a COX inhibitor and was, thus, used as a negative control in the presented experiments.

Treatment of astrocytes with LM 4108 resulted in significant main effects of treatment duration, $F(3,221) = 30.84$, $p < 0.0001$, and drug concentration, $F(6,221) = 2.37$, $p < 0.05$. Main effects analysis revealed that mean apoE levels were significantly higher at 24 hours compared to those at 48, 72, or 96 hours, $p_s < 0.01$. Moreover, significant decreases in apoE protein were observed in LM 4108-treated cells, compared to control cells, $p_s < 0.05$ (Figure 11A). Similarly, LM 4108 treatment of mixed glial cell cultures induced significant main effects of time, $F(3,221) = 5.37$, $p < 0.01$, and compound concentration, $F(6,221) = 4.58$, $p < 0.001$. Unlike astrocyte cells, mean apoE protein levels were significantly elevated at 72 hours relative to 48 or 96 hours, irrespective of drug concentration, $p_s \leq 0.05$. In addition, when compared to non-treated cells, mixed glial apoE protein levels of treated cells were found to be significantly lower, $p_s < 0.05$ (Figure 11B).

Use of another COX-2 selective indomethacin derivative revealed similar results. LM 4115 treatment induced significant main effects of time and drug concentration in both astrocytes, $F_{time}(3,139) = 29.39$, $p < 0.0001$, $F_{concentration}(6,139) = 2.23$, $p < 0.05$, and mixed glia, $F_{time}(3,215) = 16.64$, $p < 0.0001$, $F_{concentration}(6,215) = 7.28$, $p < 0.0001$. In astrocyte cell cultures, apoE protein levels decreased with time as mean levels at 24 hours were significantly greater than those at 48, 72, or 96 hours, $p_s \leq 0.05$ and mean levels at 48 hours were significantly higher than those at either 72 or 96 hours, $p_s < 0.01$. In mixed glia, mean apoE levels at 48 hours were significantly lower than mean levels at 24, 72, or 96 hours, $p_s < 0.01$. Nevertheless, in both astrocyte and

mixed glial cell cultures, treatment with LM 4115 proved to significantly reduce extracellular apoE protein levels, $p_s < 0.05$ (Figure 12A and 12B).

As a negative control, an indomethacin derivative with no COX inhibitory activity was utilized. However, analysis revealed a significant main effect of time in both astrocytes, $F(3,205) = 3.25$, $p < 0.05$, and mixed glia, $F(3,211) = 6.98$, $p < 0.001$, as well as a significant main effect of LM 4192 concentration in mixed glia, $F(6,211) = 5.39$, $p < 0.0001$. In astrocyte cell cultures, mean levels of apoE were found to be significantly higher at 24 hours than at 72 hours, $p < 0.05$. In contrast, mean levels at 48 hours were significantly lower than at 24, 72, or 96 hours in mixed glia, $p_s \leq 0.05$. In comparing treated to non-treated cells, subtle decreases in apoE protein were detected following treatment of mixed glia, $p_s < 0.05$ (Figure 13).

IL-1β and IL-6 treatment of primary rat cell cultures induced significant increases in apoE protein levels while TNF-α treatment induced significant decreases in protein levels

IL-1β treatment of astrocytes and mixed glia resulted in significant main effects of time, $F(3,183) = 4.22$, $p < 0.01$, and concentration, $F(8,108) = 4.93$, $p < 0.0001$, respectively. Specifically, astrocytes treated with IL-1β at 48 hours demonstrated lower levels than cells treated for 96 hours, $p < 0.01$. No significant differences were detected between astrocyte apoE levels of treated and non-treated cells. In contrast, mixed glia treated with IL-1β illustrated significantly elevated levels of apoE protein when compared to non-treated cells, $p < 0.05$ (Figure 14).

Similarly, IL-6 treatment of astrocytes was found to induce a significant main effect of both time, $F(3,139) = 3.55$, $p < 0.05$, and concentration, $F(6,139) = 4.09$, $p < 0.001$. Pairwise analysis of apoE levels at different time points showed significantly lower levels at 48 hours compared to 72 hours, $p < 0.01$. Contrast of treated and non-treated cells also revealed a significant induction of extracellular protein with IL-6 exposure, $p < 0.05$ (Figure 15).

In spite of the inductions seen with IL-1β and IL-6, TNF-α treatment was associated with significant reductions in apoE protein. ANOVA analysis of astrocyte data showed significant main effects of time, $F(2,66) = 4.22$, $p < 0.05$, and concentration, $F(7,66) = 5.27$, $p < 0.0001$. Mean apoE protein levels at 72 hours proved to be significantly higher than at 96 hours, $p < 0.01$. Of key interest, comparisons of treated and non-treated cells across time points revealed significant decreases in apoE protein, $p < 0.05$ (Figure 16A).

Although no main effect of time was found upon analysis of mixed glial data, a significant effect of TNF-α concentration was noted, $F(6,135) = 6.14$, $p < 0.0001$. As with astrocyte cell cultures, mixed glia exhibited decreases in apoE protein following treatment with TNF-α, $p_s < 0.05$ (Figure 16B).

Treatment of primary rat astrocytes with 17-β-estradiol induced a significant increase in apoE protein levels: positive control assay

Significant main effects of time, $F(2,164) = 9.50$, $p < 0.001$, and concentration, $F(6,164) = 2.25$, $p < 0.05$, were detected following treatment of astrocyte cell cultures. Across time, apoE levels were found to increase as protein levels at 24 hours were significantly lower than those at 48 or 72 hours, $p_s < 0.01$. In addition, treatment induced a significant increase in apoE protein, $p < 0.05$ (Figure 17).

No significant differences in primary rat astrocyte or mixed glial extracellular IL-1β protein levels were detected following treatment with NSAIDs or NSAID derivatives for 96 hours

IL-1β ELISA analysis of collected cell culture medium samples revealed no significant differences between non-treated and treated cells or between treated cells (Data not shown). Although unexpected, the failure of the IL-1β ELISA to detect any significant changes in IL-1β protein levels may have been the result of technical issues such as sample age or cell number. Minimal levels of IL-1β protein were observed, thereby suggesting that the number of cells per culture well was insufficient to allow for accumulation of protein for later quantification.

NSAID, probucol, and cytokine treatment of primary rat astrocyte cell cultures resulted in no significant changes in apoE mRNA levels

Indomethacin, ibuprofen, and aspirin treatment of primary rat astrocyte cell cultures proved to have no significant effect on apoE mRNA levels (Figures 18-20). However, high variability and low sample numbers may have limited the statistical power of the analyses. Furthermore, the indomethacin derivative, LM 4192, was found to have no significant effect on apoE mRNA levels

(Figure 21).

In addition to NSAIDs and NSAID derivatives, other compounds such as probucol and IL-1β were utilized. As with the anti-inflammatory compounds, no significant differences were detected (Figures 22 and 23).

Preliminary data suggests that indomethacin treatment of primary human microglia did not significantly affect apoE mRNA levels

Pilot data suggested that indomethacin treatment of primary human microglia derived from epilepsy patients did not significantly affect apoE mRNA levels (Figure 24).

Discussion

In the present study, treatment of primary rat astrocyte and mixed glial cell cultures with indomethacin, aspirin, and the COX-2 selective aspirin derivative, APHS, resulted in significant increases in extracellular apoE protein. Ibuprofen and the inactive aspirin derivative, APHS phenol failed to induce apoE expression. In contrast, use of indomethacin derivatives with COX-2 selective inhibitory activity and an inactive indomethacin derivative significantly reduced apoE protein levels. Moreover, exposure to IL-1β and IL-6 was followed by significant increases in apoE protein in mixed glia and astrocytes, respectively. In addition, TNF-α mediated significant decreases in protein in both astrocyte and mixed glial cell cultures (Table 2).

Main effects analysis revealed variability in the effects of time on apoE protein levels. Trends towards increased apoE protein with time, as seen with indomethacin, ibuprofen, APHS phenol, and 17-β-estradiol treatment, may have been the result of increasing cell number with time. In contrast, trends towards decreased apoE protein with time, as demonstrated by treatment with aspirin and APHS, may have been the product of an early peak in drug effect with increases in apoE protein coming early during treatment and decreasing thereafter. Finally, exposure to indomethacin derivatives resulted in a trend towards decreased protein with time, a trend that may have reflected increasing drug effect with longer incubation time. Indomethacin derivatives generally induced significant decreases in apoE protein, relative to non-treated cells; thus, drug effects at later time points may have resulted in the observed main effect of time with lower protein levels seen at 72 and 96 hours.

Consequently, the evidence suggests that commonly used NSAIDs, including indomethacin and aspirin and to a lesser extent ibuprofen, have the capacity to affect proteins beyond those involved in inflammation. As such, the results of the current study implicate apoE modulation as a potential mechanism of NSAID neuroprotection in AD. The failure of recent NSAID clinical trials to demonstrate significant quantitative benefits for AD patients has called into question the exclusivity of an inflammatory mechanism of NSAID action in AD. To date, the general hypothesis underlying the potential benefit of NSAIDs in AD has been one derived from an inflammatory perspective. The elevated levels of immune cell activation, complement, and cytokines observed in AD naturally led many to hypothesize that inhibition of these inflammatory mechanisms might provide some benefit by reducing chronic inflammation, immune-mediated cell damage, and further Aβ pathology[369,381].

Recent evidence, however, suggests that NSAID neuroprotection may not be solely the result of anti-inflammatory processes but rather a collaboration of effects, not all of which are immune in nature. Epidemiological analysis has revealed that an inverse association between AD and NSAID use exists at both low and high drug dosages[26]. In fact, it has been suggested that the low naproxen dose equivalent of less than 500 mg/day used in the study would prove relatively ineffective at suppression of brain inflammation[26]. Thus, the reported inverse association might not be the product of NSAID-mediated immunomodulation but rather the result of an, as of yet, undefined alternative pathway, one which may involve NSAID-mediated effects on apoE regulation.

To date, the results of most studies have been consistent with the hypothesis that NSAID use is associated with a *protective* effect, more so than a treatment effect following diagnosis. Various inconsistencies in NSAID benefit among diagnosed AD patients only act to emphasize the potential importance of NSAID treatment in the period prior to diagnosis, as a means of either delaying the onset of AD or halting the disease process. Thus, the inability of NSAIDs to consistently provide cognitive benefit among AD patients may be a function of patient age and severity of the disease process already in progress. Studies indicate that even in mild AD cases, a loss of neurons of up to 46% can be observed in the CA1 region[442]. In contrast, no significant decreases have been isolated in healthy, pre-clinical AD groups[442]. Consequently, NSAID-mediated anti-inflammatory activity following AD diagnosis might be moot in light of the cell loss already suffered. In the face of the severity of pathology typically present even upon initial diagnosis of AD, it seems likely that NSAIDs may be best used as preventive agents.

Although COX-1 and COX-2 protein levels significantly increase in AD[381,389] and may, therefore, progressively present a larger target for NSAID action, it is unlikely that NSAID inhibition of COX enzyme activity would account for the degree of protection reported. In spite of the fact that some reduction in inflammation is likely due to NSAID-mediated COX inhibition, various other chronic mechanisms may be more than capable of perpetuating brain inflammation. For example, existing cytokines could foster further cytokine and neurotoxin production via chronic activation of astrocytes and microglia, as well as activation of the transcription factor, NF-κB. Although NSAIDs may function to alleviate the initiation of chronic AD inflammation during the pre-clinical phase, it seems unlikely that the degree of observed neuroprotection can be solely attributed to the suppression of an elevation in immune function prior to diagnosis.

All of these factors are consistent with an alternative mechanism of NSAID neuroprotection

in AD, one that may involve apoE. NSAID-mediated increases in apoE, as demonstrated in the present study, may have implications in immune function, synaptic plasticity, and Aβ clearance. Prior studies have established that apoE has the capacity to act as an immunosuppressent via inhibition of glial cytokine secretion[33], microglial activation[321], and astrocyte activation[322]. Consequently, increased levels of apoE in persons taking NSAIDs could potentially act to temper building levels of immune mediators pre-clinically, thereby reducing levels of cytokines and immune cell activation and, ultimately, limiting the chronic inflammatory cycle. Though it might be argued that diagnosed AD patients may also benefit from daily use of NSAIDs, the immune benefit, as argued above, is likely minimal and most probably dependent on the scope of the chronic inflammatory cycle already in progress, as well as the extent of cell loss already incurred.

Though NSAID neuroprotection has been classically proposed to be the product of immune modulation, the up-regulation of apoE, as seen here, suggests that increased synaptic plasticity may very well be a key alternative mechanism of NSAID action. Previous work has demonstrated that apoE protein and mRNA levels are significantly increased following entorhinal cortex lesions[275], sciatic nerve crush injuries[8], and forebrain ischemia[276]. Furthermore, apoE has been shown to mediate a neurite extension process in an allelic-dependent manner with apoE3 and apoE4 having been proven to promote increases and decreases in neurite branching and extension, respectively[277,279]. Such allelic differences in apoE-driven plasticity have been shown to be consistent with observations detailing maximal regenerative capacity in apoE ε4-negative AD patients[317], a population of subjects also having been reported to demonstrate a less pronounced AD brain immune response[17,105].

Collectively, the evidence supports the idea that increased levels of apoE following injury or in response to aging may facilitate cholesterol transport for membrane and synapse formation, terminal proliferation, and remyelination[222,271]. Consequently, the reduced risk for AD associated with prolonged NSAID use may be, in part, the result of NSAID-mediated increases in apoE and, ultimately, a greater capacity for synaptic plasticity. Pre-clinically, heightened apoE levels may provide: (1) a greater "cognitive reserve" with which to face the potential AD neurodegenerative process and (2) enhanced recovery mechanisms for early neuronal injury. Thus, such protective mechanisms may delay the onset of full AD pathology both symptomatically and histologically.

In addition to a role in synaptic plasticity, higher concentrations of apoE may also promote increased clearance of Aβ. Numerous studies have established an allelic-specific binding between

apoE and Aβ, whereby apoE3 has been found to have higher affinity for Aβ than apoE4[302,305]. Consequently, isoform-specific binding has been proposed to contribute to apoE-mediated internalization and degradation of attached Aβ[220,305,312]. Consistent with this hypothesis, *in vitro* results have indicated that the presence of Aβ increases apoE binding and internalization in neurons[313]. Furthermore, apoE knock-in mice expressing human apoE3 or apoE4 but not mouse apoE, have been found to show markedly suppressed early Aβ deposition[314]. Therefore, elevated apoE levels pre-clinically may reduce AD risk by clearing early deposits of Aβ, potentially delaying the progression of more serious Aβ pathology.

The precise mechanisms underlying NSAID-mediated apoE induction remain unclear. Nevertheless, in an attempt to analyse the role of COX enzymes in this process, COX-2 selective and inactive NSAID derivatives were utilized. The results indicate that astrocyte treatment with the COX-2 selective aspirin derivative, APHS, and its inactive phenol resulted in a significant increase in apoE protein and a trend towards increased protein, respectively. However, the concentrations at which these effects were seen fell within the nanomolar-picomolar range. Given the fact that the IC_{50} value for APHS-mediated inhibition of COX-2 has been published to be 0.8 μM[368], it is unlikely that the apoE induction seen here was the result of COX-2-mediated processes. Moreover, the ability of the inactive APHS phenol compound to affect apoE levels also suggests of a COX-independent pathway. Furthermore, in light of an aspirin IC_{50} value of 62.5 μM[366], aspirin-mediated apoE increases at concentrations less than 1 nM provide further evidence of an alternative mechanism. Thus, the results indicate that aspirin and aspirin-like molecules, at physiological concentrations, have the ability to positively affect apoE expression.

In contrast, COX-2 selective and inactive indomethacin derivatives were found to induce significant decreases in astrocyte and mixed glial apoE protein levels. These decreases were detected over a large span of drug concentrations, generally within the micromolar-picomolar range. The published IC_{50} values for these COX-2 selective indomethacin amides have been listed as 0.12 μM and 0.060 μM[365], thereby suggesting that the drug concentrations exhibiting apoE-reducing effects may have involved some COX-inhibitory activity. The bulk of the data demonstrating reductions in apoE protein reflected treatment at concentrations far below the micromolar range, indicating the potential involvement of a COX-independent mechanism. Again, such a COX-independent mechanism would be consistent with the significant reductions in apoE levels induced by the inactive indomethacin derivative, as well as the significant increases induced

by indomethacin at concentrations far below its own IC_{50} value of 0.75 μM[365]. In addition, the indomethacin concentrations utilized were not only far below the IC_{50} value but also well within physiological concentrations described in human plasma and CSF, both of which lie in the nanomolar range[364].

Unlike aspirin, APHS, and APHS phenol, the results show that indomethacin and its derivatives induced conflicting increases and decreases in apoE protein, respectively. The mechanisms underlying the opposing effects of these compounds may require specific structural components of indomethacin, components that have been augmented in the COX-2 selective compounds. The substitution or addition of chemical side groups may have, in turn, altered subsequent structural interactions and, ultimately, the nature of the drug's effect on apoE regulation.

Thus, the ability of NSAIDs and NSAID derivatives to induce significant changes in apoE expression at physiological and sub-physiological concentrations well below their IC_{50} values, as well as the ability of inactive COX inhibitors to significantly affect apoE protein levels, lends support to the hypothesis that these compounds, most likely, mediate their apoE effects via a COX-independent pathway. One such pathway may involve NSAID effects on the transcription factors, NF-κB and PPAR-γ. Studies have established that NSAIDs including aspirin and ibuprofen, inhibit NF-κB activation[382,383,396,397,402]. NF-κB has been implicated in the expression of such mediators as IL-1, IL-6, TNF-α, and iNOS[404,405]. Consequently, NSAID-mediated inhibition of NF-κB could potentially reduce expression of pro-inflammatory cytokines and other toxins. However, the question remains as to whether NF-κB plays a role in apoE gene regulation. Though this avenue of research has yet to be explored, the fact that NSAIDs have the capacity to affect gene expression at a transcriptional level indicates the need to investigate this area further.

PPAR-γ, a member of a nuclear receptor family of transcription factors that regulate ligand-dependent transcriptional activation and repression[411], has also been implicated in NSAID activity. Indomethacin and ibuprofen have both been found to act as PPAR-γ and PPAR-α agonists[384] with the potential to inhibit expression of TNF-α, COX-2, and iNOS[381,386,387,410,412]. Thus, it has been postulated that the anti-inflammatory effects of indomethacin and ibuprofen in AD may be due, in part, to PPAR activation and inhibition, at a transcriptional level, of cytokine, COX, and neurotoxin expression[386,410]. Of particular interest, PPAR-γ agonists have also been reported to inhibit Aβ-stimulated expression of IL-6 and TNF-α[387], thereby emphasizing the potential benefit of PPAR

and PPAR agonists in Aβ-induced immunopathology.

Unlike NF-κB, however, a substantial link has been established between PPAR and lipid homeostasis, and specifically apoE regulation. Two recent studies have implicated PPAR-γ and PPAR-α in reverse cholesterol transport and cholesterol synthesis, respectively[413,414]. Specifically, increased cholesterol efflux has been observed following PPAR-γ agonist treatment of macrophages, fibroblasts, and intestinal cells[413]. Furthermore, gene analysis has revealed a PPAR response element in the proximal promoter of the human HMG-CoA synthase gene[414].

In light of the fact that NSAIDs may activate PPAR and that activation of PPAR is associated with increased cholesterol efflux, up-regulation of apoE expression may be required in order to facilitate cholesterol transport and removal. In support of this hypothesis, recent evidence has demonstrated the presence of a peroxisome proliferator response element (PPRE) located in the apoE/apoCI intergenic region[415]. In fact, treatment with a PPAR-γ agonist at micromolar concentrations has been reported to induce apoE mRNA two-fold in THP-1 cells[415]. Consequently, these results lend support to the hypothesis that NSAID-mediated increases in astrocyte and mixed glial extracellular apoE protein, as reported in the current study, may have been the product of NSAID activation of PPAR with subsequent modulation of apoE transcription.

Although the current experiments failed to show any significant changes in apoE mRNA levels following NSAID or NSAID derivative treatment, this may have been the result of low statistical power. However, it may also be hypothesized that increased mRNA stabilization rather than increased apoE mRNA quantity was the underlying mechanism of protein level changes. Thus, it must be argued that transcriptional modulation may not be the sole mechanism underlying significant increases in apoE protein.

In fact, up-regulation of extracellular apoE protein may have been the product of various other metabolic mechanisms including changes in the rates of apoE recycling, secretion, and degradation. Upon internalization of apoE via specific receptors and release of cholesterol, it has been observed that apoE may be degraded or resecreted for subsequent action[248,254,255]. Consequently, elevated levels of extracellular apoE protein may have been the result of enhanced recycling of pre-existing apoE following release of lipid particles, as opposed to *de novo* protein synthesis.

In addition, increased secretion of newly synthesized apoE protein residing in cell surface protein pools may have contributed to overall increases in extracellular apoE protein. Prior work

has established that secretion of intracellular apoE is dependent upon the addition of carbohydrate chains containing sialic acid within the Golgi apparatus[247,249,250]. Thus, an enhanced rate of apoE modification may have facilitated apoE secretion and, ultimately, increased extracellular apoE protein levels.

Moreover, reduced apoE protein degradation may have been responsible, in part, for the observed protein effect. Studies have established that newly synthesized apoE may be targeted from the Golgi network to lysosomes for degradation prior to secretion[251,252]. As such, reduced targeting of apoE protein to lysosomal compartments may have prolonged the life of the apoE protein, thereby allowing for subsequent addition of carbohydrate chains and secretion. Hence, reduced intracellular degradation could have elevated extracellular apoE protein levels, as detected in the present study.

Although such mechanisms remain speculative, *in vitro* evidence has suggested that apoE protein regulation may be mediated by such non-transcriptional mechanisms[443]. In fact, experiments have demonstrated significant differences in apoE secretion, degradation, and release from the cell surface in an allelic-specific manner[443]. *In vitro* studies have established higher apoE secretion and degradation in apoE3/3 and apoE2/2 cells, respectively[443]. Consequently, significant increases in apoE protein, as seen in the present study, may have been the product of significant changes in apoE metabolism.

In order to investigate the relationship between NSAIDs and apoE expression, primary rat astrocyte and mixed glial cell cultures were treated with pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Elevated levels of apoE were detected following IL-1β and IL-6 treatment of mixed glia and astrocytes, respectively. Both mixed glia and astrocytes, however, showed significant reductions in apoE protein following exposure to TNF-α. Main effects analysis generally revealed increases in apoE protein with longer incubation time in both rodent astrocyte and mixed glial cell cultures. These results, though not unexpected, may have been the result of increasing cell number with time.

Increased levels of apoE protein in response to IL-1β and IL-6 exposure may have been the result of gliosis in culture. Both IL-1β and IL-6 have been reported to stimulate gliosis, thereby increasing cell number and, consequently, apoE production[195,444-446]. However, the elevations in apoE protein may have been indicative of a cytokine signal for apoE-mediated remodeling following injury, as well as a means of immunoregulation.

Numerous studies have demonstrated increased expression of IL-1β and IL-6 following such injuries as trimethylin-induced neurodegeneration[447], ischemia[448,449], cortical impact injury[450], and mechanical trauma[451]. Temporally, elevated cytokine expression has been shown to be dependent upon the specific trauma inflicted; however, IL-1β and IL-6 expression have been typically identified within hours to two days following injury[449-452]. In contrast, apoE induction has been found to peak 6 days following lesion of the entorhinal cortex[453]. As pro-inflammatory cytokines, IL-1β and IL-6 may trigger macrophage activation and subsequent neuronal damage. Such damage, however, could potentially release a wide assortment of lipids, thereby requiring later apoE-mediated transport and removal. This is consistent with the temporal pattern of apoE induction with apoE mRNA levels increasing a few days after heightened cytokine expression. Subsequent apoE protein expression could facilitate cholesterol internalization for membrane and synapse formation and, ultimately, neuronal remodeling. Thus, from an AD perspective, heightened levels of cytokine expression in the brain may trigger apoE-mediated recovery mechanisms, thereby potentially countering some of the detrimental effects of prolonged cytokine exposure.

In addition to remodeling processes, increasing apoE protein levels in the period following cytokine induction can also be a means by which elevated immune activation is partially suppressed, thereby terminating the immune response. Prior studies have shown apoE to be capable of suppressing lymphocyte proliferation[292] and glial secretion of cytokines[33]. Thus, increased apoE protein levels may be indicative of an endogenous negative feedback mechanism by which the brain limits further cytokine stimulation.

Therefore, cytokine-induced increases in apoE protein may have been related to cytokine signaling for apoE-regulated immune modulation, neuronal remodeling, and recovery. Moreover, in the pre-diagnostic stage, mild elevations in cytokines may foster increased apoE-mediated recovery processes that, ultimately, act to delay AD onset.

In contrast, a reduction in apoE protein was observed following TNF-α treatment. Though the mechanism underlying this decrease in extracellular protein remains speculative, in light of the apoE induction associated with IL-1β and IL-6 treatment, TNF-α-mediated decreases could act as a counter-balancing force with which to prevent unfettered increases in apoE production. One potential mechanism may involve TNF-α effects on the transcription factor, PPAR-γ. Recent work has found IL-1β, IL-6, and TNF-α all able to decrease PPAR-γ expression in mature rat

adipocytes[454]. Specifically, TNF-α has been found to inhibit adipogenesis in adipose tissues via downregulation of adipogenic factors such as PPAR-γ[455]. Moreover, injection of TNF-α into rats has been associated with decreased hepatic PPAR-α expression[456]. As previously described, PPAR may have a positive effect on apoE expression via a PPRE in the apoE gene[415]. Consequently, TNF-α-mediated decreases in PPAR-γ expression may inhibit PPRE-mediated apoE expression, as was observed in the current study. Though both IL-1β and IL-6 have also been shown to reduce PPAR expression, two key factors that may impact each cytokine's effect on PPAR remain unclear: (1) tissue specificity of action and (2) relative inhibitory strength of IL-1β and IL-6, compared to TNF-α. The majority of experiments describing cytokine action on PPAR expression has involved adipocytes and not glia, thereby leading to the question as to whether the listed cytokines may have opposing effects on brain cells. In addition, the variability of individual cytokine strength may play a role in the differential effects of TNF-α, IL-1β, and IL-6 on apoE expression.

Although the focus of the present study was the impact of immune system regulators on apoE expression, other drugs including 17-β-estradiol and probucol were used in treatment strategies so as to examine the nature of apoE expression in the cell culture systems utilized. Epidemiologically, estrogen replacement therapy (ERT) has been found to reduce the risk of developing AD[457-459]. Though the precise mechanisms underlying this estrogen effect may be varied and complementary, it has been suggested that estrogen's involvement in dendritic spine density, synaptic excitability, cholinergic neurotransmission, and cerebrovasculature, may play some beneficial role in AD[460]. Moreover, estrogen has been shown to have an anti-inflammatory effect, whereby co-treatment of microglia with LPS and estrogen reduced microglial activation[461].

A tangible link between estrogen and apoE has also been identified in which mixed glial cell cultures of astrocytes and microglia have responded to estrogen treatment with increased apoE mRNA and protein[221]. In the present study, 17-β-estradiol treatment of primary rat astrocytes also induced significant increases in apoE protein. The functional implications of this result may include increased apoE-mediated immunosuppression, synaptic plasticity, and Aβ clearance, thereby contributing to the decreased relative risk of AD associated with ERT. In fact, studies have shown that estradiol-enhanced synaptic sprouting following entorhinal cortex lesion operates in an apoE-dependent manner as lesioned apoE-deficient mice failed to demonstrate enhanced

sprouting following estradiol exposure[462]. In addition, recent work demonstrating increased Aβ uptake by human frontal cortex microglia following estrogen treatment, ties in nicely with the hypothesis that estrogen-derived increases in astrocyte apoE could lead to increased apoE-Aβ binding and subsequent clearance[132,220].

With the link between apoE and AD having been established, other studies have assessed the potential relationship between cholesterol and AD. Various epidemiological and clinical studies have determined that high levels of serum cholesterol and atherosclerosis are both linked to AD[463,464]. Furthermore, retrospective studies have illustrated that use of statins to treat hypercholesterolemia may be associated with a decreased prevalence of AD[465,466]. As such, probucol was used in the present study to assess the relationship between a cholesterol-lowering drug and apoE levels in primary rat glial cell cultures. It has been proposed that probucol lowers plasma levels of LDL and HDL cholesterol via increased LDL catabolism and decreased flux of LDL[467,468]. In fact, published data has revealed that probucol-treated rabbits show a 2-4 fold increase in brain apoE mRNA, compared to control rabbits[422]. In addition, the cholesterol-lowering effect of probucol has been described to be apoE allele-dependent with apoE ε4 familial hypercholesterolemia patients showing greater reductions in cholesterol, compared to apoE ε3 patients[423].

In the current pilot study, initial data failed to demonstrate any significant differences in apoE mRNA levels between control and probucol-treated cells. However, this may have been the result of low statistical power. Nevertheless, recent experiments have reported a three-fold increase in rat hippocampal apoE protein levels following chronic probucol treatment[469]. Consequently, increased apoE protein levels could enhance apoE-mediated cholesterol removal, neuronal remodeling, and Aβ clearance, all of which would prove beneficial in AD pathogenesis. Although the current probucol experiments failed to support such a hypothesis, there remains strong evidence for the argument that cholesterol-lowering drugs may provide some benefit in AD via apoE-inducing mechanisms.

In contrast to the anti-inflammatory hypothesis presented here, many researchers have recently focused upon vaccination as an alternative AD immune treatment. With elevated Aβ deposition having been clearly identified in AD, it has been suggested that Aβ vaccination could invoke an antibody response against Aβ, thereby leading to immune system removal of Aβ protein and reduced pathology[470,471]. *In vivo* mouse studies have shown that active immunization of mice

overexpressing APP against human Aβ resulted in reduced Aβ plaque burden[471,472]. In addition, Aβ peptide immunization has been reported to reduce the behavioural impairments, particularly memory loss, characteristic of transgenic mice overexpressing APP[472,473]. In spite of the promising mouse model results, recent phase IIa human clinical trials have proven to be far less successful. In fact, recent reports have indicated that some AD trial patients demonstrated CNS inflammation following vaccination[474]. Moreover, it was recently disclosed at the 7th International Alzheimer's Disease Conference that Aβ vaccination resulted in severe encephalitis and paralysis among some AD patients. Though the cause of the these side-effects remains unclear, it has been suggested that the creation of Aβ antibodies could potentially lead to interactions and further immune system activation, a potentially devastating consequence[474]. Though enhancement of the immune system is still a worthwhile avenue of exploration, it is clear that there may be inherent problems to an active immunization methodology. Consequently, anti-inflammatory pathways must be examined as another viable alternative.

Summary, Conclusions, and Implications

In summary, the present study demonstrated that indomethacin, aspirin, and an aspirin derivative may induce significant increases in extracellular apoE protein following treatment of primary rat astrocytes and mixed glia. IL-1β and IL-6 treatment also resulted in significantly elevated levels of apoE protein in mixed glial and astrocyte cell cultures, respectively. In contrast, TNF-α treatment of both astrocytes and mixed glia was associated with significantly reduced apoE protein levels. Functionally, increased levels of apoE, the result of NSAID treatment, may temper building chronic inflammation, enhance Aβ clearance, and facilitate synaptic remodeling. Thus, these apoE-mediated processes may all act in concert to reduce the risk and delay the onset of AD. In addition, early cytokine synthesis may be countered by cytokine-induced apoE protein, thereby minimizing the effects of pre-clinical inflammation, as well as contributing to neuronal recovery.

Consequently, these results indicate that NSAID neuroprotection in AD may be the product of increased apoE levels, thereby indicating the need to further investigate treatment strategies targeting apoE regulation in individuals at risk for AD, as well as those in the early stages of the disease process. To date, three classes of drugs have been shown to be associated with reduced risk for AD: (1) hormones (i.e., estrogen)[457,458], (2) cholesterol-reducing drugs[465,466], and (3) NSAIDs[27,369]. Of key importance, however, is the fact that estrogen and the cholesterol-reducing drug, probucol, have both been shown to increase apoE expression[221,422]. Moreover, the results from the present study are consistent with this trend as 17-β-estradiol, indomethacin, and aspirin all increased extracellular apoE protein levels *in vitro*. Thus, it is clear that one common thread among these compounds is their ability to increase apoE expression. As such, future clinical studies should examine the effect of other apoE-modulating drugs on AD risk. In addition, future drug development projects must assess the viability of agents aimed at disease prevention via apoE-mediated pathways.

Although the presented results demonstrate a clear relationship between NSAIDs and apoE, this association must be extended to *in vivo* experiments so as to understand fully the physiological ramifications of NSAID treatment on lipid homeostasis and apoE. Future work must also concentrate on elucidating the precise mechanisms underlying NSAID-mediated apoE induction. Specifically, the role of the transcription factors, NF-κB and PPAR-γ, in apoE regulation must be pursued. Moreover, the biological apoE cascade should be delineated by exploring the

effects of NSAIDs and other inflammatory mediators on apoE receptors including the LDL receptor, LDL receptor related protein, and apoE-R2 receptor. Nevertheless, the results presented here mark a step forward in our understanding of NSAID mechanisms in AD, as well as provide useful information about potential strategies for future treatments aimed at delaying or preventing the disease.

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NSAID	COX-1	IC_{50} (μM)	COX-2	IC_{50} (μM)
Indomethacin	+++	0.050	+	0.75
COX-2 selective indomethacin analog #1	N/A	> 66	+++	0.060
COX-2 selective indomethacin analog #2	N/A	> 66	+++	0.12
Inactive indomethacin analog	N/A	N/A	N/A	N/A
Ibuprofen	+	2.1	++	1.6
Aspirin	+++	12.5	+	62.5
COX-2 selective aspirin analog	N/A	17	+++	0.8
Inactive aspirin analog	N/A	N/A	N/A	N/A

Table 1: Summary table detailing the effects of various agents on cyclooxygenase (COX)-1 and -2 activity and their respective IC_{50} values; (+) = low degree of inactivation; (+++) = high degree of inactivation

Alzheimer's Disease	↑	↑	↑	(E3) -/ (E4) ↓
AGENT	**COX-1**	**COX-2**	**PPAR**	**ApoE Protein**
Indomethacin	–	–	+	↑
COX-2 selective indomethacin analog #1	N/A	–	?	↓
COX-2 selective indomethacin analog #2	N/A	–	?	↓
Inactive indomethacin analog	N/A	N/A	?	↓
Ibuprofen	–	–	+	↑ (Trend)
Aspirin	–	–	+	↑
COX-2 selective aspirin analog	N/A	–	?	↑
Inactive aspirin analog	N/A	N/A	?	↑ (Trend)
Interleukin-1β	N/A	↑	↓	↑
Interleukin-6	N/A	N/A	↓	↑
Tumour Necrosis Factor-α	N/A	↑	↓	↓

Table 2: Summary table detailing the effects of various agents on extracellular apolipoprotein E (apoE) protein expression. COX = cyclooxygenase; PPAR = peroxisome proliferator activated receptor; N/A = non-applicable; ↑ = increased expression; ↓ = decreased expression; (+) = activation; (–) = inactivation

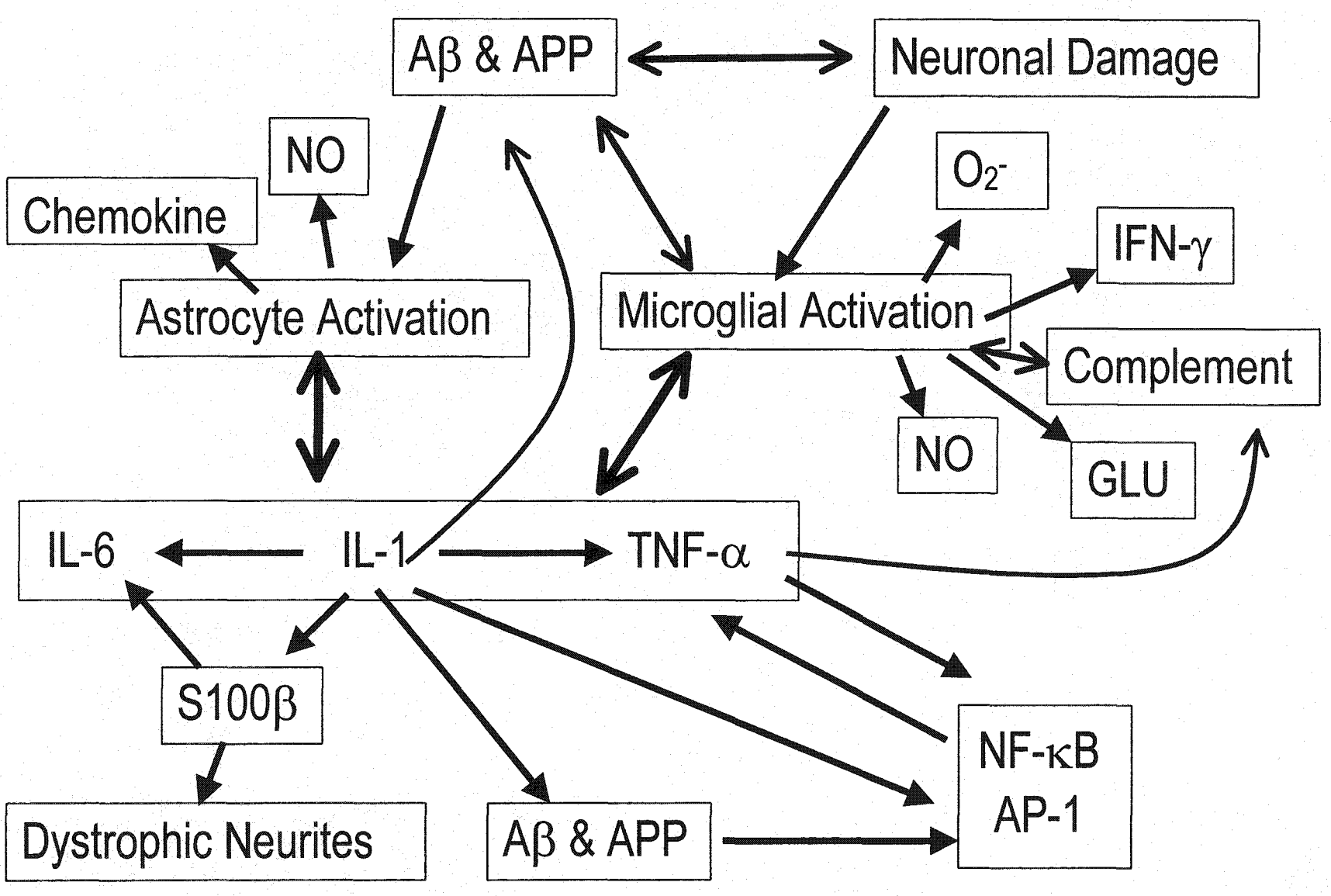


Figure 1: Summary of chronic AD inflammation. GLU = glutamate; O_2^- = superoxide anions

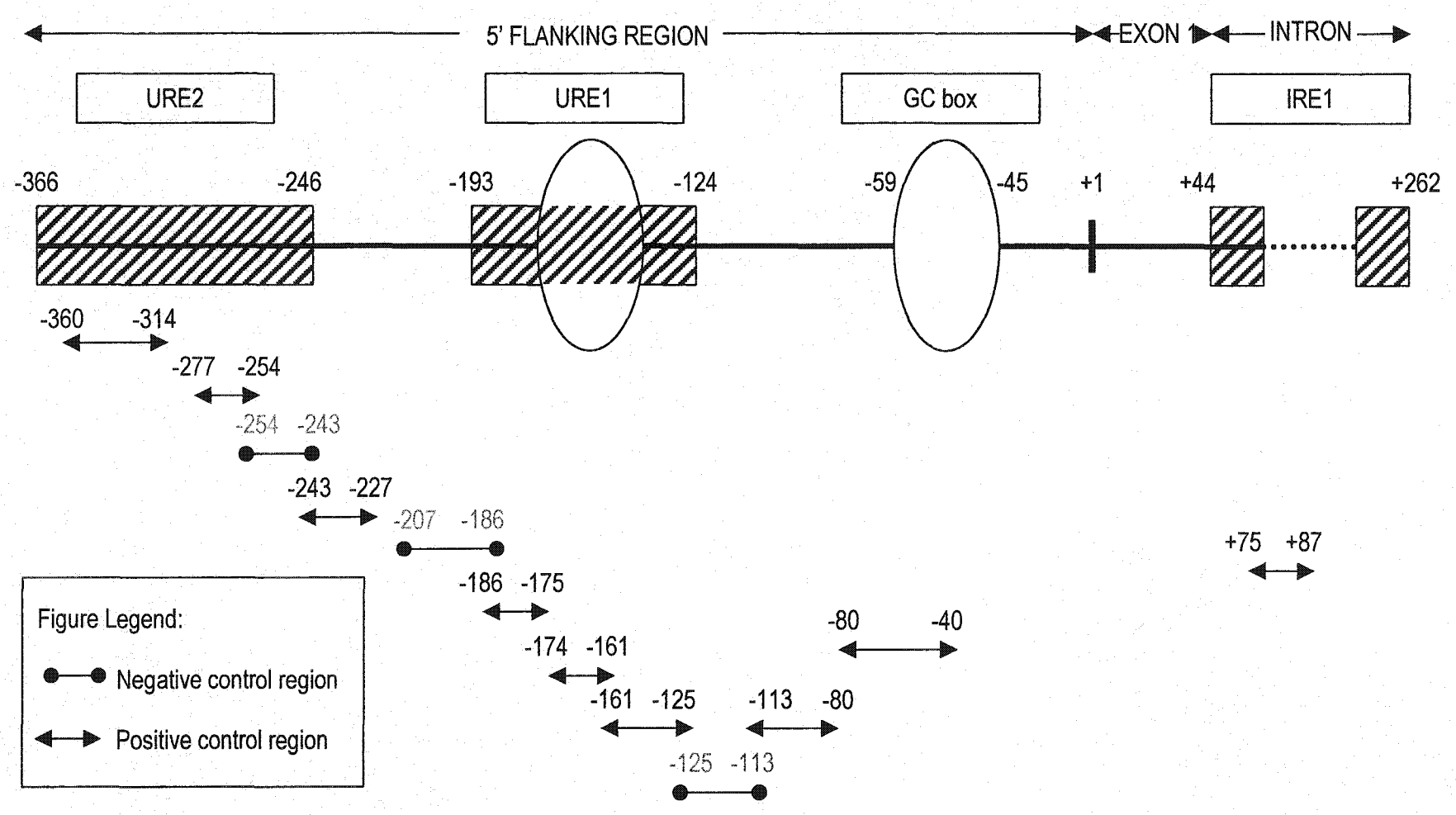


Figure 2: Mapping of the 5' flanking region and first intron of the apolipoprotein E gene. The hatched boxes reflect major gene domains that have demonstrated enhancer-like activity. The open ovals represent potential protein binding sites. In addition, the numbers indicate the nucleotide sequence in base pairs relative to the apoE transcription initiation site (+1). Finer mapping analysis is represented by the series of arrows beneath the main diagram. Arrow lines and bulb lines represent positive and negative control regions, respectively.

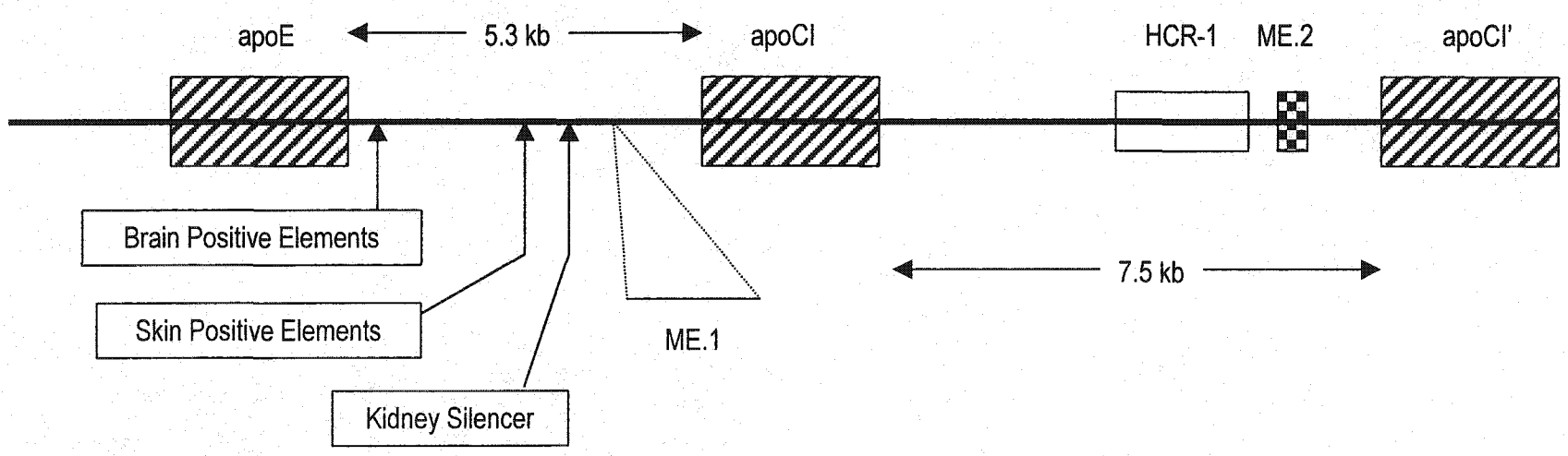
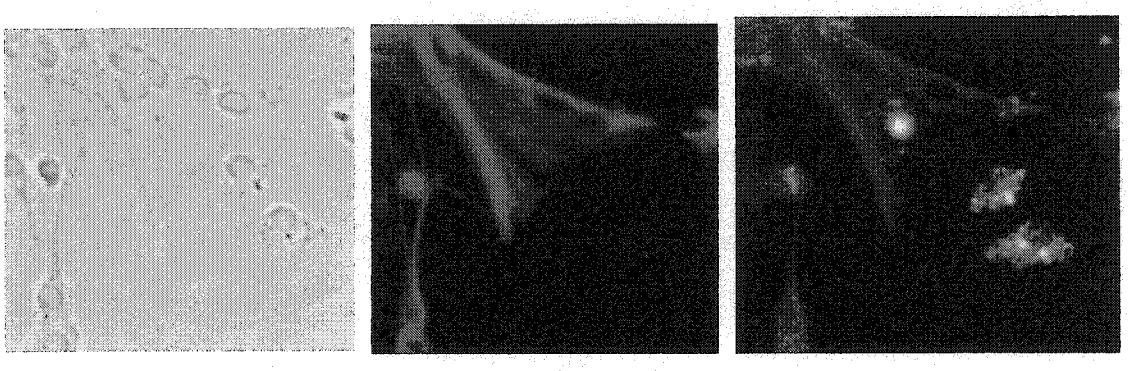
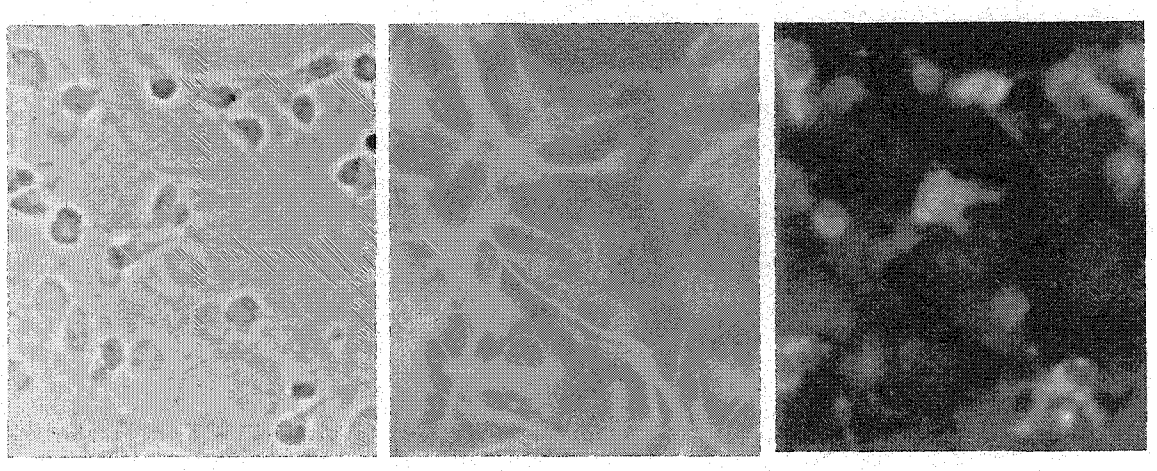


Figure 3: Mapping of the apolipoprotein E gene. The hatched boxes reflect individual apolipoprotein genes within the gene cluster. The HCR region reflects the hepatic control region while the ME.1 and ME.2 regions represent astrocyte-specific distal enhancers.



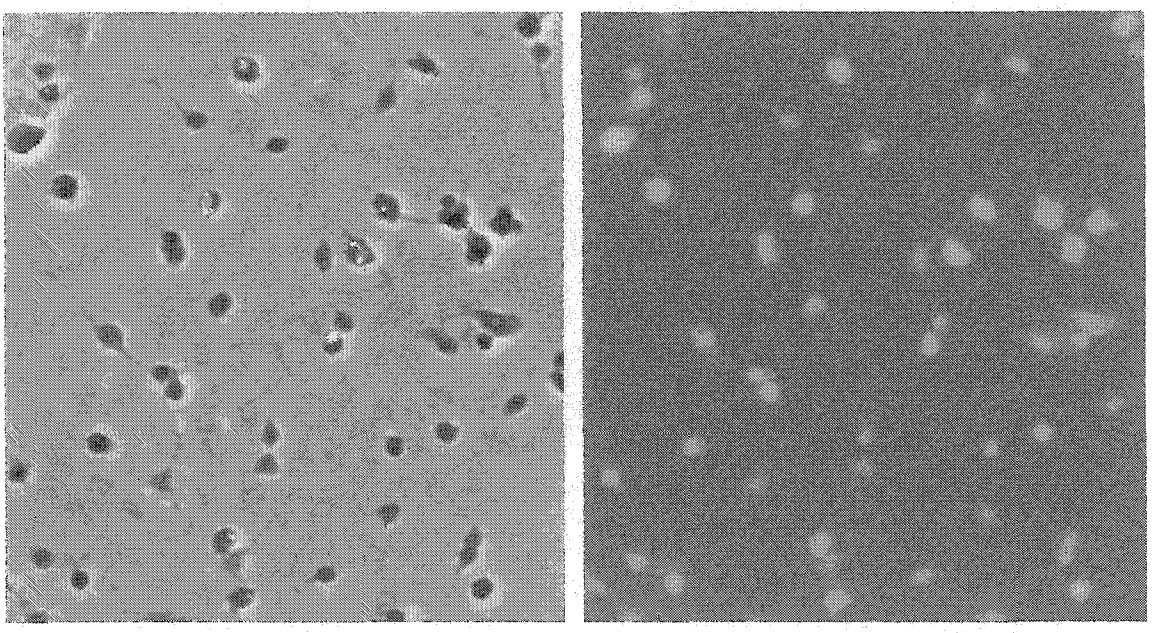
(a) (b) (c)

Figure 4: Primary rat astrocyte cell cultures fluorescent antibody labeling. Astrocyte cell cultures were labeled with fluorescent antibodies specific for the antigens, glial fibrillary acidic protein (GFAP) in astrocytes, and ED-1 in microglia. (a) Astrocyte cell cultures with no antibody labeling. (b) Astrocytes within the same cell culture sample labeled positively with anti-GFAP antibodies were identified with Texas red dye. (c) Microglia within the same astrocyte cell culture sample were isolated with anti-ED-1 antibodies as shown by the cells labeled positively with fluorescein isothiocyanate (FITC). Astrocyte cell cultures were shown to be ~95% pure with only ~5% microglial contamination.



(a) (b) (c)

Figure 5: Primary rat mixed glial cell cultures fluorescent antibody labeling. Mixed glial cell cultures were labeled with fluorescent antibodies specific for the antigens, glial fibrillary acidic protein (GFAP) in astrocytes, and ED-1 in microglia. (a) Mixed glial cell cultures with no antibody labeling. (b) Astrocytes within the same mixed cell culture sample labeled positively with anti-GFAP antibodies were identified with Texas red dye. (c) Microglia within the same glial cell culture sample were isolated with anti-ED-1 antibodies as shown by the cells labeled positively with fluorescein isothiocyanate (FITC). Mixed glial cell cultures were shown to be composed of ~70% astrocytes, 25% microglia, and 5% oligodendrocytes.



(a) (b)

Figure 6: Acridine orange staining. Primary rat astrocyte cell cultures (a) were stained with the monovalent, cationic dye, acridine orange, in order to assess cell viability following 96 hours of treatment with the COX-2 selective indomethacin derivative, LM 4108, at a concentration of 10^{-8} M. Viable cells appeared to be fluorescent green following staining (b). Both primary rat astrocyte and mixed glial cell cultures showed over 95% cell viability upon treatment with all agents used at the concentrations reported.

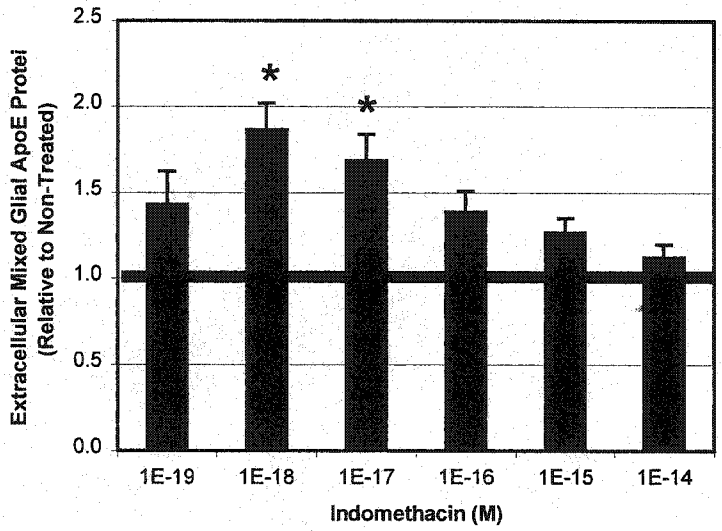


Figure 7: Mean (± standard error of the mean) mixed glial extracellular apolipoprotein E (apoE) protein following 96 hours as a function of indomethacin treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean protein values were derived from an average of $n \geq 6$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

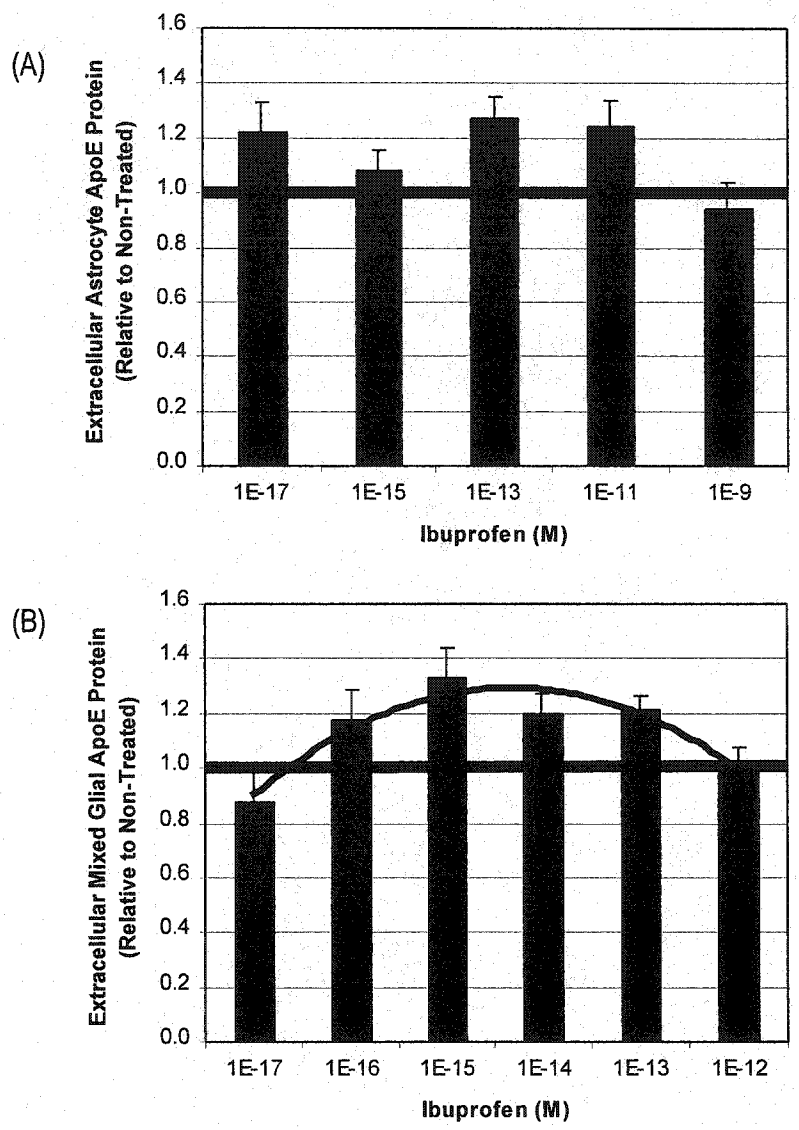


Figure 8: Mean (± standard error of the mean) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of ibuprofen treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte and mixed glial protein values were derived from an average of $n \geq 9$ and $n \geq 8$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

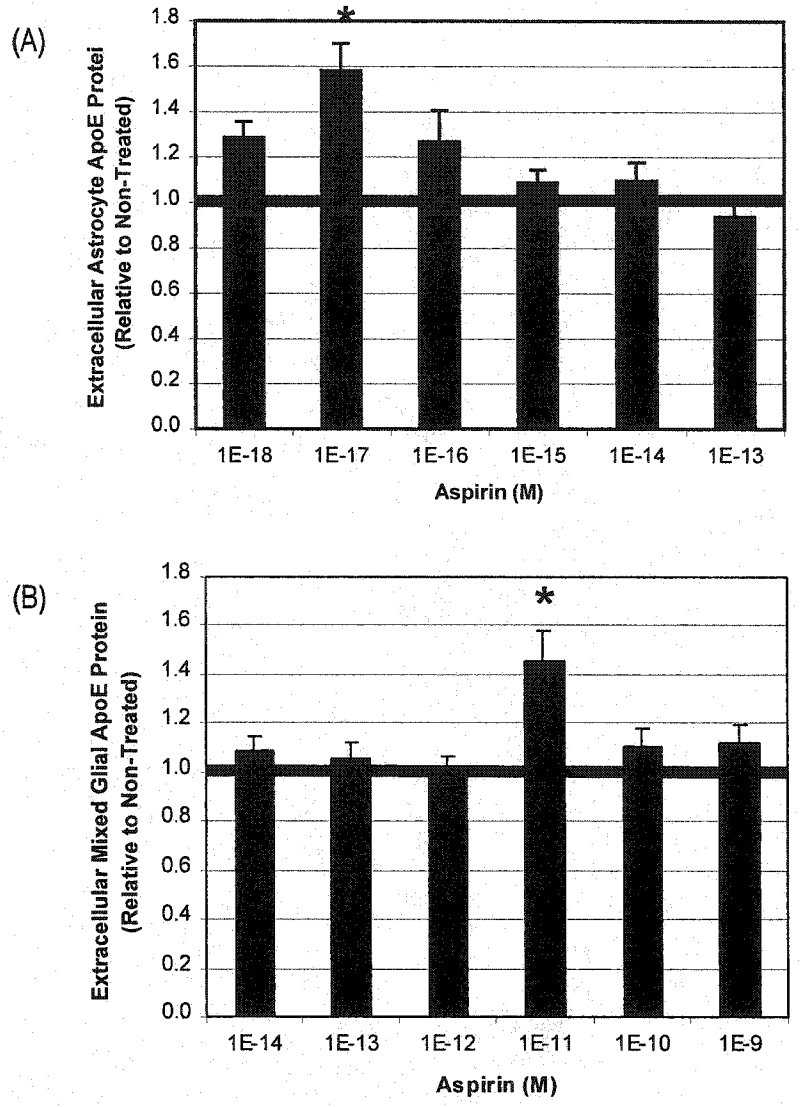


Figure 9: Mean (± standard error of the mean) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of aspirin treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte and mixed glial protein values were derived from an average of $n \geq 8$ and $n \geq 25$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

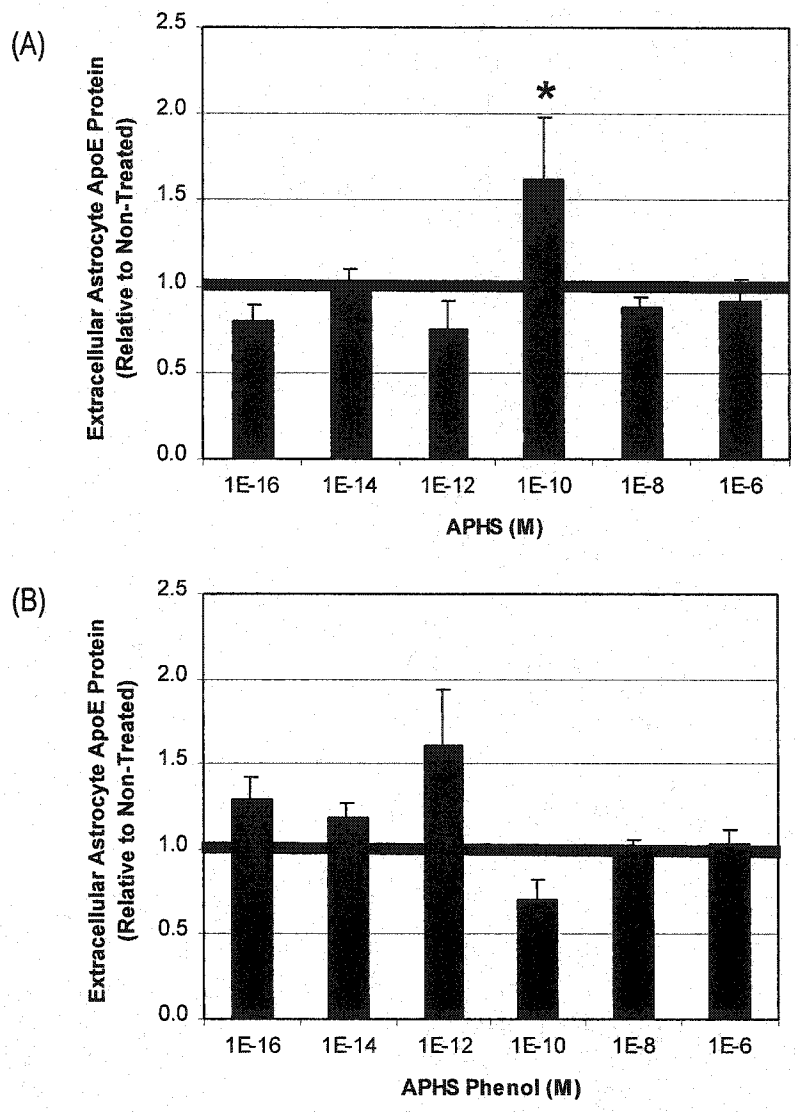


Figure 10: Mean (± standard error of the mean) astrocyte extracellular apolipoprotein E (apoE) protein after (A) 24 hours of APHS and (B) 96 hours of APHS phenol treatment. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte protein values following APHS and APHS phenol treatment were derived from an average of $n \geq 5$ and $n \geq 6$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

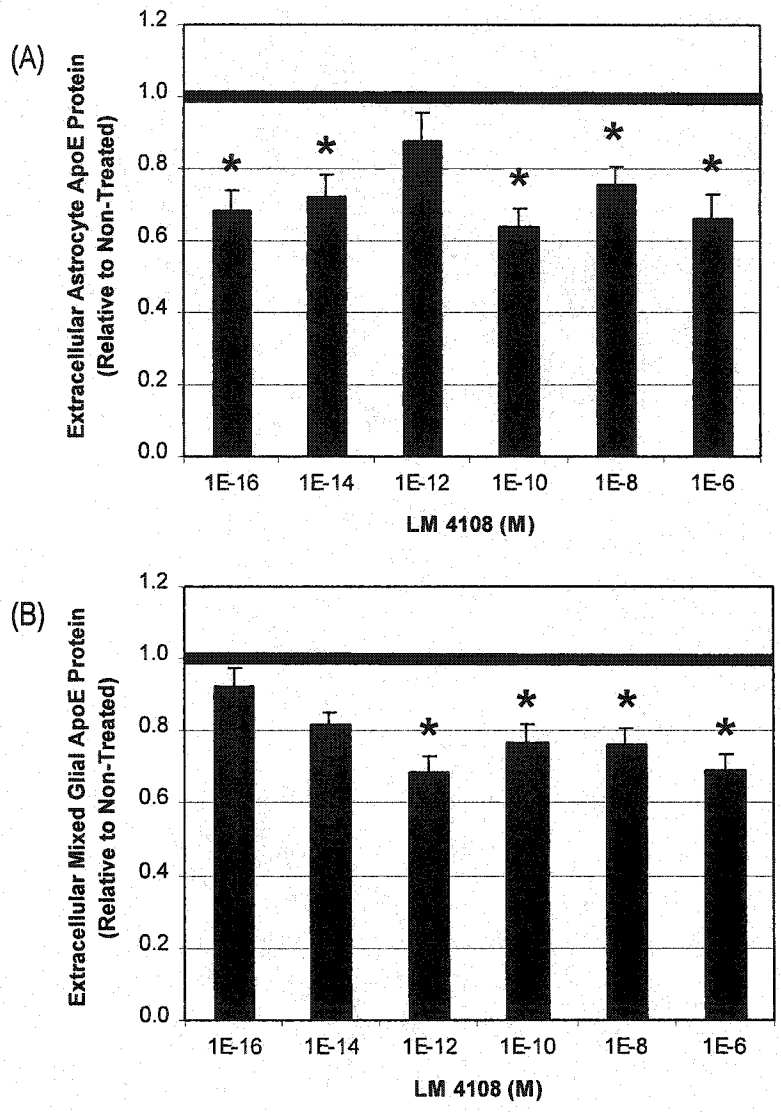


Figure 11: Mean (± standard error of the mean) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of LM 4108 treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte and mixed glial protein values were derived from an average of $n \geq 34$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

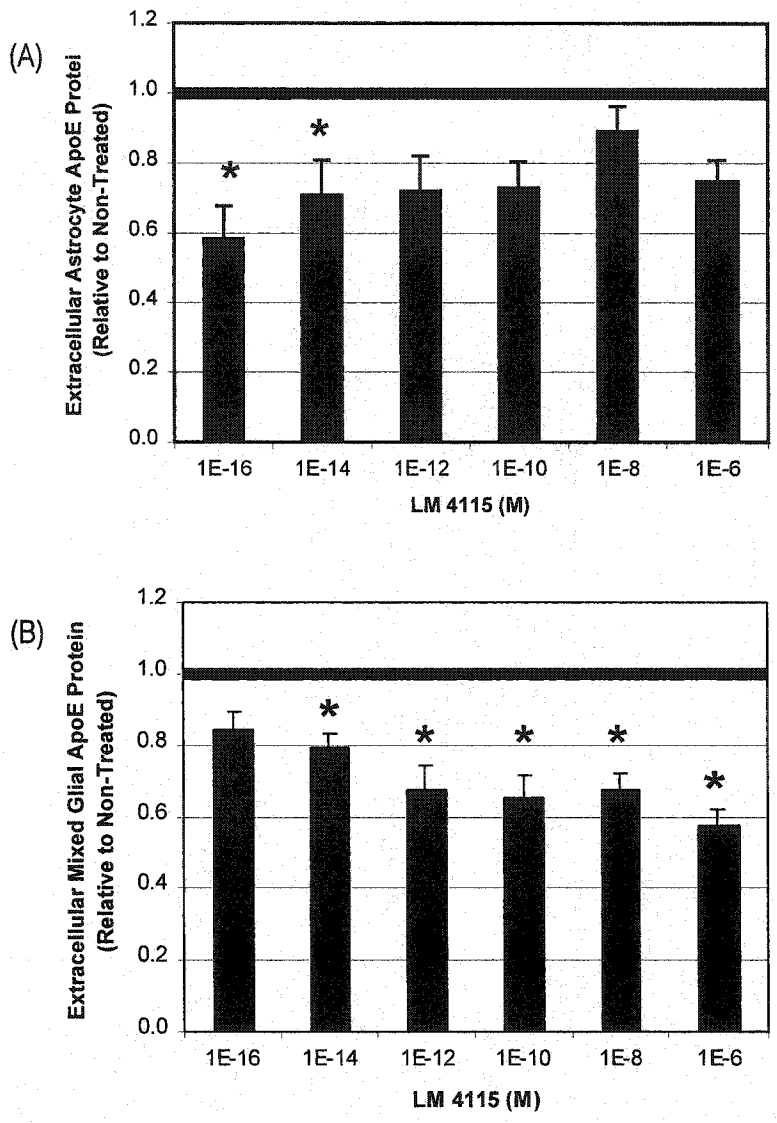


Figure 12: Mean (± standard error of the mean) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of LM 4115 treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte and mixed glial protein values were derived from an average of $n \geq 23$ and $n \geq 33$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

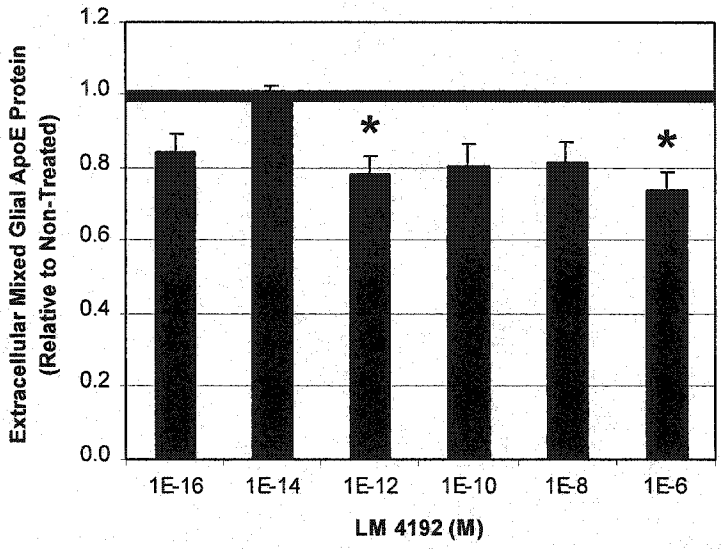


Figure 13: Mean (± standard error of the mean) mixed glial extracellular apolipoprotein E (apoE) protein as a function of LM 4192 treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean protein values were derived from an average of $n \geq 33$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

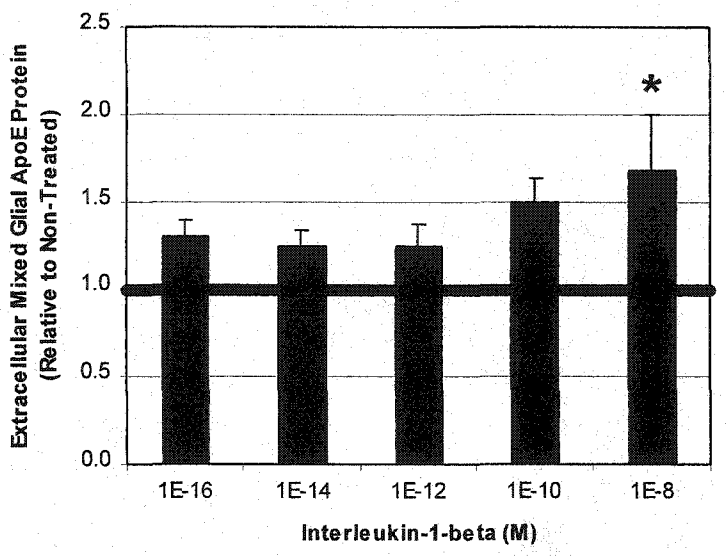


Figure 14: Mean (± standard error of the mean) mixed glial extracellular apolipoprotein E (apoE) protein as a function of interleukin-1β treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean protein values were derived from an average of $n \geq 6$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

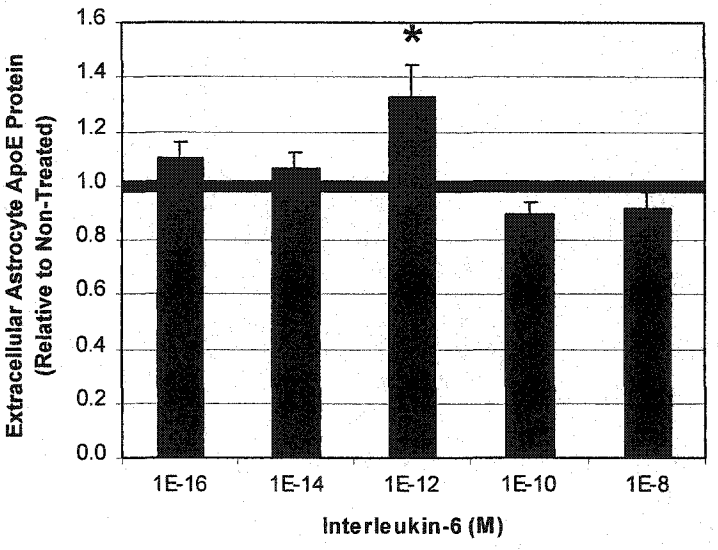


Figure 15: Mean (± standard error of the mean) astrocyte extracellular apolipoprotein E (apoE) protein as a function of interleukin-6 treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean protein values were derived from an average of $n \geq 23$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

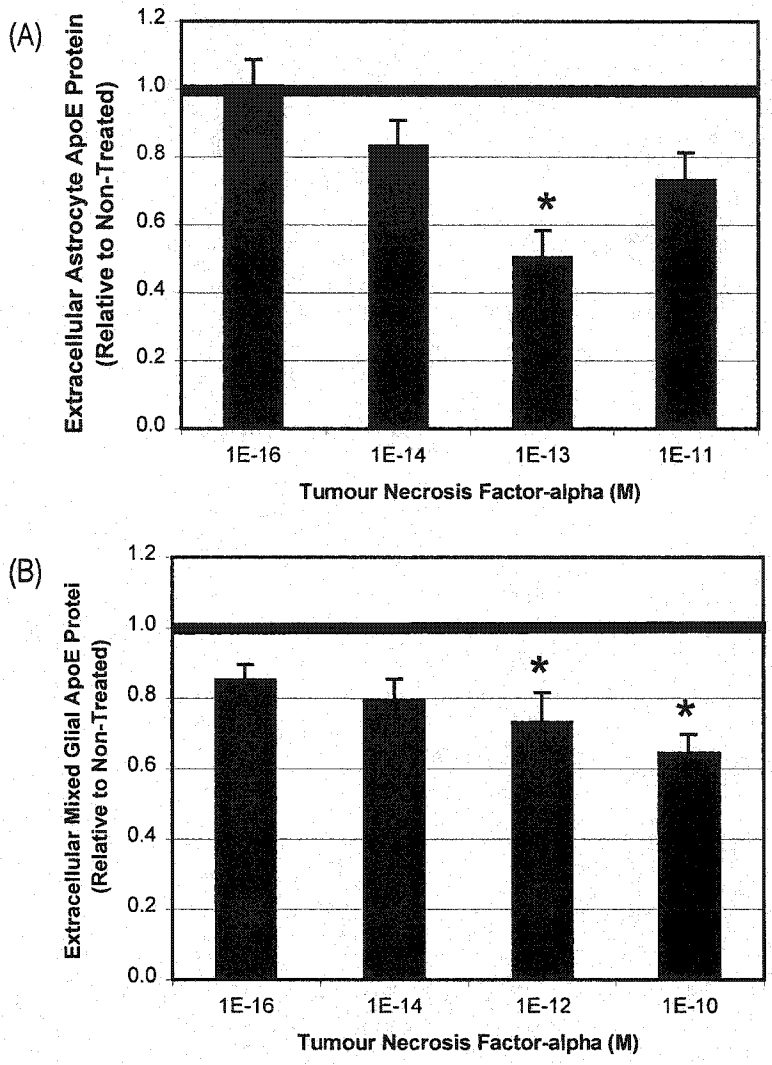


Figure 16: Mean (± standard error of the mean) (A) astrocyte and (B) mixed glial extracellular apolipoprotein E (apoE) protein as a function of tumour necrosis factor-α treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean astrocyte and mixed glial protein values were derived from an average of $n \geq 9$ and $n \geq 22$ cell culture wells, respectively. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

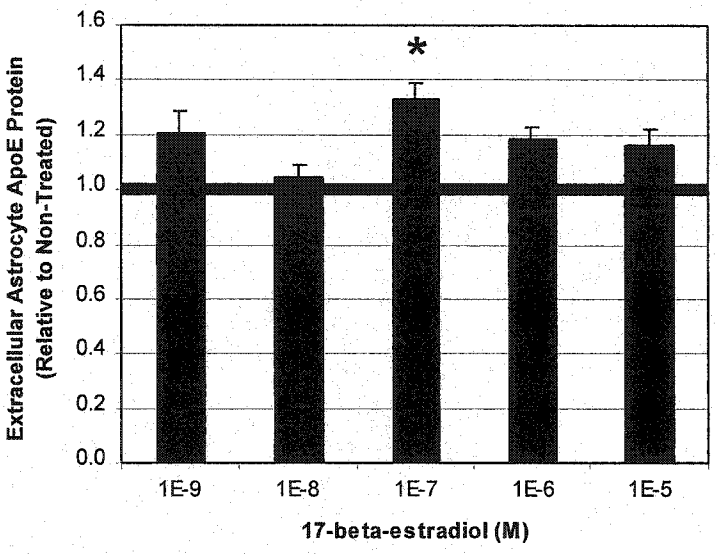


Figure 17: Mean (± standard error of the mean) astrocyte extracellular apolipoprotein E (apoE) protein as a function of 17-β-estradiol treatment concentration. All data points were expressed relative to the mean of non-treated (NT) cells. Mean protein values were derived from an average of $n \geq 25$ cell culture wells. The mean apoE protein values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in extracellular apoE protein levels, relative to those of NT cells, are indicated by * $p < 0.05$.

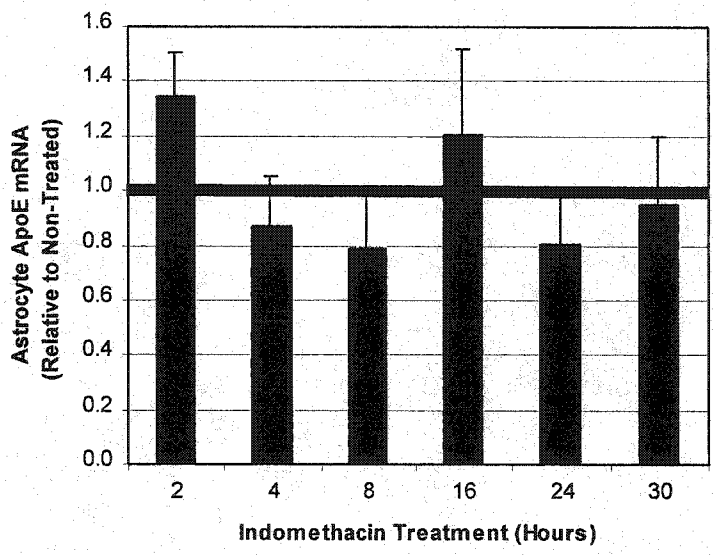


Figure 18: Mean (± standard error of the mean) rat astrocyte apolipoprotein E (apoE) mRNA as a function of the duration of indomethacin treatment at concentrations of 10^{-13} M and 10^{-15} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from an average of $n \geq 4$ cell culture flasks. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.

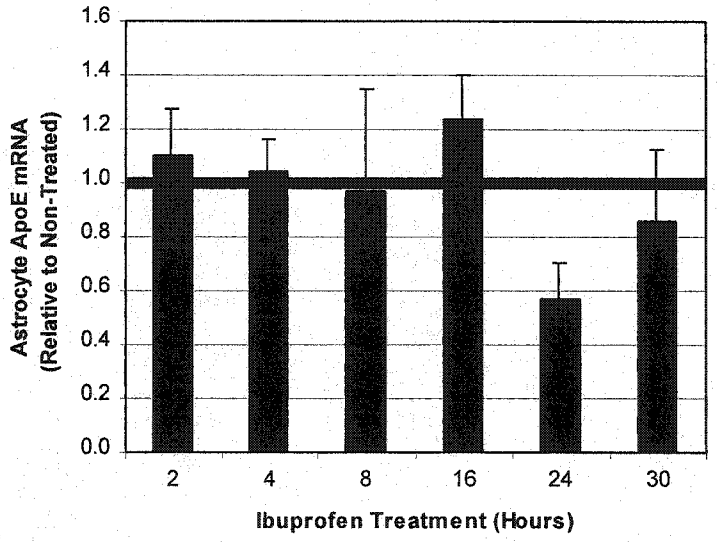


Figure 19: Mean (± standard error of the mean) rat astrocyte apolipoprotein E (apoE) mRNA as a function of the duration of ibuprofen treatment at concentrations of 10^{-13} M and 10^{-15} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from an average of $n \geq 3$ cell culture flasks. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.

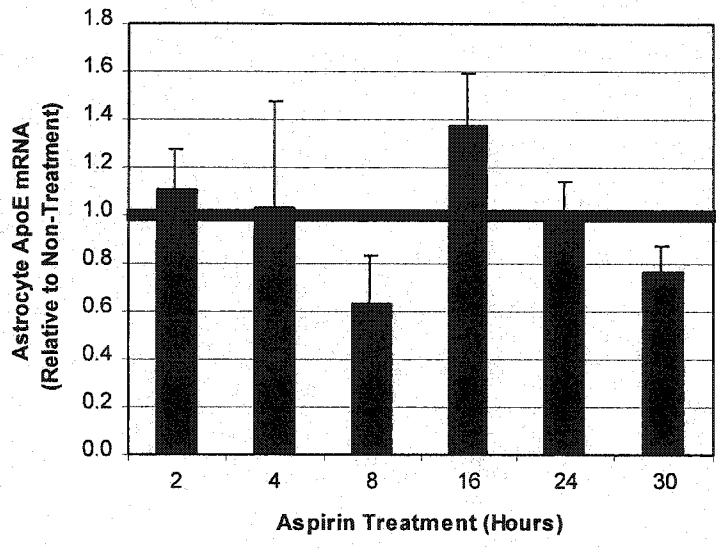


Figure 20: Mean (± standard error of the mean) rat astrocyte apolipoprotein E (apoE) mRNA as a function of the duration of aspirin treatment at concentrations of 10^{-11} M and 10^{-13} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from an average of $n \geq 5$ cell culture flasks. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.

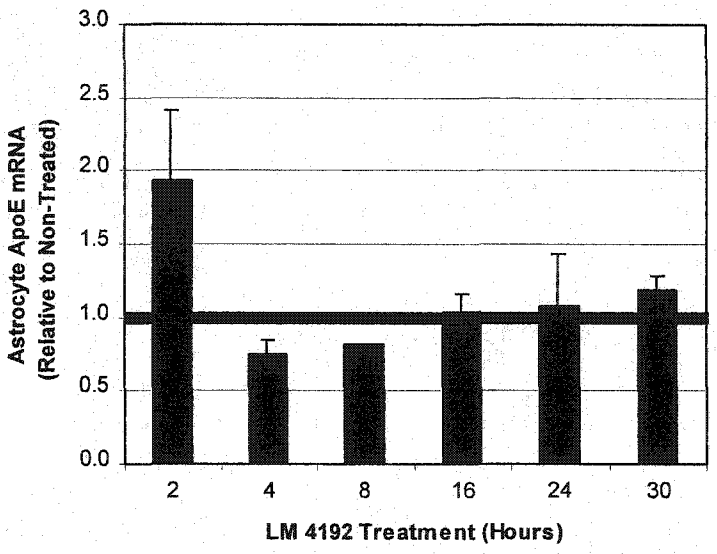


Figure 21: Mean (± standard error of the mean) rat astrocyte apolipoprotein E (apoE) mRNA as a function of the duration of LM 4192 treatment at concentrations of 10^{-13} M and 10^{-15} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from an average of n = 2 cell culture flasks. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.

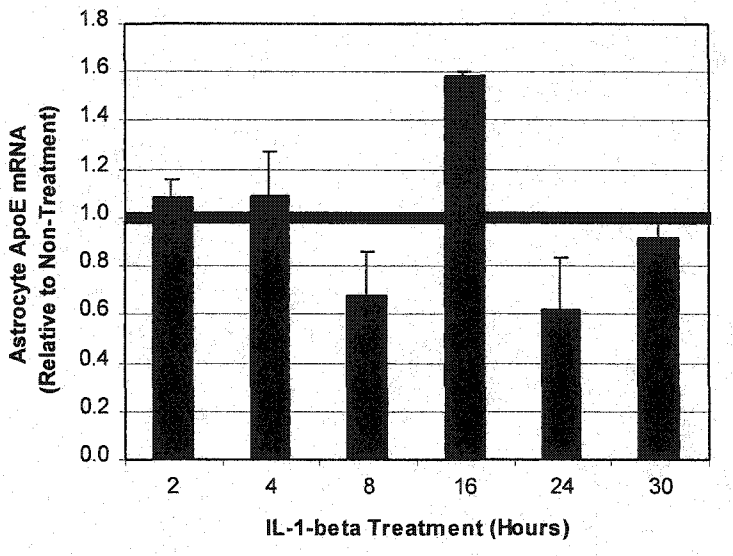


Figure 22: Mean (± standard error of the mean) rat astrocyte apolipoprotein E (apoE) mRNA as a function of the duration of interleukin-1β treatment at concentrations of 10^{-10} M and 10^{-12} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from an average of n = 2 cell culture flasks. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.

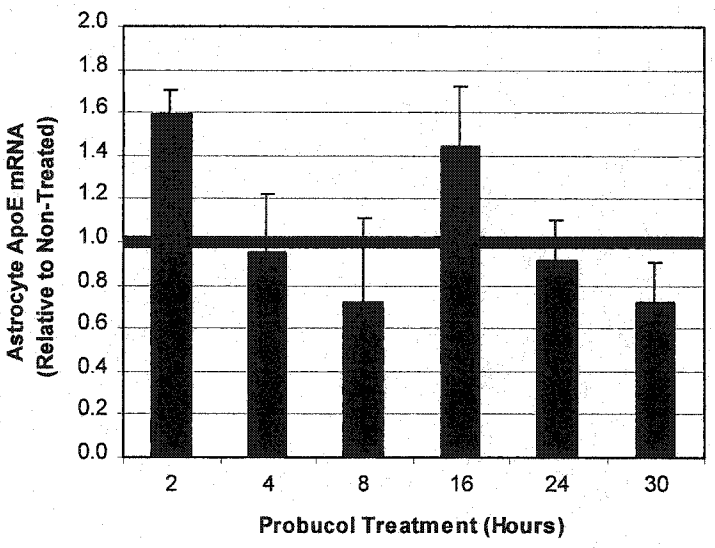


Figure 23: Mean (± standard error of the mean) rat astrocyte apolipoprotein E (apoE) mRNA as a function of the duration of probucol treatment at concentrations of 10^{-7} M and 10^{-9} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from an average of $n \geq 2$ cell culture flasks. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.

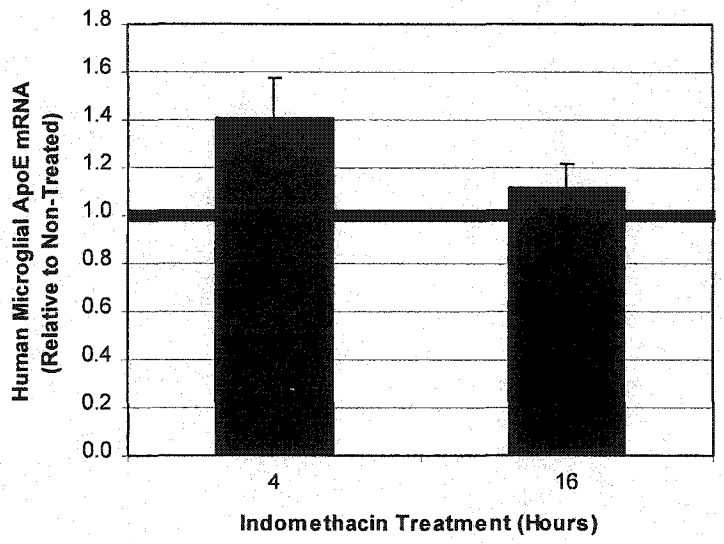


Figure 24: Mean (± standard error of the mean) human microglial apolipoprotein E (apoE) mRNA as a function of the duration of indomethacin treatment at a concentration of 10^{-13} M. All data points were expressed relative to the mean of non-treated (NT) cells. Mean apoE mRNA values were derived from n = 1 cell culture flask. The mean apoE mRNA values of treated cells were then compared via multiple 95% confidence intervals to the arbitrarily set NT population mean of 1.0, as depicted by the solid black line. Significant differences in apoE mRNA levels, relative to those of NT cells, are indicated by * $p < 0.05$.