Beta-Amyloid$_{1-42}$-Induced Intracellular Signaling Pathways, Functional Responses and Modulation by 4-Aminopyridine in Microglia

by

Sonia Franciosi

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ABSTRACT

Alzheimer's Disease (AD) is a progressive, neurodegenerative disease characterized by gradual cognitive decline and memory loss. Although research has focused on elucidating the risk factors, pathophysiologic abnormalities associated with AD and on mechanisms of impeding disease progression, results indicate that a variety of factors may contribute to AD which makes treating this disease difficult. The neuropathological hallmarks of AD include senile plaques which are composed of extracellular deposits of amyloid beta (Aβ) peptide as well as neurofibrillary tangles, neuronal loss and inflammation. Microglia, the immune cells of the CNS, are abundantly found in the vicinity of neuritic plaques. It is believed that microglia become activated in response to Aβ leading to an inflammatory response and subsequent neuronal loss associated with AD pathogenesis. Modulation of the Aβ-induced intracellular signaling and functional responses of microglia could serve as a therapeutic strategy for AD.

Full length amyloid beta, $A\beta_{1-42}$, induced distinct intracellular signaling pathways in human microglia. Electophysiological studies indicated that $A\beta_{1-42}$ acutely applied to human microglia upregulated the expression of a novel outward K^+ current, sensitive to the non-selective K^+ channel blocker 4-aminopyridine (4-AP). A similar outward K^+ current was activated by intracellular application of GTPγS which suggests that $A\beta_{1-42}$ induces an outward K^+ current in microglia via a G protein. Molecular biology studies indicated that the K^+ channel upregulated by $A\beta_{1-42}$ was likely due to Kv3.1. $A\beta_{1-42}$ also caused a transient depolarization of microglia and increased the expression of the FcγII receptor. The FcγII receptor mediated this depolarization since antibody inhibition of the FcγII receptor inhibited the $A\beta_{1-42}$-induced depolarization.

In addition to its ability to block the outward K^+ current upregulated by $A\beta_{1-42}$, several *in vitro* and *in vivo* assays indicated that 4-AP modulates $A\beta_{1-42}$-induced intracellular signaling and functional responses of microglia including neurotoxicity. Calcium spectrofluorometric studies indicated that $A\beta_{1-42}$ activated a calcium entry pathway which was blocked by 4-AP. Chronic exposure of microglia to $A\beta_{1-42}$ led to increased p38 MAP kinase expression and NFκB activation; in the presence of 4-AP, both factors were inhibited. Stimulation with $A\beta_{1-42}$ also led to the expression and production of pro-inflammatory mediators; 4-AP was effective in reducing the expression and production of these factors. Furthermore, 4-AP attenuated neurotoxicity induced by conditioned medium from $A\beta_{1-42}$ stimulated microglia. *In vivo*, injection of $A\beta_{1-42}$ into rat hippocampus caused neuronal damage and increased microglial activation. Daily administration of 4-AP was found to suppress microglial activation and exhibited neuroprotection. These results suggest that 4-AP modulation of $A\beta_{1-42}$-induced intracellular signaling pathways and functional responses in human microglia including microglial-mediated neurotoxicity serves as a potential therapeutic strategy in AD pathology.

The chemokine CXCL8 (IL-8) appears to potentiate $A\beta_{1-42}$ responses in human microglia. RT-PCR and ELISA studies indicated that CXCL8 potentiated $A\beta_{1-42}$-induced expression and production of pro-inflammatory mediators; the expression of anti-inflammatory cytokines IL-10 and $TGF\beta_1$ remained unchanged from basal levels despite treatment with stimuli. Stimulation with CXCL8 itself was effective in increasing microglial expression of pro-inflammatory mediators however, had no effect on protein levels of all these factors. CXCL8 potentiation of $A\beta_{1-42}$-induced inflammatory mediators may have particular relevance to AD brain which exhibits elevated levels of the chemokine.

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ABBREVIATIONS

AD	Alzheimer's Disease
Aβ	amyloid beta
$A\beta_{1-40}$	40-residue C-terminal variant of amyloid beta
$A\beta_{1-42}$	42-residue C-terminal variant of amyloid beta
ACT	alpha-1-antichymotrypsin
ADAM	a disintegrin and metalloproteinase
AICD	amyloid precursor protein intracellular domain
A2M	alpha-2-macroglobulin
4-AP	4-aminopyridine
ApoE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
BACE	β-site APP cleavage enzyme
BBB	blood brain barrier
°C	degrees celsius
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
Caspases	cysteine aspartyl proteases
cDNA	complimentary deoxyribonucleic acid
Cl^-	chloride ion
CNS	central nervous system
COX	cyclooxygenase
CR	complement receptor
CREB	cAMP responsive element binding protein
C-terminal	carboxy-terminal
CTF-α	carboxy-terminal fragment alpha
CTF-β	carboxy-terminal fragment beta
DAPI	4'-6'-diaminodino-2-phenylindole
DMEM	dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EOFAD	early onset familial Alzheimer's Disease
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
Fc	Fc receptor
FPR	formyl peptide receptor
Fura-2/AM	fura-2 acetoxymethylester
G3PDH	glyceraldehydes-3-phosphate dehydrogenase
GRO-α	growth-related oncogene-alpha
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
IFN-γ	interferon gamma
IL-1β	interleukin-1β
IL-4	interleukin-4

IL-6	interleukin-6
CXCL8 (IL-8)	interleukin-8
IL-10	interleukin-10
IL-13	interleukin-13
IFN-γ	interferon-gamma
i.p.	Intraperitoneal
JNK	c-jun N-terminal kinases
K^+	potassium ion
K_v	voltage-dependent potassium channel
La^{3+}	lanthanum ion
LOAD	late-onset Alzheimer's Disease
LPS	lipopolysaccharide
LRP	low density lipoprotein receptor related protein
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1 beta
mRNA	messenger ribonucleic acid
mV	millivolt
Na^{2+}	sodium ion
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NFκB	nuclear factor kappa B
NFT	neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NPPB	5-nitro-2-(3- phenylpropylamino)-benzoate
NSAID	non-steroidal anti-inflammatory drug
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
PKC	protein kinase C
PPARγ	peroxisome proliferators activated receptor gamma
PSEN1	presenilin-1
PSEN2	presenilin-2
PSS	physiological salt solution
RAGE	receptor for advanced glycation end product
RANTES	regulated upon activation, normal T cells, expressed and secreted
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
sAPPα	secreted amyloid precursor protein alpha
sAPPβ	secreted amyloid precursor protein beta
SEM	standard error of the mean
SOC	store-operated channel

SOD	superoxide dismutase
SR	scavenger receptor
TEA	tetraethylammonium
TGFβ	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
VLDL-R	very low density lipoprotein receptor
V_m	membrane potential

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DEDICATION

I would like to dedicate this thesis to my mother, father and brother Luigi.

Chapter 1: INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disease marked by gradual cognitive decline and memory loss. Both the incidence and prevalence of AD increases sharply with age (Kawas et al., 2000; Jorm and Jolly, 1998). The German doctor Alois Alzheimer first reported the disease in 1907 after he examined a patient post-mortem who had died of an unknown mental illness. Alzheimer found unusual clumps of protein or plaques in the patient's brain at autopsy. The presence of plaques, which are composed of aggregated proteins called amyloid beta protein (Aβ), and neurofibrillary tangles, which are intracellular aggregates of tau protein associated with neurofilaments, are the classical hallmarks of AD. In AD, amyloid deposition and neurofibrillary tangle formation are found predominantly in brain regions important for learning and memory processes.

Most cases of AD are late-onset sporadic forms but 5-10% of cases are familial caused by a single mutation in genes located on chromosome 1, 14 and 21. The etiology of the late-onset sporadic form of AD is unknown but likely involves multiple factors. Among these are genetic polymorphisms (Roses, 1998), mutations of genes encoding the amyloid precursor protein (APP), presenilins and apolipoprotein E (APOE), physiological and environmental risk factors. The genetic defects all result in either an increase in total levels of Aβ, or increase the more amyloidogenic form, $A\beta_{1-42}$. Overall, the neuropathological changes and clinical symptoms of both familial and age-related forms of AD are similar.

As noted above, AD is characterized by the presence of amyloid plaques and neurofibrillary tangles. Neuritic plaques in AD brain are also associated with gliosis and

inflammation, which are characteristic pathological features of both familial and sporadic forms of AD. Reactive microglia and astrocytes are found in the vicinity of neuritic plaques with microglial activation preceding astrocyte activation (Frautschy et al., 1998). Microglia, the immune cells of the CNS, are activated by Aβ and it has been proposed that inefficient phagocytosis of the peptide by microglia could lead to hyperactivation of cells and release of inflammatory mediators and neurotoxic factors thereby contributing to neurodegenerative processes and subsequent cognitive impairment in AD (Akiyama et al., 2000).

This introductory chapter provides an overview of AD, the inflammatory response in AD, the role microglia play in AD pathogenesis with particular emphasis on Aβ as a stimulus of microglia, the secretory products of stimulated microglia and finally the hypothesis and research objectives of this thesis.

1.1 ALZHEIMER'S DISEASE: GENERAL

After the initial report in 1907 by Alois Alzheimer, intense research has focused on elucidating the risk factors, pathophysiologic abnormalities associated with AD and on mechanisms of impeding disease progression. AD is characterized by progressive memory loss, deterioration of cognitive function, progressive inability to carry out daily activities as well as psychiatric and behavioural disturbances which leads to death of the individual inflicted with this disease in a period of approximately ten years. AD accounts for 60% to 70% of cases of progressive cognitive impairment in elderly patients. The prevalence of AD doubles every 5 years after the age of 60, increasing from a prevalence of 1% among those 60- to 64-years-old to up to 40% of those aged 85 years and older (Von Strauss et al., 1999).

Pathologically, AD selectively damages the frontal and temporal lobes, including the hippocampus—a structure involved in memory and visuospatial orientation (Arnold et al., 1991). In more advanced cases the pathology extends to other regions of cortex including the parietal and occipital lobes. This disease is distinguished from other dementias by the presence of amyloid plaques, neurofibrillary tangles and neuronal loss and is usually only diagnosed with certainty at autopsy. Amyloid plaques are extracellular deposits of insoluble, 8–10-nm amyloid fibrils that are polymers of Aβ (Masters et al., 1985). Neurofibrillary tangles (NFT), contain paired helical filaments of abnormally phosphorylated tau protein that occupy the neuronal cell body and apical dendrites, in distal dendrites as neuropil threads, and in the abnormal neurites that are associated with some Aβ plaques (neuritic plaques). The neuritic plaque consists of an extracellular core of amyloid fibrils intimately surrounded by dystrophic dendrites and axons, often containing paired helical filaments, as well as by activated microglia and reactive astrocytes (Cummings et al., 1998). Inflammation also plays a role in AD pathogenesis and seems to be facilitated by the presence of microglia, the immune cells of the CNS.

1.1.1 Early vs Late Onset AD

AD is usually classified according to its age of onset. When the disease occurs before 65 years of age, it is classified as early-onset ("presenile") AD, while late-onset ("senile") AD (LOAD) occurs in subjects over 65 years of age. In addition to age, inheritance of predisposing risk factors appears to play a role in disease onset. Research suggests that early onset AD is familial (EOFAD), inherited as an autosomal dominant trait. EOFAD represent only about 5% of all AD cases. On the other hand, LOAD is

more frequently sporadic, only a minority of these cases showing a clear family history and likely involves multiple factors most probably genetic susceptibility at multiple genes and interaction between these genes and/or environmental factors.

Genetic Mutations Associated with Early Onset Familial AD

Based on the observation that middle-aged patients with Down's syndrome (trisomy 21) commonly suffer from AD, researchers suspected that a gene on chromosome 21 could be involved in AD etiology. In 1987, linkage analysis in families with EOFAD identified a locus on chromosome 21 close to the APP gene (St. George-Hyslop et al., 1987). The APP protein is the precursor for Aβ, which is the major component of amyloid plaques. Therefore, APP was considered an obvious candidate gene for AD. In 1991, missense mutations in the APP gene were identified (Chartier-Harlin et al., 1991; Goate et al., 1991) and the first gene involved in the etiology of AD was recognized. Approximately twenty mutations in the APP gene have been identified and all are clustered around the β- and γ-secretase cleavage sites of the APP protein. These mutations result in an increase in the formation of $A\beta_{40}$ and/or $A\beta_{42}$, the more amyloidogenic and toxic species of Aβ (Citron et al., 1992; Suzuki et al., 1994).

The identification of APP as the first gene to be involved in AD led to the generation of transgenic animal models over-expressing normal or mutated human AD genes. Interestingly, most of the mouse models successfully mimic important features of the human disease, such as the presence of amyloid plaques, gliosis and neurodegeneration with age-related cognitive impairment, but neurofibrillary tangles are absent (Higgins and Jacobsen, 2003).

Significant linkage of EOFAD to chromosome 14 was found and later identified as the presenilin1 (PSEN1) gene as the responsible gene (Sherrington et al., 1995). Shortly after, evidence for a second gene from the presenilin family called presenilin 2 gene (PSEN2) was identified as being linked to EOFAD (Levy-Lahad et al., 1995). Presenilin activity is essential for the normal processing of APP (Citron et al., 1997). Transgenic mice over-expressing human wild-type or mutant PSEN1 have consistently shown elevated amounts of Aβ, especially $A\beta_{1-42}$ (Citron et al., 1997) and double mutants (APP/PSEN1) produce more Aβ than either transgene alone (Chapman et al., 2001). Moreover, the double transgenic mouse, PSEN2/APP, develops age-related cognitive decline associated with severe amyloidosis and inflammation in discrete brain regions (Richards et al., 2003). Mutations in the presenilins alter the γ-secretase cleavage of APP resulting in over-production of the $A\beta_{1-42}$ peptides (Citron et al., 1997). Indeed, it has been suggested that the presenilin1 protein could itself be a γ-secretase (Wolfe et al., 1999). Overall, mutations in PS genes account for greater than 55% of all EOFAD mutations.

Genetic Mutations Associated with Late Onset AD

The apolipoprotein E (APOE) gene is recognized as a major risk factor for complex forms of AD, mainly in sporadic LOAD. By genetic linkage analysis using a collection of late-onset AD families, APOE was identified as a disease locus because of its localization in the peak linkage region on chromosome 19 (Pericak-Vance et al., 1991). However, only less than 50% of LOAD cases are carriers of the ApoE ε4 allele, the genetic variant that predisposes to AD. In a study by Corder et al. (1993), a "dose-

dependent" increase in the risk of AD from 20 to 90% and the mean age of onset decreased from 84 to 68 years was found with increasing number of ε4 alleles. ApoE serves several functions involving the mobilization and redistribution of cholesterol during neuronal growth and after injury (Mahley, 1988), nerve regeneration, immunoregulation and activation of several lipolytic enzymes (Mahley and Rall, 2000; Vancea et al., 2000). The ApoE e4 allele increases amyloid deposition (Schmechel et al., 1993) however, the mechanism seems not to involve increased Aβ production but rather stabilization and decreased clearance of fibrillar Aβ deposits.

Several other candidate genes have been linked to LOAD. On chromosome 12, genes encoding alpha-2macroglobulin (A2M), an acute phase protein, its receptor the low density lipoprotein receptor-related protein1 (LRP1) and the transcription factor LBP-1c/CP2/LSF have been associated with LOAD. These genes play a role in Aβ metabolism (Narita et al., 1997; Pericak-Vance et al., 1997). Insulin degrading enzyme and urokinase plasminogen activator both located on chromosome 10 have also been associated with Aβ degradation and clearance (Vekrellis et al., 2000; Ertekin-Taner, 2002). Increased risk of LOAD has also been associated with the very low density lipoprotein receptor (VLDL-R) gene, located on chromosome 9. VLDL-R expresses a cell-surface molecule specialized for the internalization of multiple ligands, including ApoE-containing lipoprotein particles, via clathrin-coated pits (Sakai et al., 1994). Polymorphisms for inflammatory genes including pro-inflammatory cytokines, A2M, and α_1-antichymotrypsin (ACT) have also been associated with increased risk of developing AD (McGeer and McGeer, 2001).

In the majority of LOAD cases, genetic factors act as predisposing agents; increasing the risk of disease above that of the general population. They probably interact with environmental factors or with other pathologic or physiologic conditions such as traumatic brain injury, cardiovascular abnormalities, oxidative damage and diet to exert their pathogenic effect. They may also interact between themselves to further enhance the probability of inducing the disease.

1.1.2 Processing of the Amyloid Precursor Protein

Since changes in the generation or the degradation of Aβ are, according to the amyloid hypothesis (discussed in Section 1.2.1.1), believed to trigger the molecular events in the pathogenic cascade of AD, research has been carried out to better understand the normal functions performed by APP and the physiological actions of Aβ in order to more fully comprehend the disease process.

The APP gene is localized to chromosome 21 and is expressed in many cell and tissue types including endothelial cells, glia and neurons (Schmechel et al., 1988). APP exists as three major protein isoforms APP_{770}, APP_{751} and APP_{695}, however, the APP_{695} is the predominant isoform in the brain. APP exists as a transmembrane protein with a large N-terminal ectodomain and a short intracellular C-terminus and can be localized to many membranous structures in the cell such as the endoplasmic reticulum and Golgi compartments, the cell membrane and further localized to postsynaptic densities, axons and dendrites (Schubert et al., 1991, Shigematsu et al., 1992, Caporaso et al., 1994).

The cloning and characterization of APP revealed that it possessed many features reminiscent of a membrane-anchored receptor molecule and subsequent work demonstrated that full-length APP functioned as a typical cell surface G-protein-coupled

receptor (Okamoto et al., 1995) or as a secreted derivative that acts upon other cells. Neuronal APP colocalizes with β_1 integrins at point contacts, suggesting a possible role in adhesion (Yamazaki et al., 1997). It has been demonstrated that APP binds directly to extracellular matrix molecules, particularly collagen type I (Beher et al., 1996). Furthermore, it has been reported that co-localization of APP with $\beta_1$integrin results in tyrosine kinase activation and subsequent productions of pro-inflammatory mediators in monocytes and microglia (Sondag and Combs, 2004). Other studies indicate roles of APP in cell motility (Sabo et al., 2001), synaptic transmission and plasticity (Perez et al., 1997; Dawson et al., 1999).

The processing of APP involves three secretases (β, γ, α) and two distinct intracellular metabolic pathways: the non-amyloidogenic and the amyloidogenic pathways (Figure 1-1). The α and β cleavages seem to be mutually exclusive events and each liberates a large extracellular domain of the protein, differing in size by only 17 amino acids at the carboxy terminus. In the non-amyloidogenic pathway, APP is cleaved, within the Aβ sequence by α-secretase, a member of the ADAM family of metalloproteases. Cleavage of APP by α secretase generates extracellular soluble APP (sAPPα), a growth factor with neuroprotective and memory enhancing effects, as well as an intracellular C-terminal fragment (CTFα) corresponding to the 83 C-terminal amino acids of the protein (Oltersdorf et al., 1990). This α-secretase cleavage precludes the formation of Aβ peptide. The activity of α-secretase is increased by activation of protein kinase C (PKC), perhaps via phosphorylation of sites on the intracellular carboxy-terminal tail of APP in turn making APP a more suitable substrate for α secretase.

In the amyloidogenic pathway, Aβ is generated by sequential cleavages of APP by β- and γ-secretases. β-secretase, attributed to a single protein BACE (β-site APP cleavage enzymes), is expressed in high amounts in neuronal tissue and is increased in AD (Vassar et al., 1999). Intracellularly, BACE is localized primarily in the trans-Golgi network, endosomes, endoplasmic reticulum, and on the cell surface (Ling et al., 2003). The β-secretase cleavage generates sAPPβ and CTFβ corresponding to the 99 C-terminal amino acid of the protein. The CTFβ is further cleaved by γ-secretase, whose activity was shown to depend on the presence of a total of four components: presenilin, nicastrin, APH-1 and PEN-2 (Vassar et al., 1999; Edbauer et al., 2003). γ-secretase activity generates the predominant $A\beta_{1-40}$ or $A\beta_{1-42}$ fragments, as well as fragments spanning $A\beta_{1-39}$ and $A\beta_{1-43}$, together with the remaining C-terminal tail (AICD). Similarly, after α cleavage, C83 can be processed by γ-secretase to generate a shortened Aβ-like fragment termed p3, plus the same C-terminal tail (AICD). In the absence of presenilin or nicastrin, the two C-terminal fragments corresponding to C83 and C99 (CTF-α, β) accumulate and associate with the γ-secretase complex at the plasma membrane where γ-secretase is thought to be active. AICD is a very unstable peptide that upon ectopic expression in cultured cells may translocate to the nucleus and activate gene transduction (Gao and Pimplikar, 2001).

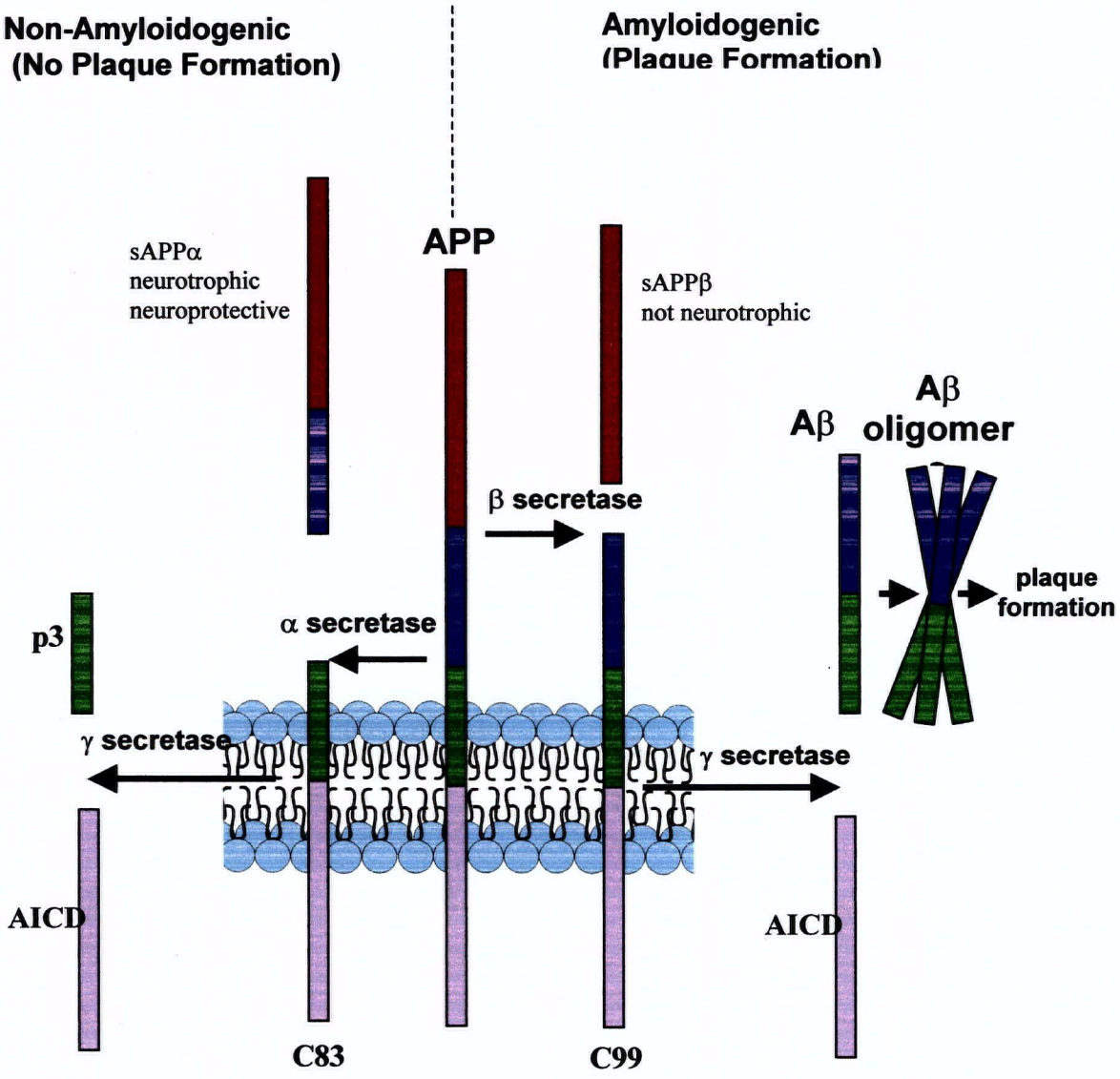

Figure 1-1. Processing of the Amyloid Precursor Protein Resulting in Aβ Formation

1.1.3 The Amyloid Peptide

Aβ is a ~4 kDa protein. Amyloid fibrils are filamentous structures with a width of ~10 nm and a length of 0.1–10 μM. Aβ exists as monomers, dimers, and higher oligomers (3-6 units and up to 24 units), while further aggregation yields protofibrils and then fully-fledged fibrils that seem to compose the bulk of the amyloid plaques in AD brain (Walsh et al., 1999). *In vitro* experiments demonstrate that aggregation into fibrils is dependent on factors which include peptide concentration, acidic pH, environment, interaction with metals and various biomolecules and oxidative stress. The aggregation process results in the conversion of a Aβ α helical filament or random coil into fibrils containing a β sheet conformation. Although $A\beta_{1-40}$ increases proportionately more in AD and may correlate better with synaptic change and cognitive deficits (Lue et al., 1999), $A\beta_{1-42}$ aggregates more readily (Snyder et al., 1994) and is also more toxic (Pike et al., 1993).

Although research has focused on the effects of Aβ extracellularly, reports also indicate that Aβ can accumulate intracellularly particularly in neurons (Walsh et al., 2000). It has been proposed that intracellular accumulation of Aβ leads to neuronal lysis and dispersion of the cell contents into the surrounding parenchyma. According to this hypothesis, this extracellular Aβ could then act as a locus for nucleation and aggregation of the soluble extracellular pool of Aβ to form plaques (D'Andrea et al., 2001). Intracellular Aβ has been found to co-localize with neurofibrillary tangles and is associated with signs of cytoskeletal degeneration. Furthermore, intraneuronal Aβ

accumulation has been associated with synaptic pathology and dysfunction (Takahashi et al., 2002; Oddo et al., 2003).

1.1.4 Cellular Dysfunction in AD

Several mechanisms of Aβ mediated neurotoxicity have been postulated however the causal relationship and the sequence in which they occur is unknown; the mechanisms include inducing mitochondrial dysfunction, oxidative stress, disturbances in Ca^{2+} homeostasis, reactive oxygen species (ROS) generation, excitotoxicity, altered synaptic plasticity, ER stress and microglial activation (discussed in Section 1.3). Some of these mechanisms are described in further detail below.

Mitochondrial abnormalities have been shown to be a very early pathological sign in AD (de la Monte et al., 2000). Several key mitochondrial enzymes including α-ketoglutarate dehyrogenase, pyruvate dehyrdogenase and cytochrome c oxidase are decreased in AD (Gibson et al., 1998; Kish et al., 1992; Mutisya et al., 1994). The APP molecule has been reported to interact with mitochondrial proteins resulting in mitochondrial dysfunction and inhibition of ATP synthesis (Anandatheerthavarada et al., 2003). Dysfunctional mitochondria are a source of reactive oxygen species (ROS) and inhibition of mitochondria has also been shown to increase the amyloidogenic processing of APP to Aβ creating conditions for further cell damage (Gabuzda et al., 1994).

Evidence for oxidative damage to proteins and DNA and increased levels of lipid peroxidation have all been described in postmortem tissue from patients with AD. Generation of ROS increases membrane lipid peroxidation resulting in impaired function of membrane ion-motive ATPases (Na^+/K^+- and Ca^{2+}-ATPases), glucose and glutamate transporters leading to membrane depolarization and a decrease in cellular ATP levels

(Mark et al., 1995; 1997). In addition to dysfunctional mitochondria, Aβ may directly generate ROS or interact with other free radical generating systems such as the microglia and other cell types in the CNS via activation of NADPH oxidase. The antioxidant enzyme superoxide dismutase (SOD) displays reduced activity in affected brain regions in AD (Zemlan et al., 1989). Aβ alone directly interacts with metal ions such as copper (II) resulting in ROS generation. In addition, binding of Aβ to copper (II), iron (III) and zinc (II) results in aggregation of the Aβ peptide (Atwood et al., 1998) promoting plaque formation and toxicity.

Increased levels of Aβ have been shown to induce oxidative stress rendering neurons vulnerable to apoptosis and excitotoxicity through dysregulation of calcium homeostasis. The ability of antioxidants such as vitamin E to prevent impairment of the membrane transporters and to stabilize cellular calcium homeostasis supports a key role for membrane lipid peroxidation in disruption of calcium homeostasis by Aβ (Goodman and Mattson, 1994). Studies of lymphocytes and lymphoblast cell lines from AD patients and age-matched normal control patients have documented alterations in cytokine and calcium signaling and increased levels of oxidative stress in immune cells from AD patients (Mattson, 2002). Furthermore, studies of the pathogenic actions of mutations in presenilins and APP that cause EOFAD have established central roles for perturbed cellular calcium homeostasis and oxidative stress in the neurodegenerative process. A direct effect of Aβ on intracellular calcium $[Ca^{2+}]_i$ has been demonstrated. Whole cell patch clamp experiments of cultured neurons provided evidence that Aβ can potentiate the activation of L-type voltage-gated calcium channels (Weiss et al., 1994; Ueda et al.,1997) rendering the neurons vulnerable to excitotoxicity (Mattson et al., 1992; Weiss

et al., 1994). This toxicity was dependent on the Aβ concentration used. As well, it has been reported that Aβ can itself form calcium-conducting pores in cell membranes (Kawahara and Kuroda, 2000; Kagan et al., 2002). In addition, to its effects on plasma membrane calcium-regulating systems, Aβ has been shown to have adverse effects on calcium regulation in the endoplasmic reticulum (ER) and mitochondria (Mattson and Chan, 2003). Moreover, it has been reported that increased amounts of $[Ca^{2+}]_i$ induced by neuronal depolarization results in intraneuronal $A\beta_{1-42}$ production and subsequent neuronal death (Pierrot et al., 2004).

Whether the mechanism of neuronal cell death in AD is necrotic or apoptotic remains unclear but could involve both processes. Apoptosis is believed to play a major role in AD pathology (Cotman and Anderson, 1995). Caspases (cysteine aspartyl proteases) initiate intracellular cascades leading to apoptotic outcomes which include proteolytic cleavage of cytoskeletal proteins and proteins of the nuclear scaffold. Apoptosis is initiated through intracellular mechanisms that often involve alterations in mitochondria or endoplasmic reticulum and by signaling through cell membrane death receptors. Some experimental studies suggest that Aβ can activate caspases through binding of extracellular Aβ to cell receptors (Dickson, 2004). Furthermore, accumulation of Aβ in ER (endoplasmic reticulum) may activate apoptotic mechanisms through mitochondrial or ER stress (Lustbader et al., 2004).

1.1.5 Amyloid Hypothesis and AD Pathology

An ongoing debate in AD research relates to the etiology and connection between amyloid plaques and neurofibrillary tangles as well as their relative contribution to the

disease. On the one hand, supporters of the "tau hypothesis" believe that the accumulation of hyperphosphorylated tau protein and resulting NFT formation leads to AD whereas supporters of the "amyloid hypothesis" believe that amyloid deposition is a central event and that amyloid plaques are the causative factor of AD. Although a number of studies have linked NFT numbers to synaptic loss and cognitive decline (Terry et al., 1991; McKee et al., 1991), Aβ deposition and levels of Aβ peptides are also correlated with cognitive decline (Naslund et al., 2000; Cummings et al., 1996). The amyloid hypothesis is more widely accepted since three mutations (APP on chromosome 21, PS1 on chromosome 14, and PS2 on chromosome 1), which underly EOFAD, lead to increased deposition of Aβ (Scheuner et al., 1996). However, the amyloid hypothesis does not take into account reports which indicate that other factors such as oxidative damage, mitotic failure, neurofibrillary tangle formation and inflammation may occur upstream of amyloid formation and deposition.

Improper metabolism of the amyloid precursor protein (as mentioned above in Section 1.1.2) with the resulting accumulation of the Aβ fragment is viewed as the central event in AD pathogenesis (Selkoe, 2000).

1.1.5.1 Amyloid Hypothesis

The hypothesis states that "AD is a pathological syndrome in which different gene defects can lead directly or indirectly to altered APP expression or proteolytic processing or to changes in Aβ stability or aggregation. These result in a chronic imbalance between Aβ production and clearance. Gradual accumulation of aggregated Aβ initiates a complex, multistep cascade that includes gliosis, inflammatory changes, neuritic/synaptic

change, tangles, transmitter loss and impaired cognitive function" (Selkoe, 2000). Key observations which support the amyloid hypothesis are summarized in Selkoe, 2000.

1.1.5.2 Classification of Amyloid Plaques

Deposits of Aβ that form in AD have been morphologically classified into four major types: diffuse plaques-most of the Aβ peptide is not aggregated into a fibrillar structure, dystrophic neurites and paired helical filaments are infrequent or absent; primitive plaques- Aβ is aggregated into amyloid and dystrophic neurites and paired helical filaments are clearly present; classic/neuritic plaques-Aβ is highly aggregated to form a central core which is surrounded by a ring of dystrophic neuritis; compact plaques-consist of a solid core of amyloid but which lack a ring of dystrophic neuritis (Armstrong, 1998). Activated microglia are mainly associated with classic/neuritic plaques with few microglia associated with compact plaques. Two hypothesis exist for plaque formation: the life history hypothesis which states that plaque formation is thought to progress from diffuse through to compact and the "independent origin" hypothesis which states that plaques develop independently from each other. Irrespective of plaque progression, it has been reported that the relative frequency of these types of deposits changes during the progression of AD, with diffuse plaques being prevalent in the preclinical stages, and fibrillar plaques increasing in frequency as the disease progresses to clinical dementia (Dickson and Vickers, 2001; Thal et al., 2000).

1.2 ROLE OF INFLAMMATION IN ALZHEIMER'S DISEASE

Whether inflammation is a cause and/or a consequence of AD pathology is still unknown. However, evidence continues to support the involvement of chronic

inflammation in AD pathophysiology (Akiyama et al., 2000). Inflammatory processes in the CNS have the potential to be both beneficial and detrimental in AD pathogenesis (Wyss-Corray and Mucke, 2002). Interestingly, amyloid plaques with little or no inflammatory changes have been reported in the brains of non-demented individuals (Arriagada et al., 1992) which indicates that the plaques themselves are not responsible for clinical symptoms of AD but rather supports the involvement of inflammation in AD pathogenesis. Support for a role of inflammation in AD pathogenesis is also based on the association of inflammatory proteins, complement, potentially cytotoxic factors and microglia in the vicinity of neuritic plaques (discussed in Section 1.2.1). Also, polymorphisms of inflammatory factors associated with neuritic plaques are genetic risks factors for AD (discussed in Section 1.2.2). Moreover, support for a role of inflammation in AD is evident from epidemiological studies suggesting that anti-inflammatory drugs can prevent or slow the progression of AD (discussed in Section 1.2.3).

1.2.1 Inflammatory Response in Alzheimer's Disease

Traditionally thought of as immunologically privileged, the CNS is known to have an endogenous immune system that is coordinated by immunocompetent cells such as the microglia. The inflammation associated with the CNS, neuroinflammation, differs from that found in the periphery. The brain lacks pain fibers, making it difficult to recognize the occurrence of inflammation and the classic signs of inflammation such as rubor (redness), tumor (swelling), calor (heat), and dolor (pain) are typically not seen in the CNS. Also, the CNS differs from the periphery in that it is isolated from other organs by the blood–brain barrier (BBB). The BBB is formed by endothelial cells that are bound together by tight junctions surrounded by a thin basement membrane (i.e. basal lamina)

which supports the ablumenal surface of the endothelium. Astrocytes are adjacent to the endothelial cell, with astrocytic end feet sharing the basal lamina preventing the entry of inflammatory cells, pathogens, and some macromolecules from blood into the brain. This barrier acts to protect neurons from the damages typically associated with inflammation. Contradictory results have been reported in both AD patients (Claudio, 1996; Caserta et al., 1998) as well as in animal models of AD (Podulso et al., 2001; Ujjie et al., 2003) as to whether the integrity of the BBB is disrupted or not. Regardless, neuritic plaques are the foci of local inflammatory responses, as evidenced by the presence of inflammatory mediators which include acute phase proteins (i.e. ACT, A2M, C reactive protein), cytokines, chemokines, cyclooxygenase, complement components and proteases (McGeer and McGeer, 1999; Eikelenboom et al., 1998; Akiyama et al., 2000).

Aβ peptide itself has been shown to induce a local inflammatory response since reports indicate that Aβ binds to the complement factor C1 and activates the classical pathway of the complement system (Rogers et al., 1992). The C3 and C5 fragments formed by complement activation, represent chemotactic and activation factors for microglia and astrocytes which have specific receptors for these fragments. The membrane attack complex composed of C5b, C6, C7, C8 and C9 can then affect surrounding cells cytotoxically or even cytolytically (McGeer and McGeer, 1999). Overactivation of the complement system is believed to play a role in AD pathogenesis since increases in complement protein (McGeer et al., 2000) and the presence of MAC (the membrane attack complex) on the surface of neurons have been detected in AD brain

(Webster et al., 1997). Overactivation of complement could lead to amplification of an inflammatory response.

The presence of pro-inflammatory cytokines including IL-1, IL-6, and TNF are of particular interest since it has been shown in acute brain injury that these same cytokines may exacerbate lesion size and neuronal loss by their impact on already compromised neurons (Allan and Rothwell, 2001). These results would suggest that the presence of these cytokines could exacerbate the loss of neurons.

Studies also indicate that some of these inflammatory proteins increase amyloid formation. The activated C1q complement component increases the aggregation of Aβ and plays a role in Aβ clearance by microglia (Webster et al., 1994; Rogers et al., 2002). Also, transgenic APP mice crossed with ACT transgenic mice had twice the amyloid load and plaque density compared with mice with the APP transgene alone (Nilson et al., 2001). Furthermore, it has been shown that IL-1 together with other cytokines can regulate APP synthesis and Aβ production *in vitro* (Goldgaber et al., 1989; Blasko et al., 1999).

1.2.2 Inflammatory Polymorphisms Increase Risk of AD

Polymorphisms for inflammatory genes including pro-inflammatory cytokines (IL-1, TNF-α, IL-6), A2M, and ACT have also been associated with increased risk of developing AD (McGeer and McGeer, 2001). The polymorphisms are in the noncoding regions of these genes (the promoter and untranslated regions). The polymorphisms are fairly common ones in the general population so there is a strong likelihood that any given individual will inherit one or more of the high-risk alleles. Those alleles which

favour increased expression of the inflammatory mediators are more frequent in AD patients than in controls.

1.2.3 Anti-Inflammatory Therapy as Treatment in AD

Support for a role of inflammation in AD pathogenesis is also evident from epidemiological studies which indicate that non-steroidal anti-inflammatory drug (NSAID) use reduces the risk of developing AD, slows the progression and decreases the severity of dementia (McGeer et al., 1996; In 't Veld et al., 2001). It is generally accepted that NSAIDs exert their beneficial effects in AD via inhibition of COX thereby inhibiting a wide range of inflammatory responses however, other mechanisms for NSAID action have been reported. These include activation of peroxisome proliferator-activated receptor γ (PPARγ), NFκB inhibition (Dodel et al., 1999), direct effects on amyloid processing (Weggen et al., 2003) and altering Aβ aggregation (Thomas et al., 2001; Agdeppa et al., 2003).

Differences exist in the efficacy of NSAIDs for use in treatment of AD and these differences may be due to the differing mechanisms of action of COX inhibition (COX-2 vs COX-1/non-selective COX-1/2). Clinical results favouring the use of COX-1 inhibitors and/or mixed COX-1/COX-2 inhibitors such as indomethacin and ibuprofen may be a result of COX-independent effects since these agents are capable of activating peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear receptor which has been shown to inhibit the expression of wide range of pro-inflammatory genes (Landreth and Heneka, 2001). The differing results of epidemiological studies versus recent clinical trials may also indicate that NSAIDs may not work in established AD,

although for preclinical AD or mild cognitive impairment, a protective effect may be present (Aisen, 2002).

Interestingly, NSAID treatment was associated with less *in vivo* microglial activation, as assessed by class II major histocompatibility complex (MHC) staining (Mackenzie and Munoz, 1998). NSAIDs suppressed Aβ-stimulated proinflammatory and neurotoxic responses by microglia (Combs et al., 2000). Also, NSAIDs have been shown to reduce IFN-γ/lipopolysaccharide induced microglial neurotoxicity (Klegeris et al., 1999). These studies collectively support the concept that inflammation mediated by microglia is an important component of AD pathophysiology, and strategies to control microglial activation could provide new therapeutic approaches for the treatment of AD.

Studies using other anti-inflammatory agents such as low-dose prednisone for 1 year (Aisen et al., 2000) and hydroxychloroquine for 18 months (Van Gool et al., 2001) failed to demonstrate benefit in the treatment of AD. On the other hand, epidemiological studies have shown that estrogen replacement therapy is associated with improvement in cognitive performance, protection against cognitive decline and a decreased incidence of AD (Paganini-Hill, 1994; Costa et al., 1999). It has been reported that a possible mechanism of estrogen anti-inflammatory activity may be through inhibition of microglial activation (Bruce-Keller et al., 2000; Vegeto et al., 2001).

1.3 MICROGLIA AND ALZHEIMER'S DISEASE

1.3.1 Microglia

Microglia, which are the immune cells of the central nervous system (CNS), were first identified and characterized by Pio del Rio-Hortega who published his findings in 1932 (del Rio-Hortega, 1932). Microglia represent approximately 10-20% of all glial

cells in the CNS. Evidence from immunocytochemical studies using macrophage-specific markers have shown that microglia originate from monocytes entering the CNS at early stages of embryonic development (Perry et al., 1985; Ling and Wong, 1993). Other views include microglia as being derived from mesenchymal progenitor cells or that they are of neuroectodermal origin; the cells either originate from glioblasts or the germinal matrix (Kaur et al., 2001). Microglia are broadly classified based on two types of morphologies: ramified (resting) or ameboid (activated). In the resting state, microglia demonstrate a down-regulated process bearing phenotype however, are capable of becoming rapidly activated in response to pathological events. This would indicate that despite their apparent quiescence, microglia remain vigilant. Activated microglia demonstrate macrophage like properties: rounding of cells, lacking processes, upregulation of cell surface markers such as MHC Class II and complement receptors (indicative of their antigen presenting capacity) and often become reactive when carrying out phagocytosis (Kreutzberg, 1996; McGeer et al., 1988). *In vitro*, microglia possess an ameboid morphology immediately after isolation and then diversify into an inhomogenous population of both ameboid and ramified cells (Walker et al., 1995).

Microglia respond quickly to a variety of signaling molecules and are typically the first cells to become activated at a site of injury (Gehrmann et al., 1995). Because of their responsiveness to an array of stimuli, microglia play a role in various immune functions in the brain which include host defence, neuroprotection, repair processes, phagocytosis, initiation and propogation of an inflammatory response (Banati and Graeber, 1994). Microglia express several cell surface receptors which couple stimuli to microglial functions including receptors for Fc and complement on their surface;

receptors implicated in microglial phagocytosis and cell lysis. A striking feature of microglial reactivity is the ability to synthesize and secrete a large number of substances, which, alone or in concert with factors derived from other brain or hematogenous cells, may have a crucial role in host defence or in the establishment or maintenance of brain damage. These secretory products include growth factors, cytokines, coagulation and complement factors, enzymes, reactive oxygen species and neurotoxins. Chronic activation of microglia leads to production of toxic substances which has been suggested as a factor contributing to neurodegenerative diseases (McGeer and McGeer, 1995). Moreover, many of the secretory products of activated microglia such as pro-inflammatory cytokines and chemokines are autocrine leading to chronic activation of these cells and attraction of more microglia to the site of injury.

1.3.2 Role of Microglia in AD Pathogenesis

Activated microglia cluster around amyloid plaques and microglial release products are thought to mediate a significant portion of neurotoxicity (Yates et al., 2000; Bamberger and Landreth, 2001; Benveniste et al., 2001; Combs et al., 2001; Meda et al., 2001). Factors released from dying neurons, such as ATP, could fuel cytokine release from microglia and aggravate consequences for neural cells. Thus, microglia play a significant role in the pathogenesis of AD ultimately leading to the cognitive decline which is associated with this disease.

1.3.3 Aβ as a Stimulus of Microglia

The presence of activated microglia in the vicinity of neuritic plaques may simply be a non-specific inflammatory response. However, this glial population appears to favor

amyloid-containing plaques, indicating a specific interaction between Aβ and microglia. Of note is the fact that activated microglia are associated with neuritic plaques containing dystrophic neurites and high levels of Aβ (Rogers et al., 1988; Itagaki et al., 1989). In contrast, reactive microglia are not found within diffuse plaques, which are considered clinically benign Aβ deposits. The clustering of microglia at neuritic plaques could be due to activation of microglia by chemotactic activation by Aβ, activation by release products from damaged neurons or may be due to the presence of other pro-inflammatory mediators in the vicinity of plaques.

1.3.3.1 Receptors for Aβ in Microglia

Several receptors in microglia have been proposed with varying abilities to bind fibrillar and nonfibrillar forms of Aβ. They include the receptor of advanced glycation end products (RAGE) (Yan et al., 1996) and the scavenger receptor class A (SR-A) (El Khoury et al., 1996) and class B (SR-B) (Husemann et al., 2001). The RAGE receptor has been implicated in Aβ induced secretion of pro-inflammatory mediators and chemotaxis (Lue et al., 2001b) and in the internalization of Aβ by microglia (Yan et al., 1996). The SRs are thought to mediate adhesion and clearance of aggregated Aβ, as well as the production of reactive oxygen species (Husemann et al., 2002). A cell surface receptor complex composed of $\alpha_6\beta_1$ integrin, CD47 and CD36 (an SR-B) also reportedly binds fibrillar Aβ in microglia (Bamberger et al., 2003). The binding of Aβ to this receptor complex results in the activation of tyrosine kinase-based signaling cascades and subsequent activation of microglia (McDonald et al., 1997; Combs et al., 1999). The formyl peptide receptor (FPR), which is coupled to a pertussis toxin-sensitive G_i-protein,

calcium and phosphoinositide-specific phospholipase C, binds both soluble and fibrillar Aβ, mediates chemotactic effects of Aβ as well as the production of pro-inflammatory mediators by microglia (Lorton et al., 2000). CD36, also involved in a receptor complex for Aβ, mediates the binding of fibrillar Aβ and activation of the downstream Src kinase family members, Lyn and Fyn, and the mitogen-activated protein kinase p44/42 (p44/42 MAPK) resulting in reactive oxygen species and chemokine production (Moore et al., 2002). The serpin enzyme complex (Boland et al., 1996), heparan sulfate proteoglycans (Scharnagl et al., 1999), $\alpha_5\beta_1$-integrin (Matter et al., 1998), LRP and insulin receptor have also been reported to bind Aβ in microglia (Verdier et al., 2004).

1.3.3.2 Stimulatory Effects of Aβ on Microglia

There is extensive literature documenting the fact that Aβ serves as a potent microglial activator; stimulation of microglia with Aβ results in the expression (Lue et al., 2001a) and production of a repertoire of factors including proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, the chemokines CCL3 (MIP 1α), CCL2 (MCP-1) and CXCL8 (IL-8) (Meda et al., 1996; 1999; Walker et al., 2001), the complement component C3 (Haga et al., 1993), ROS (Meda et al., 1995) and glutamate (Klegeris et al., 1994; McDonald et al., 1997). Aβ stimulation of microglia also leads to the production of excitotoxins, an unidentified neurotoxin and proteases (Giulian et al., 1995; 1997; Combs et al., 2001). It is believed that Aβ alone is not toxic to neurons rather the microglia are necessary for neuronal killing in AD (Giulian et al., 1997). Furthermore, Aβ stimulation of microglia enhances the expression of the co-stimulatory molecule CD40, a receptor which plays an important regulatory role in

immune responses of microglia (Tan et al., 1999). Aβ also acts as a chemotactic agent for microglia (Davis et al., 1992).

Numerous researchers have described the ability of microglia to phagocytose and internally degrade Aβ deposits (Frautschy et al., 1992; Shaffer et al., 1995), a process that may be important for plaque evolution. There is evidence that complement opsonizes Aβ fibrils, facilitating their removal by microglial phagocytosis (Rogers et al., 1992). Surprisingly however, while microglia can clear even purified plaque material *in vitro*, plaque-removing activity is not obvious in human brain specimen. Microglia are believed to be overwhelmed and incapable of removing plaque material resulting in a continued inflammatory response, the emission of microglia-attracting and activating signals, and the release of potentially neurotoxic agents at high rates (Cotman et al., 1996).

Aβ can synergistically act with inflammatory mediators to enhance IL-1β, IL-6, TNF-α, CCL3 (MIP-1α), CCL4 (MIP-1β), and CXCL8 (IL-8) production (Cooper et al., 2000; Yates et al., 2000). Aβ-induced release of inflammatory mediators by microglia can then drive neurotoxic cascades that in turn recruit more microglia at the site of Aβ plaques with subsequent amplification of inflammatory reactions. Interestingly, in cultures of mouse microglia and human monocytes, Aβ did not alter the expression of the anti-inflammatory cytokine TGF-β1 (Meda et al., 1999). These results would suggest that an imbalance between proinflammatory cytokine and possible inhibition could contribute to the pathogenesis of AD.

The microglia themselves may be a source of Aβ production in the AD brain. Although microglia have been shown to secrete Aβ under the influence of Aβ or pro-

inflammatory stimuli (Bitting et al., 1996), microglia do not appear to express mRNA for APP *in vivo* (Scott et al., 1993). These results would indicate that microglia as a potential source of Aβ is debatable and requires further research. The production of Aβ by microglia would still be a small part of total Aβ in the brain since the major source of Aβ production comes from neurons. Therefore, microglia cells are not necessary for the production of Aβ plaques. However, evidence suggests that microglia assist in the conversion of non-fibrillar Aβ to fibrillar Aβ, and consequently, the development of neuritic plaques from diffuse plaques (MacKenzie et al., 1995; Sasaki et al., 1997).

1.3.4 Secretory Products of Microglia

Microglial activation is accompanied by secretion of a plethora of substances (McGeer and McGeer, 1995). These include complement, acute phase proteins, cytokines, chemokines, prostaglandins, proteases, and reactive oxygen species. Since microglia are often the source and recipients of cytokine signals, autocrine loops may serve to maintain microglia in an activated state. Some of the secretory products of Aβ stimulated microglia are discussed further below.

1.3.4.1 Pro-Inflammatory Cytokines

Reactive glia produce a variety of molecules that trigger and contribute to chronic inflammation. Termed the "cytokine cycle", pro-inflammatory cytokines participate in a spectrum of cellular and molecular signaling that continuously feedback with potential neurodegenerative consequences (Griffin et al., 1998).

IL-1β: *In vitro*, IL-1β has been shown to be produced by both rodent and human microglia and in response to Aβ (Lue et al., 2001a; Meda et al., 1999). IL-1β release

may in turn trigger production of other cytokines in an autocrine fashion (Benveniste et al., 1992). In particular, IL-1 has been reported to induce the expression and production of the chemokines MIP-1α and MIP-1β (McManus et al., 1998) and TNF-α in microglia (Chao et al., 1995). The activity of IL-1 is blocked by a naturally occurring receptor antagonist (IL-1ra) that binds to the Type I IL-1 receptor, but does not initiate signal transduction. In turn, secretion of IL-1 by microglia can have profound effects on astrocytes since in addition to the induction of a variety of inflammatory and cytotoxic mediators, IL-1 acts as a mitogen for rodent astrocytes (Giulian et al., 1994) and induces a stress response in human astrocytes (Lee et al., 1995). IL-1β has also been directly implicated in neuronal degeneration (Griffin and Mrak, 2002).

In AD, overexpression of IL-1β is a consistent feature found within cortical regions from post-mortem brains. Double-labeling immunohistochemical studies have localized IL-1β to plaque-associated microglia (Griffin et al., 1989; Griffin et al., 1995). IL-1β overexpression appears to occur early in plaque evolution evident in diffuse, non-neuritic Aβ deposits (Griffin et al., 1995). IL-1β in particular may be involved in the growth of dystrophic neurites around plaques (Sheng et al., 1996), excessive production and processing of amyloid precursor protein as well as the synthesis of most of the known plaque associated proteins (Akiyama et al., 2000). Furthermore, IL-1β has been linked to increased acetylcholinesterase resulting in decreased acetylcholine (Li et al., 2000) thereby contributing to cognitive decline.

TNF-α: Microglia are a prominent source of TNF-α in the brain. Depending on the stimulus, even a short exposure can be very effective and result in a strong and lasting release response (Hanisch, 2002). Thus, in CNS insults, rapid induction of microglial

TNF-α production could critically influence subsequent events. Microglia can also release soluble TNF-R (sTNF-R), an antagonist of circulating TNF-α, and hence affect the available TNF-α pool and probably regulate potential TNF-α consequences. While TNF-α seems to play a central role in glial functions (Benveniste, 1992), its effect on neuronal cell viability is controversial both *in vitro* (Cheng et al., 1994; Chao and Hu, 1994; Giulian et al., 1993) and *in vivo* (Gary et al., 1998; Barone et al., 1997). However, reports indicate that TNF-α is essential for Aβ-induced neurotoxicity (Viel et al., 2001). TNF-α has autocrine actions in microglia since in addition to acting as a stimulus of microglia, it also induces proliferation of microglia (Dopp et al., 1997; Thery and Mallat, 1993) and enhances IFN-γ-induced nitric oxide production (Merrill et al., 1993). Although, TNF-α is regarded as a pro-inflammatory cytokine, some down-regulating effects on microglia have been reported such as inducing the expression and release of the anti-inflammatory cytokine IL-10 (Sheng et al., 1995).

Immunohistochemical studies show an increase in microglial TNF-α localized to senile plaques, suggesting its participation in Aβ-induced inflammation (Dickson et al., 1993). As well, TNF-α is elevated in the serum, CSF and cerebral cortex of AD patients (Fillit et al., 1991; Tarkowski et al., 1999). TNF-α reportedly induces neuronal production of $A\beta_{1-42}$ (Blasko et al., 2001) which would then lead to increased amyloid plaque formation.

IL-6: Microglia release IL-6 in early phases of CNS insults (Raivich et al., 1999). Subsequently, IL-6 may act on astrocytes to involve these cells in the orchestration of attempts for tissue repair (Raivich et al., 1999). Interestingly, IL-6 can have both pro- and anti-inflammatory outcomes (Raivich et al., 1999). On the one hand,

like IL-1β and TNF-α, IL-6 is considered a proinflammatory cytokine with actions which include initiating and coordinating inflammatory responses, inducing acute phase proteins, increasing vascular permeability, lymphocyte activation and antibody synthesis (Akiyama et al., 2000). As well, IL-6 is autocrine in microglia since it is a major mediator of microglial activation (Raivich et al., 1996; Streit et al., 2000). On the other hand, there is also evidence that IL-6 may also have anti-inflammatory actions; for example, IL-6 can inhibit glial production of TNF-α induced by IFN-γ, IL-1β and the bacterial endotoxin lipopolysaccharide (LPS) (Benveniste, 1992). IL-6, as well as IL-1β, have been suggested to modulate APP synthesis (Vandenabeele and Fiers, 1991).

Plasma IL-6 concentrations in AD subjects have been found to be significantly elevated compared to control cases (Shibata et al., 2002). Histologic studies have demonstrated IL-6 immunoreactivity co-localized with diffuse plaques lacking neuritic pathology (Hull et al., 1996) and a strong immunohistochemical staining for IL-6 within senile plaques of AD patients (Strauss et al., 1992). Increased levels of IL-6 mRNA are expressed in glial cells surrounding amyloid plaques in AD transgenic mice (Mehlhorn et al., 2000; Tehranian et al., 2001). IL-1 and IL-6 could promote the synthesis and processing of APP, thus inducing further Aβ production and deposition (Buxbaum et al., 1992; Mrak and Griffin, 2000).

1.3.4.2 Anti-Inflammatory Cytokines

Interleukin-4 (IL-4), -10, -13, and transforming growth factor-β (TGF-β) share features of anti-inflammatory, immunosuppressive and neuroprotective actions. Much of these outcomes can be attributed to a downregulation of microglial production of cytokines, e.g., IL-1β and TNF-α, or the attenuation of their secondary release effects.

For example, IL-10 has been reported to inhibit IL-6 production in microglia (Heyen et al., 2000). IL-4 and IL-13 also interfere with IL-1 bioactivity by enhancing IL-1Ra synthesis (Dinarello, 1997a, 1997b). In addition, these cytokines can alter microglial cell surface marker expression (Chao et al., 1993; Suzumura et al., 1994; Raivich et al., 1999; Sawada et al., 1999).

TGF-β, a factor with multiple biological activities in many cells and playing roles in various tissue developments and immune responses, reduces proinflammatory cytokine and chemokine production. TGF-β is also a potent chemoattractant for microglia (Yao et al., 1990). Most notably, TGF-β has been reported to reduce AD plaque load in an animal model of AD through a phagocytotic mechanism mediated by microglia (Wyss-Coray et al., 2001). Moreover, TGF-β_1 has been shown to be protective against neuronal cell damage (Flanders et al., 1998).

1.3.4.3 Cyclooxygenase

Cyclooxygenase (COX) catalyses the formation of prostanoids comprised of prostaglandins, prostacyclin, and thromboxanes from arachidonic acid and is a major target of non-steroidal anti-inflammatory drugs (NSAIDs). COX exists in two isoforms: constitutive (COX-1) and inducible (COX-2). COX-2 is rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules and is considered a major player in inflammatory reactions and instrumental in neurodegenerative processes of several acute and chronic diseases such as AD.

The concept of a pathogenic role of COX in AD is derived from epidemiological studies reporting an association between long-term NSAID use and reduced risk of AD

(discussed in Section 1.2.3), although not every investigation has proved the same protective effect (Aisen, 2002).

Histological analyses of COX levels in AD brains have produced conflicting results. Several studies reported increased neuronal COX-2 immunoreactivity compared to control brain tissues (Yasojima et al., 1999) whereas other studies report a decrease in the number of COX-2-positive neurons with increasing severity of dementia (Yermakova et al., 2001; Hoozemans et al., 2002a). However, elevated levels of prostaglandin E2 (PGE_2), a product of COX activity, have been reported in CSF of AD patients relative to control (Ho et al., 2000; Montine et al., 1999). Interestingly, PGE_2 downregulates microglial activation, self-limiting the inflammatory process (Minghetti and Levi, 1998).

1.3.4.4 Chemokines

Chemokines belong to a superfamily of small (8–14 kDa) secreted proteins that were initially identified as regulators of leukocyte trafficking during inflammatory responses. A table summarizing the new nomenclature of chemokines is presented in Table 1-1. The new chemokine nomenclature uses CC, CXC, XC or CX3C, indicating the class to which the chemokine belongs, followed by the letter "L" (for ligand) and then a number which corresponds to that already in use to designate the genes encoding each chemokine.

Table 1-1. New Nomenclature for Chemokines (adapted from the IUIS/WHO Subcommittee on Chemokine Nomenclature, 2003)

Systematic Name	Original Ligand Name (Human)
CXC Chemokines	
CXCL1	GROα
CXCL2	GROβ
CXCL3	GROγ
CXCL4	PF4
CXCL5	ENA78
CXCL6	GCP-2
CXCL7	NAP-2
CXCL8	IL-8
CXCL9	Mig
CXCL10	IP-10
CXCL11	I-Tac
CXCL12	SDF-1α/β
CXCL13	BCA-1
CXCL14	BRAK/bolekine
CXCL15	Unknown
CXCL16	-
C Chemokines	
XCL1	Lymphotactin/ SCM-1α/ATAC
XCL2	SCM-1β
CX_3C Chemokines	
CX3CL1	Fractalkine
CC Chemokines	
CCL1	I-309
CCL2	MCP-1/MCAF/TDCF
CCL3	MIP-1α/LD78α
CCL3L1	LD78β
CCL4	MIP-1β
CCL5	RANTES
CCL6	unknown
CCL7	MCP-3
CCL8	MCP-2
CCL9/10	Unknown
CCL11	eotaxin
CCL12	Unknown
CCL13	MCP-4
CCL14	HCC-1
CCL15	HCC-2/Lkn-1/MIP-1δ
CCL16	HCC-4/LEC/LCC-1
CCL17	TARC
CCL18	DC-CK1/PARC/AMAC-1
CCL19	MIP-3β/ELC/exodus-3
CCL20	MIP-3α/LARC/exodus-1
CCL21	6Ckine/SLC/exodus-2
CCL22	MDC/STCP-1
CCL23	MPIF-1/CKβ8/CKβ8-1
CCL24	Eotaxin-2/MPIF-2
CCL25	TECK
CCL26	Eotaxin-3
CCL27	CTACK/ILC
CCL28	MEC

Microglia can produce several chemokines including CXCL1 (growth-related oncogene-alpha: GRO-α), CCL3/4 (macrophage inflammatory protein-1alpha/beta: MIP-1α/β), CCL2 (monocyte chemoattractant protein-1; MCP-1), CCL5 (regulated upon activation, normal T cells, expressed and secreted: RANTES) and CXCL8 (interleukin-8: IL-8) in response to experimental stimulation by bacterial agents, Aβ peptides, as well as cytokines, such as TNF-α and IL-1 (Ehrlich et al., 1998; Peterson et al., 1997; Janabi et al., 1998). This would suggest that activated microglia could serve in further microglial recruitment. Via chemokines, microglia can also affect neurons and astrocytes and orchestrate the migration of leukocytes. Stimulation of microglia with chemokines results largely in the recruitment and migration of microglia to sites of injury. More recently, the functional repertoire of chemokines has expanded to include fundamental roles in other cellular processes such as survival and proliferation of neurons and glia (Ambrosini and Aloisi, 2004).

The chemokine CX3CL1 (fractalkine), occurring in soluble and membrane-bound forms, is unique in that it is predominantly expressed on neurons, whereas the CX3CL1 receptor CX_3CR1 mainly associates with microglia (Harrison et al., 1998; Nishiyori et al., 1998). Therefore, disturbances in CX3CL1 levels may be sufficient to trigger or enhance microglial activation. Neuronal CX3CL1 may not only support microglial survival (Boehme et al., 2000), it also decreases microglial activation (Zujovic et al., 2000). CX3CL1 can induce microglial migration (Maciejewski-Lenoir et al., 1999). Moreover, microglia are also a source of CX3CL1 (Zujovic et al., 2000), allowing for autocrine signaling in these cells.

Under normal CNS conditions, low levels of chemokines are detected. However, under pathological conditions such as in AD, levels of chemokines are elevated (Xia et al., 1999). Elevated levels of CXCL8 (IL-8) (Galimberti et al., 2003) and CXCL8 (IL-8) receptors (Xia et al., 1997) have been detected in AD brain. Also, increased CCL2 (MCP-1; Ishizuka et al., 1997) and CCL4 (MIP-1β; Xia et al., 1998) immunoreactivity is evident in AD and localized to plaque associated microglia. Interestingly, the expression of the chemokine receptors CCR3 and CCR5 was also increased in plaque associated microglia which could explain how microglia are strongly recruited to sites of plaque pathology.

1.3.4.5 Cytotoxic Products

In addition to secretion of an unidentified neurotoxin in response to Aβ (Giulian et al., 1995) as well as pro-inflammatory cytokines (TNF-α, IL-1β; Giulian et al., 1993; Griffin and Mrak, 2002), activated microglia are capable of releasing several potentially cytotoxic factors *in vitro* including reactive oxygen intermediates, NO, proteases, arachidonic acid derivates, excitatory amino acids and quinolinic acid.

In either human or rat brain, activated microglia are the most abundant source of oxygen free radicals. Large amounts of superoxide anions (O_2^-) are generated on the microglial external membrane and subsequently released into the surroundings (Klegeris and McGeer, 1994; Colton and Gilbert, 1987). Activated microglia are also capable of producing hydrogen peroxide (H_2O_2) and reactive nitrogen intermediates (Hensley et al., 1998). H_2O_2 released by activated glia, though itself fairly innocuous, can be altered by peroxidases to form hypochlorous acid (HOCl), which is highly toxic to cells. Peroxynitrite ($ONOO^-$) formed through the interaction of NO with O_2^- can result in both

nitration of tyrosines and nitrosylation of cysteines within both enzymes and structural proteins. Activation of microglial and ensuing $ONOO^-$ production has been linked to Aβ toxicity in cultured neurons (Xie et al., 2002). Whereas physiological levels of NO may influence synaptic efficacy by regulating neurotransmitter release (Brenman and Bredt, 1997; Garthwaite, 1991), excess NO is toxic to neurons. However, the cytotoxic properties of microglia are subject to considerable species variation. For example, NO production is established for rat, but not in human microglia (Colton et al., 2000; Lee et al., 1993). In addition, iNOS was not found in stimulated human microglia (Zhao et al., 1998; Walker et al., 1995).

Proteases contribute in various functions of microglia. For example, cathepsin E and cathepsin S, endosomal/lysosomal proteases, have been shown to play important roles in the major histocompatibility complex (MHC) class II-mediated antigen presentation of microglia by processing of exogenous antigens and degradation of the invariant chain associated with MHC class II molecules, respectively (Nakanishi, 2003). Some members of cathepsins are also involved in neuronal death after being secreted from microglia and clearance of phagocytosed Aβ peptides. Tissue-type plasminogen activator, a serine protease, secreted from microglia participates in neuronal death, enhancement of NMDA (N-methyl-D-aspartate) receptor-mediated neuronal responses, and activation of microglia to contribute to further neurodegeneration (Tsirka, 2002; Nakanishi, 2003).

Derivatives of APP, including Aβ, can stimulate glutamate release from microglia (Barger and Basile, 2001; Klegeris and McGeer, 1997). Glutamate is an excitatory amino acid. Glutamate neurotoxicity mediated by activation of NMDA receptors and

subsequent Ca^{2+} influx has been suggested to be involved in neurodegeneration in AD (Koh et al., 1990; Mattson et al., 1992). NMDA receptor antagonists are efficacious neuroprotectants (Piani et al., 1992). Similarly, quinolinic acid, also a NMDA agonist, is an endogenous neurotoxic metabolite of the tryptophan-kyneurine pathway and is produced predominantly by microglia in response to stimuli including Aβ and IFN-γ (Guilleman et al., 2003; 2005). Binding of quinolinic acid to NMDA receptors leads to neurodegeneration through both excitotoxic (Stone, 1993) and oxidative (Santamaria et al., 2001) mechanisms. A table summarizing the secretory products of human microglia stimulated with Aβ is presented in Table 1-2. A schematic diagram illustrating the interactions between microglial secretory products and surrounding neurons is shown in Figure 1-2.

Table 1-2. Summary of some secretory products of Aβ stimulated human microglia.

Cytokines
IL-1α (Veerhuis et al., 2003)
IL-1β (Lue et al., 2001a; Nagai et al., 2001)
IL-8 (Lue et al., 2001a; Nagai et al., 2001)
IL-6 (Lue et al., 2001a)
TNF-α (Lue et al., 2001a; Nagai et al., 2001)
Chemokines
MIP-1α (Lue et al., 2001a; Nagai et al., 2001)
MCP-1 (Lue et al., 2001a)
Complement
C3 (Veerhuis et al., 1999)
Cytotoxic Products
Quinolinic Acid (Guilleman et al., 2003)
Superoxide Anion (Lue and Walker, 2002)
Growth Factors
macrophage colony stimulating factor (M-CSF) (Lue et al., 2001a)
nerve growth factor (NGF) (Heese et al., 1998)

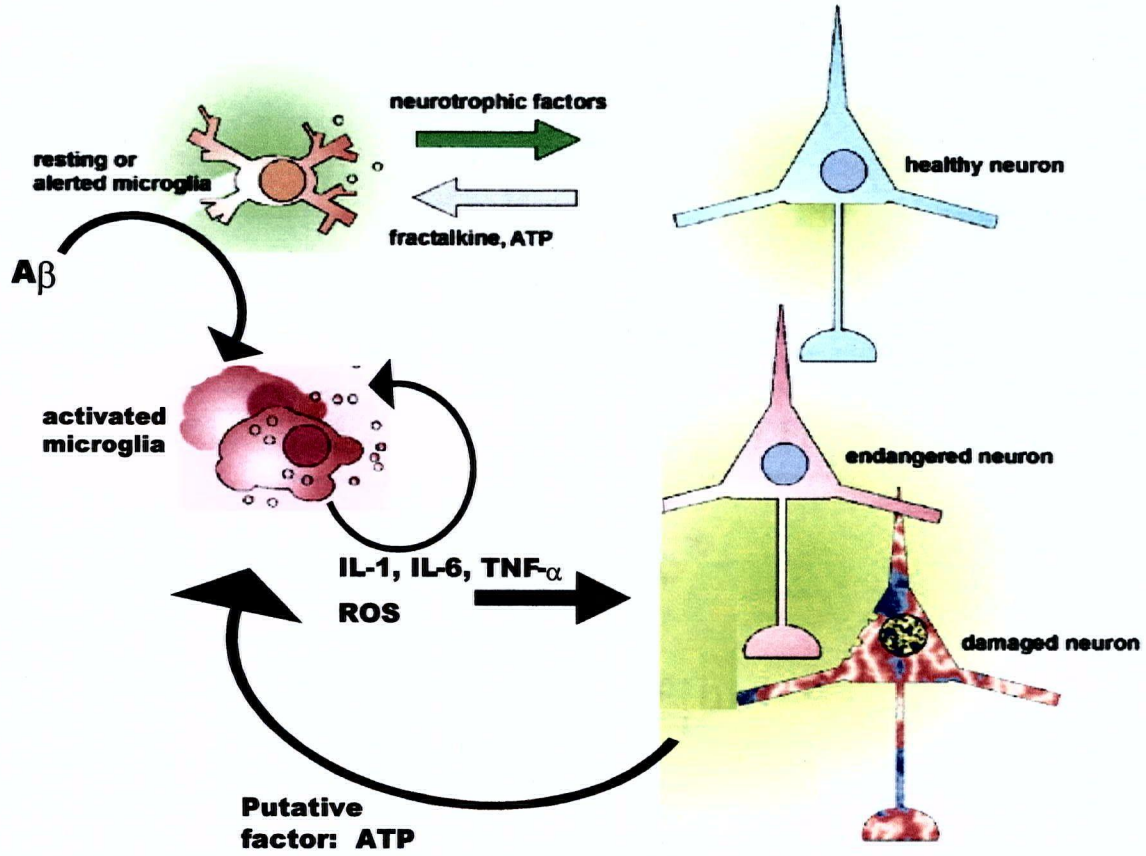

Figure 1-2. Simplified schematic illustrating the interaction between microglia and neurons of the CNS

Healthy neurons may inform microglia about their normal activity by releasing factors such as the chemokine fractalkine. Resting microglia could support neuronal function and survival by production of neurotrophic factors. Upon transformation of microglia from resting to activated in response to surrounding stimuli such as Aβ, microglia may also produce cytokines and other factors (reactive oxygen intermediates) that are potentially toxic to neurons as well as induce further recruitment of microglia (adapted from Hanisch, 2002).

1.3.5 Signal Transduction Pathways in Microglia

In vitro studies have shown that exposure of microglia to Aβ results in activation of a host of intracellular signaling pathways linked to effector functions of microglia such as secretion of inflammatory and neurotoxic substances. The tyrosine kinases Lyn, Syk, and FAK as well as p38 and ERK members of MAPK were first shown to be activated on exposure of microglia to Aβ resulting in the generation of superoxide radicals (McDonald et al., 1997; 1998). In particular, Aβ stimulated the rapid, transient activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2, p38 MAPK and downstream MAPK effectors RSK1 and RSK2 and phosphorylation of the transcription factor cAMP response element-binding (CREB) protein, providing a mechanism for Aβ-induced changes in gene expression. Also, it was reported that Aβ activation of Lyn and Syk initiated a signaling cascade resulting in a transient release of intracellular calcium, activation of PKC and the calcium-sensitive tyrosine kinase PYK2 resulting in the production of neurotoxic substances, cytokines and reactive oxygen species (Combs et al., 1999). In addition to Lyn and Syk, activation of the transcription factor NFκB was required for TNF-α production by microglia (Combs et al., 2001).

Other signaling pathways such as the CD36-dependent signaling cascade involves, Lyn and Fyn (also a tyrosine kinase), and the MAPK p44/42 resulting in reactive oxygen and chemokine production and microglial migration (Moore et al., 2002). Moreover, the Aβ-induced phosphorylation and translocation of MARCKS (Myristoylated alanine-rich C kinase substrate) and MARCKS-related protein (MRP), proteins implicated in membrane-cytoskeletal alterations underlying microglial adhesion,

migration, secretion, and phagocytosis, were reportedly dependent on a tyrosine kinase-PKC-delta signaling pathway in microglia. (Murphy et al., 2003; Nakai et al., 2001).

The expression and secretion of IL-1β by Aβ stimulated microglia involves rapid activation of three different MAPKs (p38, ERK1/2, and JNK) and NFκB activation (Kim et al., 2004; Kang et al., 2001). Furthermore, IL-1β production and chemotaxis of microglia has been linked to activation of a G protein coupled receptor and calcium increase (Lorton et al., 1997; Tiffany et al., 2001).

Moreover, induction of an outward K^+ current in rat microglia attributed to activation of voltage-dependent Kv1.3 and Kv1.5 channels (Chung et al., 2001) and increases in $[Ca^{2+}]_i$ have been reported in response to Aβ stimulation (Silei et al., 2000; Korotzer et al., 1995).

1.3.6 Treatments for Alzheimer's Disease

The most widely used therapy for AD is the acetylcholinesterase inhibitors which lead to increased acetylcholine and subsequent reduction in cognitive impairment associated with this disease. Although acetylcholinesterase inhibitors are helpful in treating the symptoms of the disease, they have no effect to prevent or delay disease progression. Other current therapeutic approaches include antioxidants, NSAIDs to reduce inflammation (discussed in Section 1.2.3), NMDA receptor antagonists, methods of inhibiting amyloid generation and aggregation as well as increasing amyloid removal.

The secretases that produce Aβ are considered potential therapeutic targets for the treatment of AD. Treatment of mice with a γ-secretase inhibitor resulted in reduced Aβ levels in the brain and attenuated Aβ deposition (Dovey et al., 2000). Compounds with

effects to block γ-secretase without blocking other γ-secretase targets, which could be lethal, have been developed (Petit et al., 2001) and are currently being tested in clinical trials.

Epidemiological data indicate that cholesterol may play a role in AD pathogenesis. Retrospective studies of individuals taking HMG-CoA (β-hydroxy- β-methylglutaryl-coenzyme A) reductase inhibitors or statins show a large reduction in the risk for developing AD (Wolozin et al., 2000). *In vitro* and *in vivo* studies indicate that statins and other cholesterol-lowering agents decrease Aβ levels and Aβ deposition (Refolo et al., 2001; Simons et al., 1998), whereas high-cholesterol diets in APP transgenic mice increase Aβ deposition (Refolo et al., 2000). *In vitro*, statins seem to promote a favourable shift toward the non-amyloidogenic pathway of APP processing leading to increased secretion of the neurotrophic sAPPα peptide and decrease in Aβ production (Buxbaum et al., 2001; Puglielli et al., 2001). These results would indicate that cholesterol lowering drugs may be of therapeutic benefit in the prevention and treatment of AD.

Active immunization with synthetic $A\beta_{1-42}$ has been shown to be effective in transgenic models of AD to significantly reduce Aβ plaques (Schenk et al., 1999; Bard et al., 2000). Using the approach of $A\beta_{1-42}$ immunization, specific $A\beta_{1-42}$ antibodies are elicited and these antibodies move across the BBB resulting in the removal of amyloid plaques and improved cognitive performance (Kotilinek et al., 2002). Although the mechanism through which antibodies to Aβ decrease plaque burden is not clear it is believed to involve the disruption of Aβ fibrils, prevent Aβ fibril formation, block the toxic effects of Aβ aggregates and enhance clearance of plaques by microglia (Golde,

2003). In a recent clinical trial, administration of the vaccine in patients with AD resulted in discontinuation of the trial after signs of meningoencephalitis developed in about 6% of the treated patients. The follow-up in patients receiving the vaccine showed that 60% produced antibodies against amyloid-containing plaques and patients with higher antibody titer had a slowing in cognitive loss (Hock et al., 2002; 2003). Due to the side effect observed in AD patients receiving the vaccine, Aβ vaccines with differing doses of Aβ peptide/anti-Aβ peptide adjuvants are being explored. As well, alternative Aβ vaccination methods such as the Aβ gene vaccination method have been proposed (Qu et al., 2004).

In my study, I have used the approach of blocking inflammation by inhibiting microglial mediated inflammatory responses using a modulator of Aβ-induced intracellular signaling pathways in these cells.

1.3.7 Rationale for Proposed Research

A chronic inflammatory reaction exists in AD brain (Akiyama et al., 2000). Activated microglial cells are localized to neuritic plaques and are a significant source of inflammatory mediators (McGeer and McGeer, 1995). Thus, selective modulation of microglial signaling pathways could be an effective strategy to lessen inflammation in AD.

The activation process induced by Aβ in microglia has been associated with specific cell surface receptors (discussed in Section 1.3.3.1) and intracellular signaling pathways (discussed in Section 1.3.5) which include membrane K^+ current expression, altered intracellular calcium, activation of tyrosine kinases, p38 MAP kinase and activation of transcription factors (NFκB, CREB). These intracellular signaling pathways

are intimately linked to particular cellular functions of microglia (discussed in Section 1.3.3.2) which include proliferation, phagocytic activity and motility. In terms of actions on bystander cells such as neurons a critical cellular role of microglia is the production of neurotoxins (discussed in Section 1.3.4.5). Therefore, it is likely that microglial inflammatory responses in AD are dependent on a complex pattern of altered membrane currents, intracellular calcium and changes in other intracellular signal transduction pathways. Therapeutic approaches focused on modulation of stimulus-induced signaling and microglial-mediated inflammatory responses could serve as a therapeutic strategy in neurodegenerative diseases.

As noted above, changes in membrane current patterns are an important signaling pathway in microglia. Based on these results, pharmacological modulation of activated channels could be a novel and effective maneuver to inhibit microglial mediated inflammatory responses. My preliminary data indicated that acute application of $A\beta_{1-42}$ to human microglia induced a novel outward current consistent with activation of a K^+ channel. As well, a novel finding in this work was that intracellular application of the non-hydrolyzable analogue of GTP, GTPγS, induced an outward K^+ current similar in properties to the outward K^+ current induced with acute $A\beta_{1-42}$. Based on these results, I used the non-selective K^+ channel inhibitor, 4-aminopyridine (4-AP), to investigate properties of this current. Preliminary experiments showed that 4-AP acted to inhibit the outward current induced by acute $A\beta_{1-42}$. This finding led to a detailed investigation of 4-AP as a pharmacological modulator of Aβ-stimulated microglia. Interestingly, 4-aminopyridine has been previously used as a therapy in treatment of AD patients (Wesseling et al., 1984; Davidson et al., 1988). This compound passes through the BBB

whereas other non-selective K^+ channel blockers such as TEA do not (Soni and Kam, 1982).

Since 4-AP blocked the channel upregulated by acute $A\beta_{1-42}$, I wanted to carry out further experiments as to whether 4-AP would be an effective modulator of other signaling pathways and functional responses of human microglia. As pointed out above, Ca^{2+} along with other second messengers are important signaling factors in microglia. Work done by the Landreth laboratory (Section 1.3.5) indicated that an increase in intracellular Ca^{2+} was involved in response to Aβ in rodent microglia in addition to the activation of several other intracellular signaling factors such as tyrosine kinases, members of MAPK and NFκB (McDonald et al., 1997; 1998; Combs et al., 2001). Many of these second messengers are linked to cytotoxic secretory products of microglia. I wanted to investigate the effects of Aβ on Ca^{2+} and other intracellular signal transduction factors such as p38 MAPK and NFκB in human microglia and whether 4-AP modulated these second messengers. Some studies were also undertaken to investigate if the Fc receptor might be involved in response to the Aβ peptide.

Aβ stimulated microglia produce a host of factors implicated in inflammation and neurodegeneration as stated above (Griffin et al., 1998). The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the inducible enzyme COX-2 are important modulators of inflammatory processes (Benveniste, 1992; O'Neill and Ford-Hutchinson, 1993) and increased levels of these factors have been observed in AD brain (Griffin et al., 1998; Pasinetti and Aisen, 1998; Ho et al., 1999). It was therefore of interest to determine in this research program whether 4-AP had effects to modulate the expression and production of these pro-inflammatory mediators.

I then carried out a series of studies to examine 4-AP actions *in vivo*. Since *in vitro* results suggested that 4-AP is a potential modulator of $A\beta_{1-42}$-induced intracellular signaling pathways and functional responses in human microglia, 4-AP was tested for potential anti-inflammatory and neuroprotective effects *in vivo*. The effects of 4-AP administration on microglial activation and neuronal toxicity induced by a local injection of $A\beta_{1-42}$ into the rat hippocampus was investigated.

In a separate set of studies, I also examined if the pro-inflammatory effects of Aβ could be potentiated by another stimulus of microglia, the chemokine CXCL8 (IL-8). The rationale for this study was that in a thorough gene expression study, CXCL8 (IL-8) was reported as the most prominent factor expressed by Aβ stimulated human microglia (Walker et al., 2001). Moreover, increased levels of CXCL8 (IL-8) have been observed in brains of AD patients (Galimberti et al., 2003). Since CXCL8 (IL-8) is secreted (Ehrlich et al., 1998) by stimuli such as Aβ (Nagai et al., 2001) and in turn CXCL8 (IL-8) stimulates chemotaxis of microglia (Cross and Woodroofe, 1999), CXCL8 (IL-8) is autocrine in these cells. Potentiation of Aβ induced responses by stimuli such as CXCL8 (IL-8) would be relevant to inflammatory actions of microglia in AD brain.

In summary, my research program will determine the effects of $A\beta_{1-42}$ on the intracellular signaling pathways (ion channel expression, Ca^{2+}, p38 MAPK, NFκB) and functional responses induced by Aβ (expression and production of pro-inflammatory factors, neurotoxicity), both in the presence and absence of the modulator 4-AP. As well, the *in vivo* effects of 4-AP will be determined on $A\beta_{1-42}$-induced microglial activation and neurotoxicity. Furthermore, the potentiating effects of the chemokine IL-8 on $A\beta_{1-42}$-

induced functional responses in human microglia will be investigated. A model of the research presented in this thesis is shown in Figure 1-3.

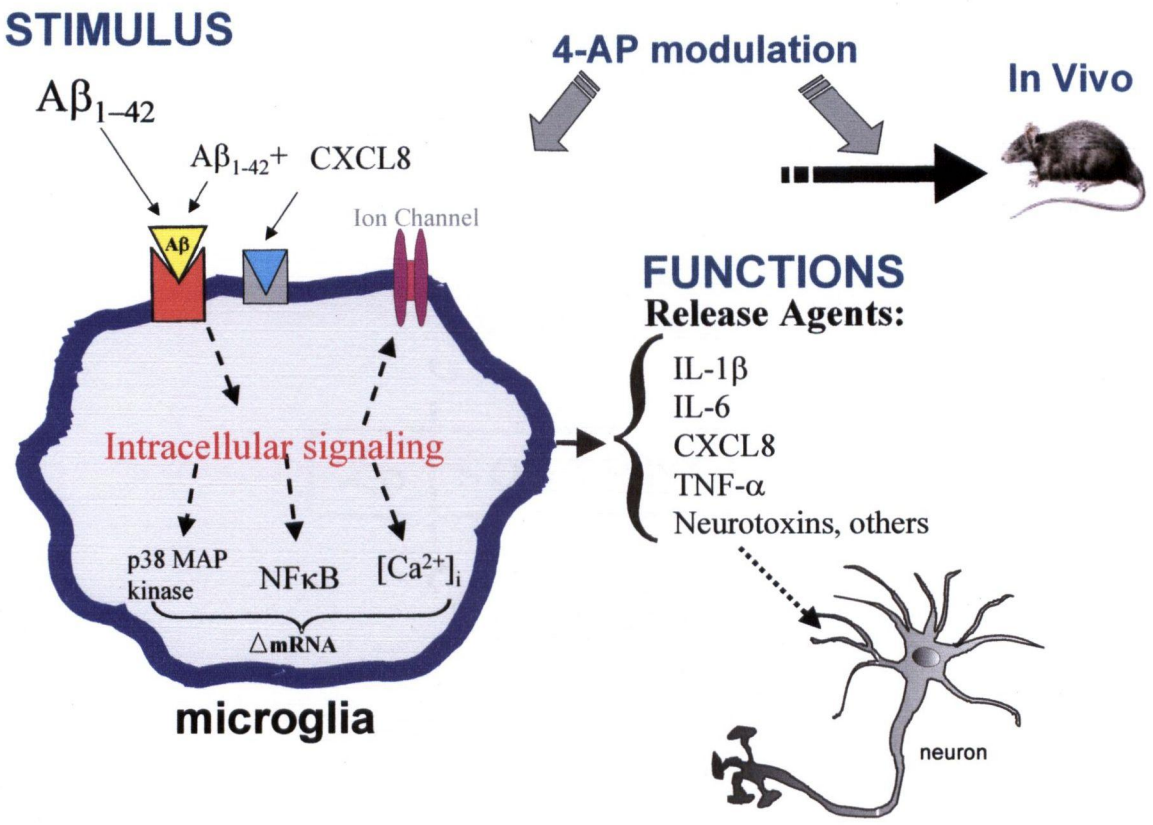

Figure 1-3. Simplified schematic diagram illustrating the research presented in this thesis.
Overall, some intracellular signaling pathways and functional responses shown in the diagram were examined in $A\beta_{1-42}$ stimulated human microglia. Some evidence suggests that Fc receptors may be involved in Aβ actions.

1.4 RESEARCH HYPOTHESIS

Microglia are involved in inflammatory responses that mediate neuronal degeneration associated with Alzheimer's Disease (AD). Signaling pathways in microglia regulate cellular functional responses both under normal and pathological conditions. The hypothesis of the proposed research is that changes in membrane currents, $[Ca^{2+}]_i$, intracellular signal transduction pathways are involved in the signaling pathways of $A\beta_{1-42}$ and are coupled to microglial functional responses including secretion of substances that would potentiate an inflammatory response as well as lead to neurotoxicity both *in vitro* and *in vivo*. Additionally, modulation of these signaling pathways could serve a site for potential therapeutic intervention in decreasing microglial-induced neuronal toxicity and subsequent neuronal loss associated with AD. One such agent, the non-selective K^+ channel blocker 4-AP, has been studied in detail as a modulator of Aβ-stimulated human microglia.

1.5 SUMMARY OF RESEARCH OBJECTIVES

The overall thesis objective is to investigate pharmacological modulation by 4-AP of $A\beta_{1-42}$-induced intracellular signaling pathways and functional responses including peptide-induced microglial mediated neurotoxicity.

The specific objectives are listed below:

1. to identify $A\beta_{1-42}$-induced Ca^{2+} signaling pathways, second messenger activation and alterations in membrane potential and currents in human microglia.
2. to investigate 4-AP as a modulator of $A\beta_{1-42}$-induced membrane currents, intracellular calcium $[Ca^{2+}]_i$ levels, second messengers (p38 MAPK, NFκB) and for effects on functional responses of human microglia such as expression and production of inflammatory mediators (TNF-α, IL-1β, IL-6, CXCL8 (IL-8) and COX-2) and potential neurotoxicity.
3. To determine the effects of 4-AP *in vivo* on $A\beta_{1-42}$-induced microglial activation and neurotoxicity.
4. To determine the potentiating effects of CXCL8 (IL-8) on $A\beta_{1-42}$-induced expression and production of inflammatory mediators (TNF-α, IL-1β, IL-6, CXCL8 (IL-8) and COX-2).

The specific objectives 1 to 4 are presented in thesis chapters 3 to 5.

Chapter 2: MATERIALS AND METHODS

2.1 ISOLATION OF HUMAN MICROGLIA

Human microglia were prepared according to procedures reported previously (Satoh et al., 1995). Briefly, embryonic brain tissues 12-18 weeks gestation were incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin and DNase (40 μg/mL) for 30 min at 37°C. Enzyme treated tissues were dissociated into single cells by gentle pipetting. Dissociated cells were then cultured into Dulbecco's Modified Eagle's Medium (DMEM) containing 5% horse serum, 5 mg/mL glucose, 20 ng/mL gentamicin and 2.5 μg/mL amphotericin B. After 7-10 days of growth in culture flasks, freely floating microglia were collected from a medium of mixed cell cultures and plated on glass coverslips. The purity of the microglial cultures was in excess of 98% as determined by immunostaining with the cell specific markers CD11b or ricinus communis agglutinin-1. Use of embryonic human tissues was approved by the Clinical Screening Committee for Human Subjects of the University of British Columbia.

2.2 ELECTROPHYSIOLOGICAL STUDIES OF HUMAN MICROGLIA AND RECORDING SOLUTIONS

Procedures used in whole-cell patch clamp studies have been described previously (McLarnon et al., 1997). Briefly, one-day plated coverslips were placed on the stage of an inverted microscope (Nikon TMS). I used one-day plated coverslips since microglia plated for more than five days express an inactivating outward K^+ current in response to a depolarizing step not present in cells one to two days post-plating (McLarnon et al., 1997). Coverslips were plated at low density since only one cell/coverslip was used and

only large ameboid cells were chosen since they were easier to patch. Typical density of coverslips and ameboid morphology of microglia used in whole cell patch clamp experiments is shown in Figure 2-1. An amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) was used to record macroscopic currents as well as membrane potential (V_m). Patch pipettes were fabricated using Corning glass no. 7052 with resistances in the range of 2-4 MΩ. Capacitance and series resistance were compensated manually on the amplifier. The whole-cell configuration was used and data sampled at 5 kHz with the low-pass filter set at 1 or 2 kHz.

Figure 2-1. Representative photomicrograph of microglia used in electrophysiological experiments.
Microglia cells one-day post-plating in normal bath solution displayed both ramified (elongated, spindle-shaped) and ameboid (rounded) morphologies. Coverslips were plated at low density and only large ameboid cells (indicated with arrows) were used in whole-cell patch clamp experiments. (x100 magnification; scale bar = 20 μm).

Protocols used in voltage clamp experiments were generated by computer and consisted of applying a depolarizing step from a holding potential (V_H) of –60 mV in order to study outward K^+ currents. All voltage clamp experiments in Chapter 3 were carried out with 500 μM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) in bath solution in order to remove any chloride contribution to the overall whole cell current. Some current clamp experiments were also carried out to determine the effects of $A\beta_{1-42}$ on V_m. Both resting membrane potential recordings and stimulus-induced changes in V_m were recorded in these experiments. Data were recorded on disk and analyzed off-line using pClamp 6.0 software. All experiments were performed at room temperature (20-22°C).

The normal bath solution contained (in mM): NaCl (140), KCl (5), $CaCl_2$ (1), $MgCl_2$ (1), glucose (10), HEPES (10); pH adjusted to 7.3. The pipette solution contained (in mM): KCl (140), NaCl (10), $MgCl_2$ (1), EGTA (0.5), ATP (1), HEPES (10); pH adjusted to 7.3. In experiments where elevated extracellular K^+ was applied, 40 mM KCl was used to replace an equivalent amount of NaCl. Stimuli were applied in the bath solution and perfused onto the cell using a gravity fed system with the exception of GTPγS which was applied intracellularly through the recording electrode (discussed below; Chapter 3).

In order to determine whether a G protein was involved in inducing an outward K^+ current in microglia as has been previously shown in macrophages (McKinney and Gallin, 1992), the non-hydrolyzable analogue of GTP, GTPγS (10 μM), was applied intracellularly through the recording electrode (Figure 2-2). Upon rupture of the cell membrane in the whole-cell patch clamp mode, GTPγS diffuses from the electrode into

the cell where it irreversibly activates G protein coupled receptors to induce second messenger signaling in microglia.

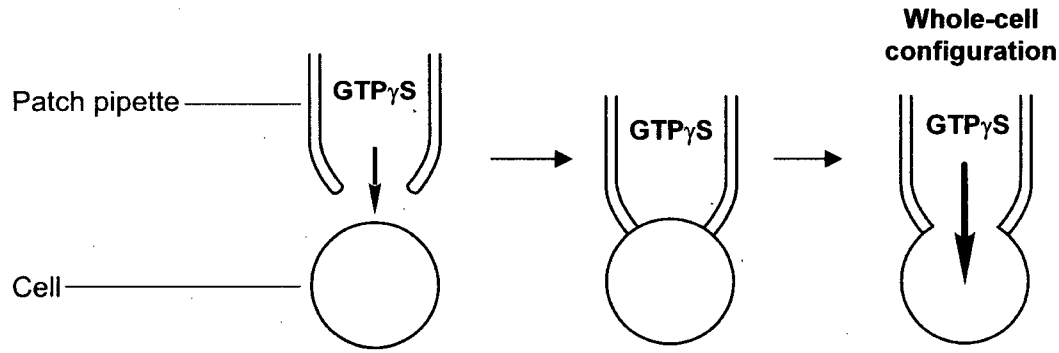

Figure 2-2. Intracellular application of GTPγS via the recording electrode. Upon rupture of the cell membrane in the whole-cell patch clamp mode, GTPγS diffuses into the cell where it exerts its intracellular effects.

2.3 CALCIUM SPECTROFLUOROMETRIC STUDIES

Levels of intracellular calcium $[Ca^{2+}]_i$ in human microglia were monitored using calcium spectrofluorometry. Cells plated on glass coverslips were loaded with 1 μM fura-2/AM (Molecular Probes, Eugene, OR) with the solubilizing agent 0.02% pluronic acid in standard physiological solution (PSS). The PSS solution contained (in mM): NaCl (126), KCl (5), $MgCl_2$ (1.2), $CaCl_2$ (1), D-glucose (10) and HEPES (10) at pH 7.4. In experiments performed with no Ca^{2+} in the extracellular solution, a Ca^{2+}-free PSS was used where the $CaCl_2$ was replaced with 1 mM EGTA. Following the wash period in dye-free solution, the coverslips were mounted on the stage of a Zeiss Axiovert inverted microscope containing a x 40 quartz objective lens. The cells were then exposed to alternating wavelengths of 340 and 380 nm UV light at intervals of 6 seconds and emission light was passed through a 510 nm filter (bandwidth of 20 nm). The signals were acquired from a digital camera (DVC-1300 Camera, Photometrics) and recorded using an imaging system (Empix, Mississauga, ON) as fluorescence ratios of 340/380 every 6 seconds. Increases in $[Ca^{2+}]_i$ were expressed as 340/380 fluorescence ratios since unherent uncertainties exist in conversion of ratios into $[Ca^{2+}]_i$ using an *in vitro* calibration (Takahashi et al., 1999). Stimuli were applied acutely using a micropipette and solutions exchanged using a vacuum suctioning system. All experiments were carried out at room temperature (20-22°C).

2.4 IMMUNOCYTOCHEMICAL STUDIES OF HUMAN MICROGLIA

2.4.1 Determination of p38 MAP Kinase and NFκB Immunoreactivity

The effects of stimuli on phosphorylated p38 MAP kinase (phospho-p38) in human microglia were determined using immunocytochemistry according to a similar procedure described previously in microglia (Tikka and Koistinaho, 2001). Briefly, following pre-incubation in serum-free medium for 48 hrs, cells were treated for 30 min with $A\beta_{1-42}$ (5 μM), 2 mM 4-AP alone, or $A\beta_{1-42}$ in combination with 4-AP following a 30 min preincubation with 4-AP, and $A\beta_{42-1}$. After treatment, cells were fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS, washed in PBS and permeabilized in 0.2% Triton X-100 containing 5% NGS in 0.1M PBS/0.5% BSA (BPBS) solution for 25 min. The cells were then incubated in rabbit anti-human phospho-p38 (1:250 dilution, Cell Signaling) containing 5% NGS in BPBS at 4°C for 48 hrs. As a negative control, the phospho-p38 primary antibody was omitted. Following wash in PBS, cells were incubated with Alexa Fluor 488 anti-rabbit IgG secondary antibody (1:100, Molecular Probes, Eugene, OR, USA) containing 5% NGS in BPBS at room temperature for 2 hr. Following a wash in PBS, cells were incubated in 4'-6'-diaminodino-2-phenylindole (DAPI, Molecular Probes) at 1 μg/mL in PBS to visualize nuclei and determine cell numbers in the field of view. Cells were then washed in water and mounted onto glass slides using gelvatol, examined under a Zeiss light microscope and photographed using a cooled CCD camera. Cells positively stained with phospo-p38 MAP kinase were determined from four representative fields in four independent experiments (approximately 600 cells/treatment group) and the ratio of phosho-p38 positive cells to total number per field was determined (at x200 magnification). As a positive control, a

similar procedure was carried out with cells stimulatd with LPS for 30 min in three independent experiments. Values are expressed as means ± SEM and statistical significance was determined using one-way ANOVA and Newman-Keuls multiple comparison post-test ($p < 0.05$).

In resting cells, the transcription factor NFκB is retained in the cytoplasm in an inactive form by binding to a family of inhibitory molecules: IκBα or IκBβ (Christman et al., 2000). Activation of NFκB results in rapid phosphorylation and degradation of IκBs allowing the active complex p65/p50 of NFκB to be released, translocate to the nucleus and transactivate target genes. To investigate the effect of 4-AP on $A\beta_{1-42}$-induced NFκB activation, a similar immunocytochemical procedure as described for the determination of p38 MAPK activation was used to determine the effects of 8 hr stimulation with $A\beta_{1-42}$ (5 μM), 4-AP alone, $A\beta_{1-42}$ in combination with 2 mM 4-AP following a 30 min preincubation with 4-AP, 4-AP alone and $A\beta_{42-1}$ on the nuclear translocation of p65 as has been described previously in microglia (Nakajima et al., 1998). The primary antibody used for this set of experiments was rabbit anti-human p65 (1:250 dilution, Santa Cruz, CA), in order to target the active p65 NFκB subunit and cells with intense staining localized to the nucleus were considered positively stained cells. Results were obtained from four representative fields in five independent experiments (approximately 600 cells/treatment group) and summarized as mean ± SEM. Positive control experiments consisted of stimulating microglia with LPS for 12 hrs and determining the number of positive p65 cells as described above in six independent experiments. As a negative control, experiments were carried out with p65 primary antibody omitted.

2.4.2 Determination of COX-2 Immunoreactivity

COX-2 production was determined in microglia using immunocytochemistry. Following 48 hr pre-incubation in serum-free medium, cells were stimulated for 24 hrs with stimuli. In experiments where 4-AP was used as a modulator, cells were pre-incubated with 4-AP for 30 min prior to addition of stimuli and 4-AP was maintained in solution subsequent to addition of stimuli. After treatment, cells were fixed with 4% PFA in 0.1 M PBS, washed in PBS and permeabilized in 0.2% Triton X-100 containing 10% goat serum in 0.1M PBS/0.5% BSA (BPBS) solution for 20 min. The cells were then incubated in rabbit anti-human COX-2 (1:200 dilution, Cayman Chemical, Ann Arbor, MI) containing 10% goat serum in BPBS at 4°C for 72 hrs. As a negative control, the COX-2 primary antibody was omitted. Following wash in PBS, cells were incubated with Alexa Fluor 488 anti-rabbit IgG secondary antibody (1:100, Molecular Probes, Eugene, OR, USA) containing 5% goat serum in BPBS at room temperature for 1 hr. Following a wash in PBS, cells were incubated in DAPI (Molecular Probes) at 1 μg/mL in PBS to visualize nuclei and determine cell numbers in the field of view. Cells were then washed in water and mounted onto glass slides using gelvatol, examined under a Zeiss light microscope and photographed using a cooled CCD camera. The number of cells positively stained with COX-2 was determined (at x200 magnification) and expressed as a ratio of COX-2 positive cells to total number from four representative fields (approximately 150 cells/field). As a positive control, cells were treated with LPS for 12 hrs and the number of COX-2 positive cells determined as described above in three independent experiments.

2.5 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION OF HUMAN MICROGLIA

Human fetal microglia were seeded into poly-L-lysine coated 12-well plates at a density of approximately 5 x 10^4 cells/well. Following pre-incubation in serum-free conditions for 48 hrs in order to promote a resting state, human microglia were then treated for 8 hours with stimuli. In experiments where 4-AP was used, cells were pre-incubated with 4-AP for 30 minutes prior to addition of stimuli and subsequently maintained in solution following addition of stimuli. Total RNA was isolated using TRIzol (GIBCO-BRL, Gathersburg, MD), subjected to DNase treatment and then processed for the first strand complimentary DNA (cDNA) synthesis using Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL). cDNA products were then amplified by PCR using a GeneAmp thermal cycler (Applied Biosystems, Foster City, Ca). The PCR reaction buffer included cDNA, sense and antisense primers and Taq polymerase. Specific sense and antisense primers with the expected product size are listed in Table 2.1. PCR consisted of an initial denaturation step of 95°C for 6 minutes followed by a 30-40 cycle amplification program consisting of denaturation at 95°C for 35 s, annealing at 59°C for 1 min and elongation at 72°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a reaction standard. The amplified PCR products were identified by electrophoresis using 1.5% agarose gels containing ethidium bromide and then visualized under UV light. The intensities of each band were measured by densitometry using the NIH Image J 1.24 software (National Institutes of Health, Bethesda, Maryland, USA). The band intensities of PCR products in control and with stimuli were measured and expressed as relative mRNA levels (mRNA values normalized to G3PDH).

Table 2-1. Primer Sequences for RT-PCR

Product	Sequence	Size (b.p.)
COX-2 sense	5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'	305
COX-2 antisense	5'-AGATCATCTCTGCCTGAGTATCTT-3'	
IL-1β sense	5'-AAAAGCTTGGTGATGTCTGG-3'	179
IL-1β antisense	5'-TTTCAACACGCAGGACAGG-3'	
IL-6 sense	5'-GTGTGAAAGCAGCAAAGAGGC-3'	159
IL-6 antisense	5'-CTGGAGGTACTCTAGGTATAC-3'	
CXCL8 sense	5'-ATGACTTCCAAGCTGGCCGTG-3'	301
CXCL8 antisense	5'-TATGAATTCTCAGCCCTCTTCAAAA-3'	
TNF-α sense	5'-CAAAGTAGACCTGCCCAGAC-3'	490
TNF-α antisense	5'-GACCTCTCTCTAATCAGCCC-3'	
IL-10 sense	5'-AGATCTCCGAGATGCCTTCAGCAGA-3'	194
IL-10 antisense	5'-CCTTGATGTCTGGGTCTTGGTTCTC-3'	
$TGF\beta_1$ sense	5'-TTGCAGTGTGTTATCCGTGCTGTC-3'	185
$TGF\beta_1$ antisense	5'-CAGAAATACAGCAACAATTCCTGG-3'	
Kv1.1 sense	5'-GTTAGGGGAACTGACGTGGA-3'	482
Kv1.1 antisense	5'-CTGAGCAGGAGAGGAAACCAG-3'	
Kv1.2 sense	5'-GGGACAGAGTTGGCTGAGAA-3'	513
Kv1.2 antisense	5'-GGAGGATGGGATCTTTGGAC-3'	
Kv1.3 sense	5'-GCGACGAGAAGGACTACCC-3'	513
Kv1.3 antisense	5'-TGCTGCTGAAACCTGAAGTG-3'	
Kv1.5 sense	5'-GAGGACGAGGAGGAAGAAGG-3'	528
Kv1.5 antisense	5'-CAAGCAGAAGGTGATGATGG-3'	
Kv1.6 sense	5'-GGGAGTCAGGAGGAAGAGGA-3'	569
Kv1.6 antisense	5'-ATGCTGGGAAAAAGCGAAT-3'	
Kv2.1 sense	5'-ACAGAGCAAACCAAAGGAAGAAC-3'	385
Kv2.1 antisense	5'-CACCCTCCATGAAGTTGACTTTA-3'	
Kv3.1 sense	5'-TCCTGAACTACTACCGCACG-3'	620
Kv3.1 antisense	5'-GAACTCTACCTTGTTGGGGC-3'	
G3PDH sense	5'-CCATGTTCGTCATGGGTGTGAACCA-3'	251
G3PDH antisense	5'-GCCAGTAGAGGCAGGGATGATGTTC-3'	

2.6 ENZYME LINKED IMMUNOSORBENT ASSAY

ELISA kits (R & D Systems, Minneapolis, MN, USA) were used to determine the production of TNF-α, IL-6, IL-1β and CXCL8 (IL-8) in culture supernatants from human fetal microglia stimulated for either 24 hrs (in the case of CXCL8 (IL-8) potentiation of $A\beta_{1\text{-}42}$ effects; Chapter 5) or 48 hrs (in the case of 4-AP modulation of $A\beta_{1\text{-}42}$ effects;

Chapter 4) after pre-incubation in serum-free medium for 48 hrs. In experiments where 4-AP was used as a modulator of $A\beta_{1-42}$ effects, cells were incubated with 4-AP for 30 min prior to addition of stimuli and maintained in solution following addition of stimuli. The kits were able to detect low levels of the cytokines (as low as 4.4 pg/mL of TNF-α, 1 pg/mL IL-1β, 0.7 pg/mL IL-6 and 10 pg/mL CXCL8 (IL-8)). After incubation of microglia with stimuli, cell culture supernatants were collected and stored at -70°C.

2.7 DETERMINATION OF FcγRII EXPRESSION IN HUMAN MICROGLIA

Microglia were plated into 96-well culture plates (1 x 10^5 cells/well) in 200 µl of DMEM containing 5% horse serum. $A\beta_{1-42}$ was applied in duplicate wells. Cells were treated and incubated in 5% CO_2, 95% air at 37°C with $A\beta_{1-42}$ for 30 min and 48 hr following pre-incubation in serum-free medium for 48 hrs. Expression of Fcγ receptor type II (FcγRII) was measured following procedures described previously (Klegeris et al., 2000). In brief, cells were fixed by air-drying overnight and then blocked with 3% BSA in PBS at room temperature for 2 hr. Subsequently, the plates were incubated with monoclonal FcγRII antibody (1:500 dilution in blocking solution) for 1 hr. In negative control experiments, the FcγRII primary antibody was omitted. Following incubation with FcγRII antibody, cells were briefly washed four times in PBS and then incubated with goat anti-mouse IgG alkaline phosphatase conjugate diluted 1:4000 in blocking solution for 1 hr. After incubating the cells with substrate buffer containing 1 mg/ml of Sigma 104 phosphate substrate in 0.1 M diethanolamine buffer, pH 9.8, optical densities (OD) were measured every 10 min for 1 hr using a 405-nm filter. The OD of the negative control well was then subtracted from the OD of untreated and treated wells at

the corresponding time point. The change in OD over 60 min in treated samples was normalized to that of untreated. Values were obtained from n= 4 independent experiments for 30 min treatment and n=6 independent experiments for 48 hr treatment; each experiment was repeated in duplicate.

2.8 MICROGLIAL MORPHOLOGY AND CELL VIABILITY

Human microglia were pre-incubated in serum-free medium for 48 hrs prior to addition of stimuli in these studies (immunocytochemistry, RT-PCR, ELISA, FcR, neurotoxicity) in order to promote a resting state. This procedure yields a homogenous population of cells with a ramified, process-bearing morphology. Following pre-incubation in serum-free medium, microglia were exposed to inflammatory agents for periods of up to 48 hrs which often yielded a rounding of cells lacking processes indicative of an ameboid morphology. Our laboratory routinely uses DAPI staining to examine the viability of microglia with different treatments (Choi et al., 2003) and I found no evidence of nuclear condensation or fragmentation under any of the experimental conditions indicating no loss of cell viability in the present experiments.

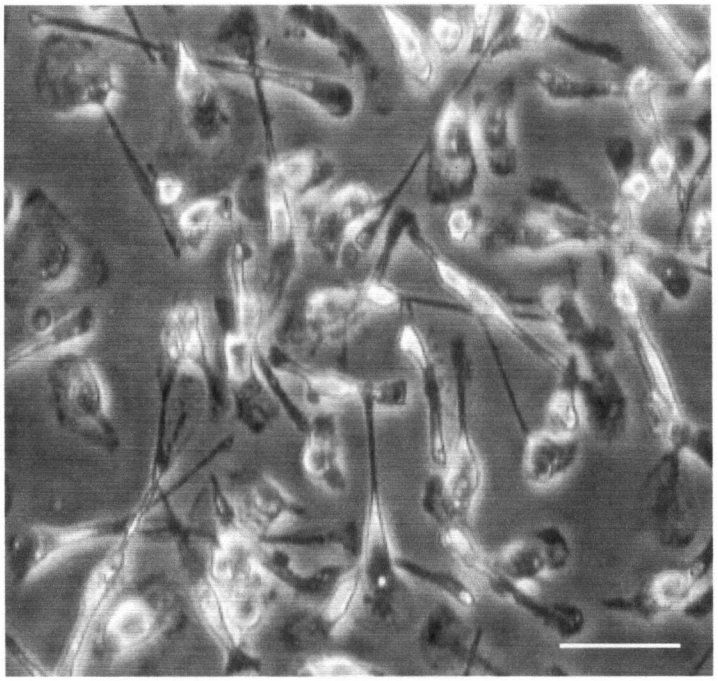

Figure 2-3. A representative photomicrograph of cultured human fetal microglia. Both ramified and ameboid microglia were present one-day post-plating (at x100 magnification; scale bar = 50 μm).

2.9 DETERMINATION OF MICROGLIAL-MEDIATED NEUROTOXICITY

2.9.1 Preparation and Treatment of Human Fetal Microglia

Human fetal microglia were seeded into 24-well plates at 1.5 x 10^5 cells per well. Following 48 hr pre-incubation in serum-free medium, cells were treated with $A\beta_{1-42}$ (5 μM), 4-AP alone, or $A\beta_{1-42}$ in combination with 2 mM 4-AP following 30 min preincubation with 4-AP, 4-AP alone and $A\beta_{42-1}$. After 48 hours incubation with stimuli, cell free supernatants were transferred to primary rat hippocampal neurons plated on glass coverslips. Figure 2-4 illustrates the procedures carried out for the determination of microglial mediated neurotoxicity including control experiments which contained conditioned medium without microglial exposure.

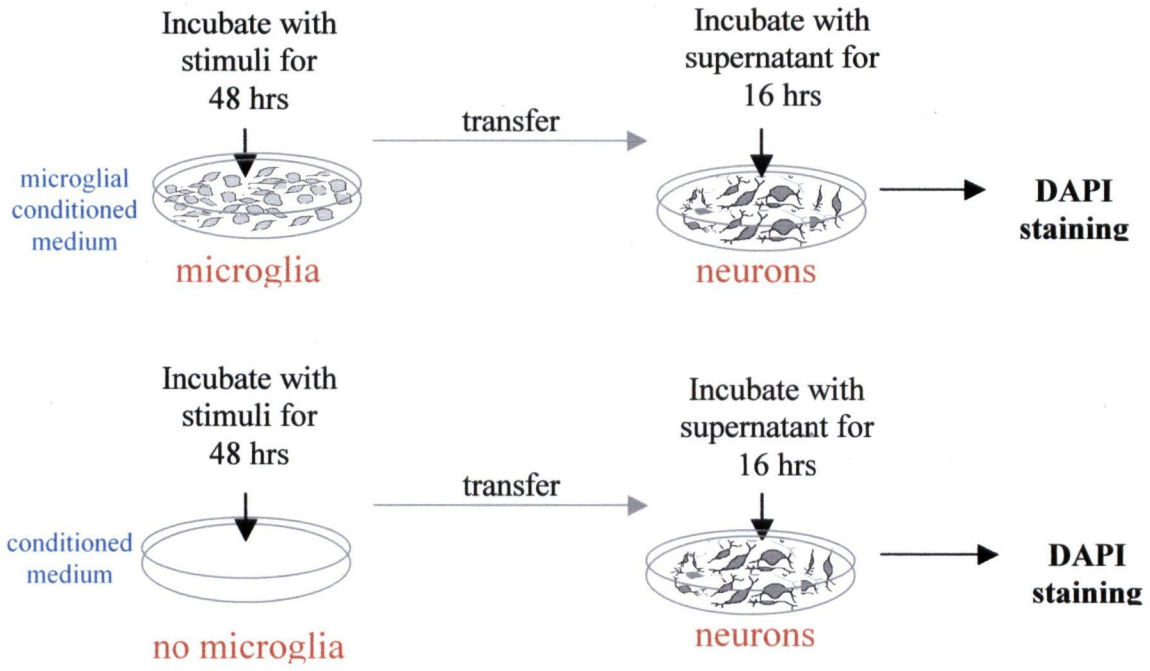

Figure 2-4. Experimental procedure used to determine the effects of microglial conditioned medium on neuronal survival.
Following incubation of primary human fetal microglia with stimuli for 48 hrs, microglial supernatant (microglial conditioned medium) was transferred to cultures of primary rat hippocampal neurons. Neurons were incubated with microglial conditioned medium for 16 hrs and subsequently stained with DAPI and visualized under a fluorescent microscope. A similar procedure was used to determine the effects of stimuli but lacking microglia (unconditioned medium); these experiments served as controls.

2.9.2 Isolation of Primary Rat Hippocampal Neurons

Isolation of primary rat hippocampal neurons has been described previously (Sheldon et al., 2004a). Briefly, 2 to 4 day-old Wistar rats were anaesthetized, decapitated and the hippocampi removed. The hippocampi were then enzymatically treated and mechanically dissociated and the resulting cell suspension was plated at a density of 5-7 x 10^5 neurons cm^{-2} onto glass coverslips coated with poly-D-lysine and laminin. The initial growth medium was DMEM/F-12 supplemented with 10% fetal bovine serum (Invitrogen Canada, Burlington, Ont., Canada). After 24 hr, this medium was changed to serum-free Neurobasal Medium A (Invitrogen Canada). Cultures were then fed every 4-5 days by half-changing the existing medium with fresh Neurobasal Medium A. Glial proliferation was inhibited 48 hr after initial plating by adding 5-10 mM cytosine arabinoside (Sigma-Aldrich). Primary neurons were used 12-15 days after plating since at this time point cultured hippocampal neurons are more susceptible to toxicity (Sheldon et al., 2004b).

Primary hippocampal neurons were treated with microglial conditioned medium or medium controls (conditioned medium not exposed to microglia) for 16 hrs.

2.9.3 Determination of Neuronal Damage from Microglial Conditioned Medium

Following treatment, cells were fixed with 4% PFA in 0.1 M PBS. Following a wash in PBS, cells were incubated in DAPI (Molecular Probes) at 1 μg/mL in PBS to visualize nuclei. Cells were then washed in water and mounted onto glass slides using gelvatol, examined under a Zeiss light microscope and photographed using a cooled CCD camera. The percentage of damaged neurons was determined by counting the number of

condensed or fragmented nuclear stained neurons by the overall number of positively DAPI stained neurons (at x200 magnification). Control experiments consisted of incubating neurons with conditioned medium without microglial exposure. Data are presented as mean ± SEM and significance determined by one-way ANOVA and Newmann-Keuls post-hoc multiple comparison test ($p < 0.05$).

2.10 *IN VIVO* STUDIES OF $A\beta_{1-42}$ MEDIATED MICROGLIAL ACTIVATION AND NEURONAL DAMAGE; EFFECTS OF 4-AP ADMINISTRATION

2.10.1 Injection of $A\beta_{1-42}$ Into CA1 Region of Rat Hippocampus and Administration of 4-AP

The procedure used for $A\beta_{1-42}$ injection *in vivo* is routinely used in our laboratory and has been published previously by other laboratories (Giovannini et al., 2002; Kowall et al., 1991; Weldon et al., 1998). Briefly, male Sprague-Dawley rats (250-280g; Charles River, Canada) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and then mounted in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). $A\beta_{1-42}$ or $A\beta_{42-1}$ (1 nmol in 2 µl) was injected slowly (0.2 µl/min) into the granule cell layer of the dentate gyrus of the hippocampus (AP: -3.6 mm, ML: -1.8 mm, DV: -3.2 mm, according to the atlas of Paxinos and Watson, 1986) using a 10 µL Hamilton syringe. A diagram illustrating the site of injection of $A\beta_{1-42}$ into the dentate gyrus of rat hippocampus is shown in Figure 2-5. 4-AP (Sigma, St. Louis, MO) was dissolved in 0.9% saline and administered i.p. at 1 mg/kg 15 minutes prior to $A\beta_{1-42}$ injection followed by once-daily injections of 1 mg/kg for 7 days. The doses of 4-AP used in this study are based on previous studies using 4-AP in rats (Haroutunian et al., 1985; Casamenti et al., 1982).

All animal experiments were approved by the Animal Care Committee of the University of British Columbia.

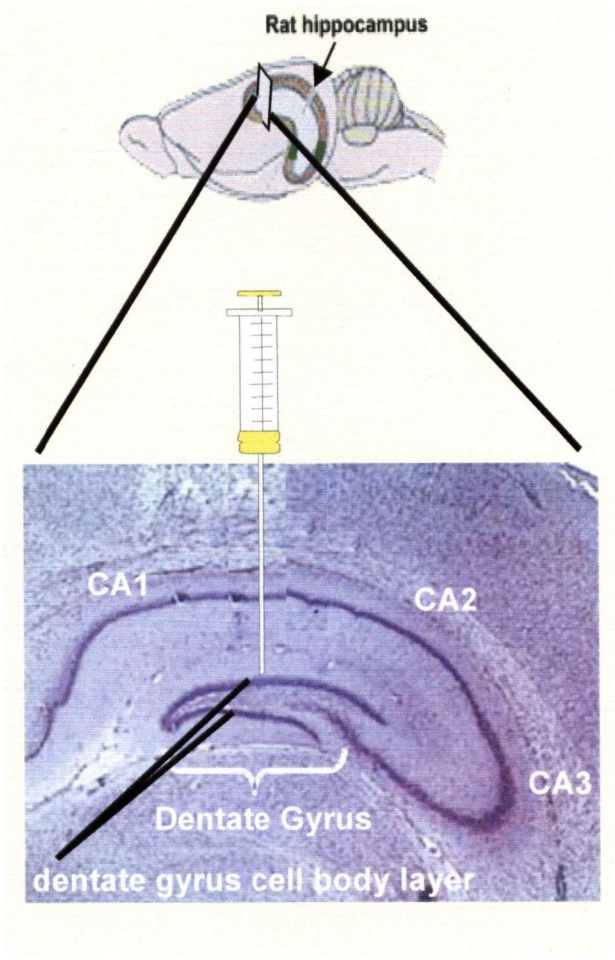

Figure 2-5. The Dentate Gyrus of the Rat Hippocampus.
The syringe indicates on a coronal section of the hippocampus, the upper cell body layer of the dentate gyrus, where $A\beta_{1-42}$ was injected.

2.10.2 Determination of $A\beta_{1-42}$-Induced Neuronal Damage and Microglial Activation *In Vivo*

Seven days post-$A\beta_{1-42}$ injection, anesthetized rats were transcardially perfused with heparinized cold saline followed by 4% PFA. Brains were postfixed overnight in the same fixative and then placed in 30% sucrose for cryoprotection. Serial coronal sections (40 μm) through the hippocampus were cut on a cryostat. For immunohistochemistry, free-floating brain sections were permeabilized with 0.2% Triton X-100 and 0.5% BSA in 0.1 M PBS for 30 min, blocked in PBS containing 0.5% BSA and 10% normal goat serum (NGS) for 30 min and then incubated overnight at room temperature in PBS containing 5% NGS and primary antibodies. The following primary antibodies were used: mouse anti-NeuN (1:1000, Chemicon, Temecula, CA) for neurons and mouse anti-ED1 (1:500, Serotec, Oxford) for activated microglia. For controls, primary antibodies were omitted. The following day, sections were incubated with biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) and visualized with ABC elite system (1:200; Vector Laboratories), and developed in 3,3'-diaminobenzidine (DAB) kit (Sigma). The number of NeuN- or ED1-positive cells in the superior blade of the dentate granule cell layer was conducted on three consecutive sections. Representative photomicographs were taken (at x200 magnification) and counting was performed using a Zeiss Axioplan 2 flourescent microscope (Zeiss) equipped with a DVC camera (Diagnostic Instruments) and Northern Eclipse software (Empix Imaging). All quantitative analyses were carried out in a blinded manner with values expressed as means ± SEM.

2.11 PEPTIDES AND REAGENTS

2.11.1 Preparation of $A\beta_{1-42}$ and $A\beta_{42-1}$

The amyloid beta peptide ($A\beta_{1-42}$) and reverse peptide ($A\beta_{42-1}$) were purchased from California Peptide (Napa, CA) and fresh stock solution prepared according to a method described previously (Walker et al., 2001) with slight modifications. Briefly, $A\beta_{1-42}$ was prepared by first dissolving the peptide in 35% acetonitrile (purchased from Sigma, St. Louis, MO), diluted to 1.5 mM with sterile water, and then to 500 μM with incremental additions of PBS with vortexing in-between additions. The Aβ solution was subsequently incubated at 37°C for 18 hrs to promote Aβ fibrillization and aggregation and then stored at -20°C. The final working concentration used in experiments was 5 μM $A\beta_{1-42}$. A similar procedure was followed for preparation of reverse peptide $A\beta_{42-1}$. The vehicle control was prepared as described for the preparation of $A\beta_{1-42}$ with omission of the peptide.

Electron microscopy was used to confirm the aggregation of $A\beta_{1-42}$ in solution. Briefly, the Aβ sample (5 μL) was placed on Formvar-coated copper grids (Ted Pella Inc., Redding, CA) for 1 min. Excess solution was removed and then negatively stained with 2% acqeous uranyl acetate for 1 min, washed and then air dried. The grids were then examined on a JEM-100CX electron microscope (JEOL, Japan) at 80 kV. Figure 2-6 is a representative photomicrograph of A) $A\beta_{1-42}$ fibrils before heating and B) aggregated $A\beta_{1-42}$ after heating at 37°C for 18 hrs. Before heating, immature fibrils appeared occasionally as a matrix-like meshwork however, after heating, these fibrils tended to appear as globular structures approximately 0.2-0.3 μM in width. Since the same procedure was used to dissolve the different lot numbers of Aβ peptide used, it was

assumed that similar aggregation was achieved between the different batches of Aβ peptide used in experiments.

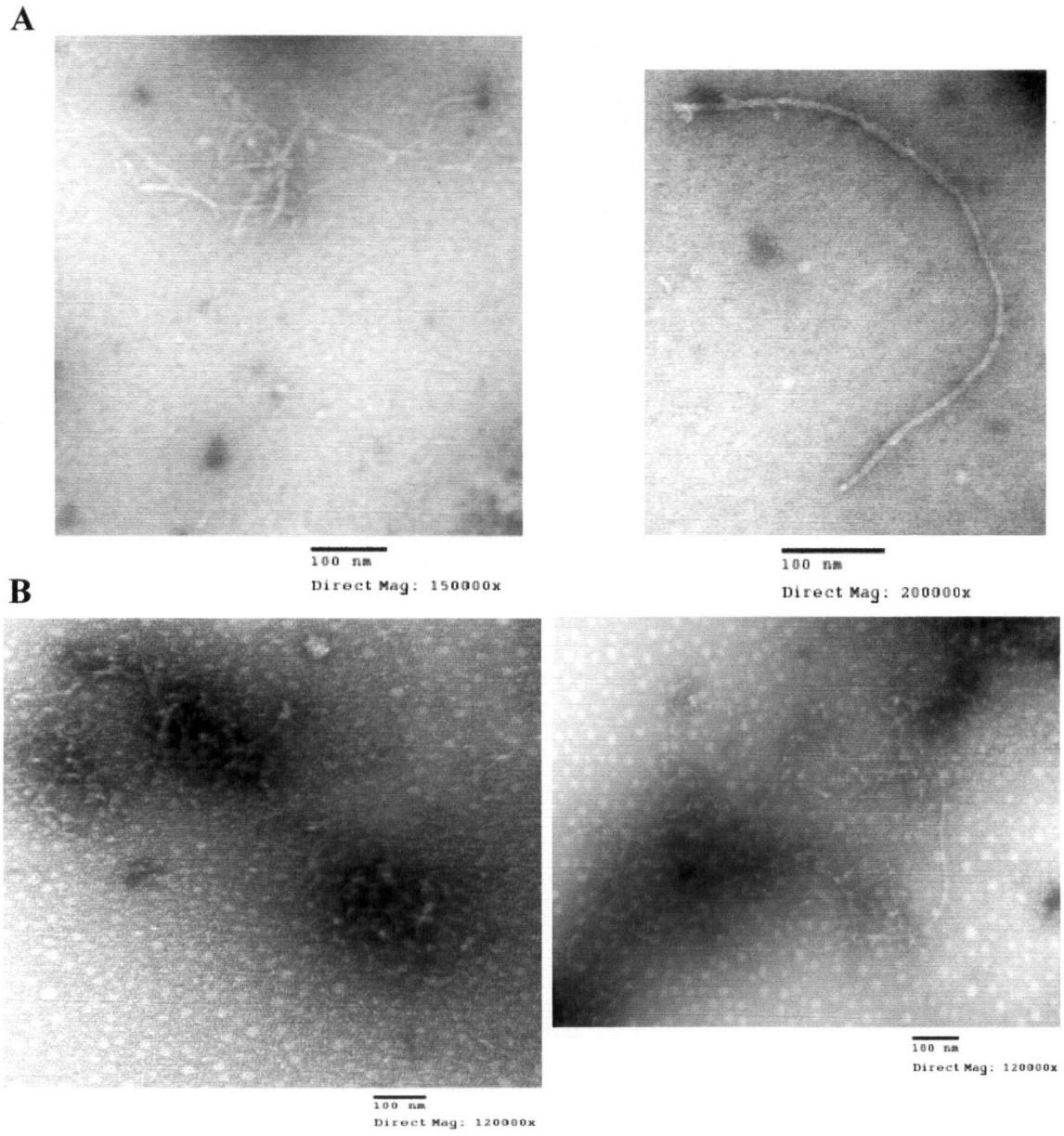

Figure 2-6. Representative photomicrographs of the $A\beta_{1-42}$ preparation before and after heating.
Negative staining results indicate that A) Aβ fibrils exists before heating and that B) Aβ aggregates form after incubation at 37°C for 18 hrs.

2.11.2 Reagents

4-aminopyridine (4-AP), guanosine-5'-O-(3-thiotriphosphate) (GTPγS), iberiotoxin, lanthanum (La^{3+}), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), SKF96365, and tetraethylammonium (TEA) were purchased from Sigma (St. Louis, MO) and apamin was a generous gift from Dr. Church, UBC. The FcγRII receptor antibody was purchased from Beckman-Coulter (Miami, FL) and CXCL8 (IL-8) was purchased from Peprotech (Rocky Hill, NJ).

2.12 STATISTICAL METHODS

Data are presented as mean ± SEM. Statistical comparisons were made using a Student's t-test to determine significant differences between control and treatment groups with significance set at $p < 0.05$. In cases where more than two groups were compared, a one-way ANOVA was used, followed by Neuman-Keuls post hoc multiple comparison test with significance set at $p < 0.05$. All statistical tests were carried out using GraphPad Prism 3.0 software.

Chapter 3: EFFECTS OF $A\beta_{1-42}$ ON MEMBRANE POTENTIAL, MEMBRANE CURRENTS and FcγRII EXPRESSION IN HUMAN MICROGLIA

In this chapter, the acute effects of $A\beta_{1-42}$ on microglial membrane potential, membrane current expression and longer term effects on microglial expression of the FcγRII receptor are determined. The activation process in microglia has been associated with altered signaling which include membrane K^+ current expression (Norenberg et al., 1992; Fischer et al., 1995). Whole-cell electrophysiology was used to determine the acute effects of $A\beta_{1-42}$ on membrane currents and membrane potential (V_m). Since, FcγRII is a receptor coupled to a non-selective cationic channel and functional responses of microglia, the expression of FcγRII was also determined using an antibody binding study.

3.1 RATIONALE

Microglial cells are capable of expressing ion channels that selectively conduct K^+, H^+ or Cl^- (Eder, 1998). Other currents in microglia, such as inward Na^+ and voltage-gated L-type Ca^{2+} have occasionally been reported however, the presence of these currents in microglia is not common (Eder, 1998). Under resting conditions, microglia express an inward rectifier solely (Ilschner et al., 1995; Kettenmann et al., 1990; McLarnon et al., 1997). The upregulation of currents in microglia is dependent on several factors including the state of activation of microglia, the presence of stimuli and time of adherence to surfaces. Incubation with LPS, a potent activator of microglia, for periods of 12 hrs leads to the expression of a transient outward K^+ current in response to a depolarizing step in rodent microglia (Norenberg, 1992; Norenberg et al., 1994). This current was attributed to the upregulation of Kv1.3 channels (Norenberg et al., 1993).

The induction of K^+ currents appears to be highly correlated with the activation state of these cells since under resting conditions in culture (ramified or ameboid morphology) microglia do not express outwardly rectifying K^+ currents but are activated in response to a stimulating agent.

Another outward K^+ current can be induced in rodent microglia when GTP (guanosine 5'-O-(3-thiotriphosphate) is applied intracellularly through the patch pipette indicating that G proteins can regulate the upregulation of currents in microglia (Ilschner et al., 1995). The rapid expression of this current indicated that it was constitutively present in the cell membrane. A current with similar properties to the GTP-induced current was activated with external application of complement (Ilschner et al., 1996) and ATP (adenosine triphosphate) (Kettenmann et al., 1993; Walz et al., 1993). Human microglia also express a high conductance calcium activated K^+ channel (BK-K_{Ca}) sensitive to both V_m (membrane potential) and $[Ca^{2+}]_i$ (intracellular calcium) (McLarnon et al., 1997).

Rodent microglia express both proton and anion currents. The induction of proton currents is not correlated with the functional state of microglia (Klee et al., 1999) rather the currents are increased with cell swelling (Morihata et al., 2000). Proliferation, stretch or swelling of microglia is accompanied by activation of anion currents (Schlichter et al., 1996; Eder, 1998). A high conductance anion channel sensitive to V_m has been reported in human microglia (McLarnon et al., 1997).

As described in Section 1.4.3.1.1, numerous receptors have been described as mediating Aβ actions in microglia. However, only a few of these receptors are coupled to signal transduction systems which could lead to alterations in membrane currents (i.e.

a receptor complex (consisting of $\alpha_6\beta_1$ integrin, CD47, CD36), FPR and CD36 alone. The Fcγ receptor in microglia has received recent attention since it is involved in microglial mediated phagocytosis of opsonized Aβ which was thought to be an effective therapy in reducing levels of Aβ in AD brain (Bard et al., 2000). The Fcγ receptor in particular is directly coupled to and activates a non-selective cationic channel in macrophages (Young et al., 1983a; 1983b) and activates signaling pathways in microglia which include phosphatidylinositol 3-kinase (PI-3K), extracellular signal regulated kinases (ERK) and mitogen-activated protein kinases (MAPK) activation (Song et al., 2004). The FcγRII class of receptors appeared to be the most highly expressed class in human microglia (Lue et al., 2002). Microglia in the brains of AD and non-demented individuals constitutively express all classes of Fcγ receptors: Fcγ RI, II, III, with expression being greater in AD than non-demented individuals (Akiyama and McGeer, 1990; Peress et al., 1993).

The acute effects of $A\beta_{1-42}$ on human microglial membrane potential and current expression have been presented in this chapter. Since cell potential and membrane currents are important signaling events in the activation process of microglia, I carried out a series of whole-cell patch clamp experiments to determine the effects of $A\beta_{1-42}$ acutely on V_m and outward currents and I determined the involvement of the FcγII receptor in mediating $A\beta_{1-42}$ effects on cell potential.

3.2 RESULTS

3.2.1 Effect of $A\beta_{1-42}$ on Membrane Current Expression; Voltage Clamp Studies

These experiments were designed to examine effects of full length $A\beta_{1-42}$ on membrane currents induced by depolarizing stimuli. Unless noted, microglia were held at a potential of –60 mV. A representative current response to a depolarizing step from a holding potential of –60 mV to + 20 mV under unstimulated conditions is shown in Figure 3-1. Currents elicited under unstimulated conditions in response to a depolarizing step were generally small (range 40 to 70 pA) and attributed to leak currents. This result is consistent with previous work on human (McLarnon et al., 1997; 1999) and studies on rodent (Ilschner et al., 1995; Kettenmann et al., 1990; Norenberg et al., 1994) microglia where outward K^+ currents in unstimulated microglia in response to a depolarizing step were generally absent. Within minutes of acute application of $A\beta_{1-42}$ to human microglia, a rapidly activating, non-inactivating outward current was observed in response to a depolarizing step (Figure 3-1). To inhibit the contribution of any anionic currents present in microglia to the outward current, all membrane current experiments were performed with bath solution containing NPPB (500 μM). Overall, the amplitude of the current induced with acute application of $A\beta_{1-42}$ in response to a depolarizing step in human microglia increased fourteen-fold (to 850 ± 102 pA, n=7 cells) from control values (mean amplitude 59 ± 6 pA, n=7 cells). Experiments were designed to determine the specificity of the outward current. The analysis of tail currents (see below) indicated that the outward current was due to K^+.

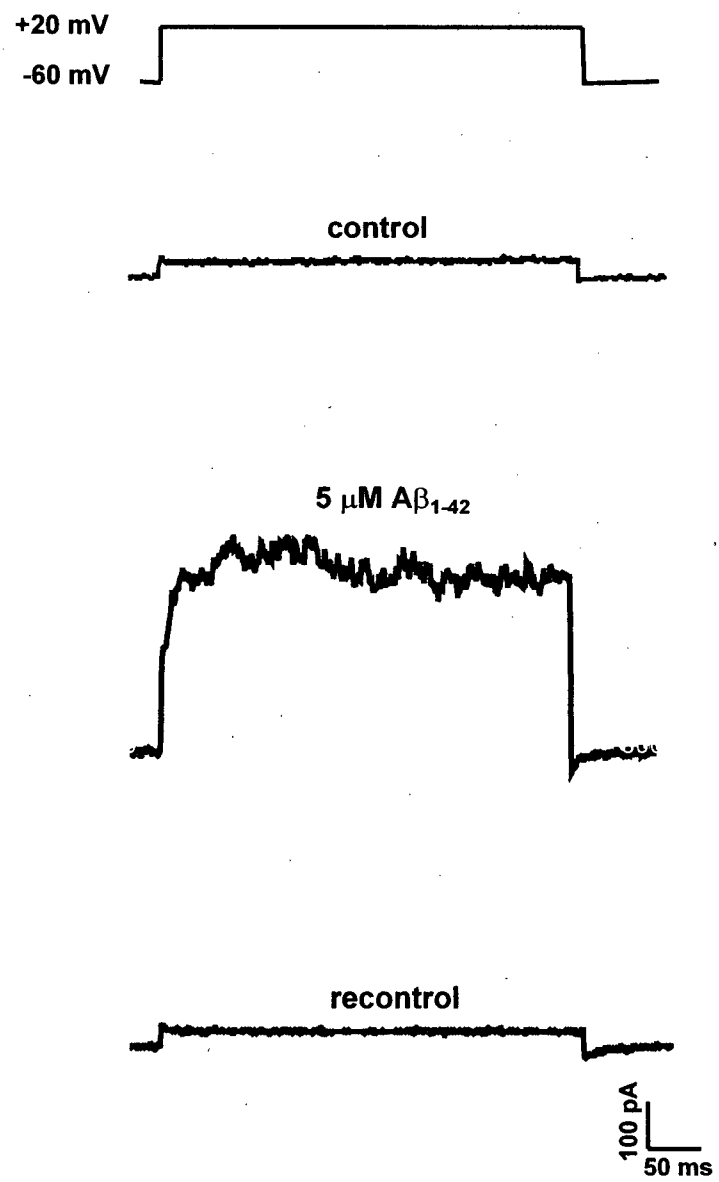

Figure 3-1. Typical current induced with acute application of $A\beta_{1-42}$ to human microglia

In control, a small current representative of leak current was elicited with a depolarizing step to +20 mV from a V_H of −60 mV. Following acute application of $A\beta_{1-42}$ (5 μM), a large outward K^+ current was evident in response to a depolarizing step and wash-off of the peptide decreased the current to control level in response to a depolarizing step. The figure is a representative recording from one cell.

3.2.1.1 The $A\beta_{1-42}$-Induced Outward Current is Attributed to K^+

In order to determine the current's threshold for activation, a series of depolarizing steps in 10 mV increments was applied from –60 mV to a maximum depolarization of +20 mV. A typical family of I/V is presented in Figure 3-2. A plot of current amplitude versus step potential (I/V plot) indicated that the current was outwardly rectifying with a threshold of activation near –40 mV. Overall, the current's mean threshold for activation was determined to be -33.7 ± 2.4 mV ($n = 4$ cells).

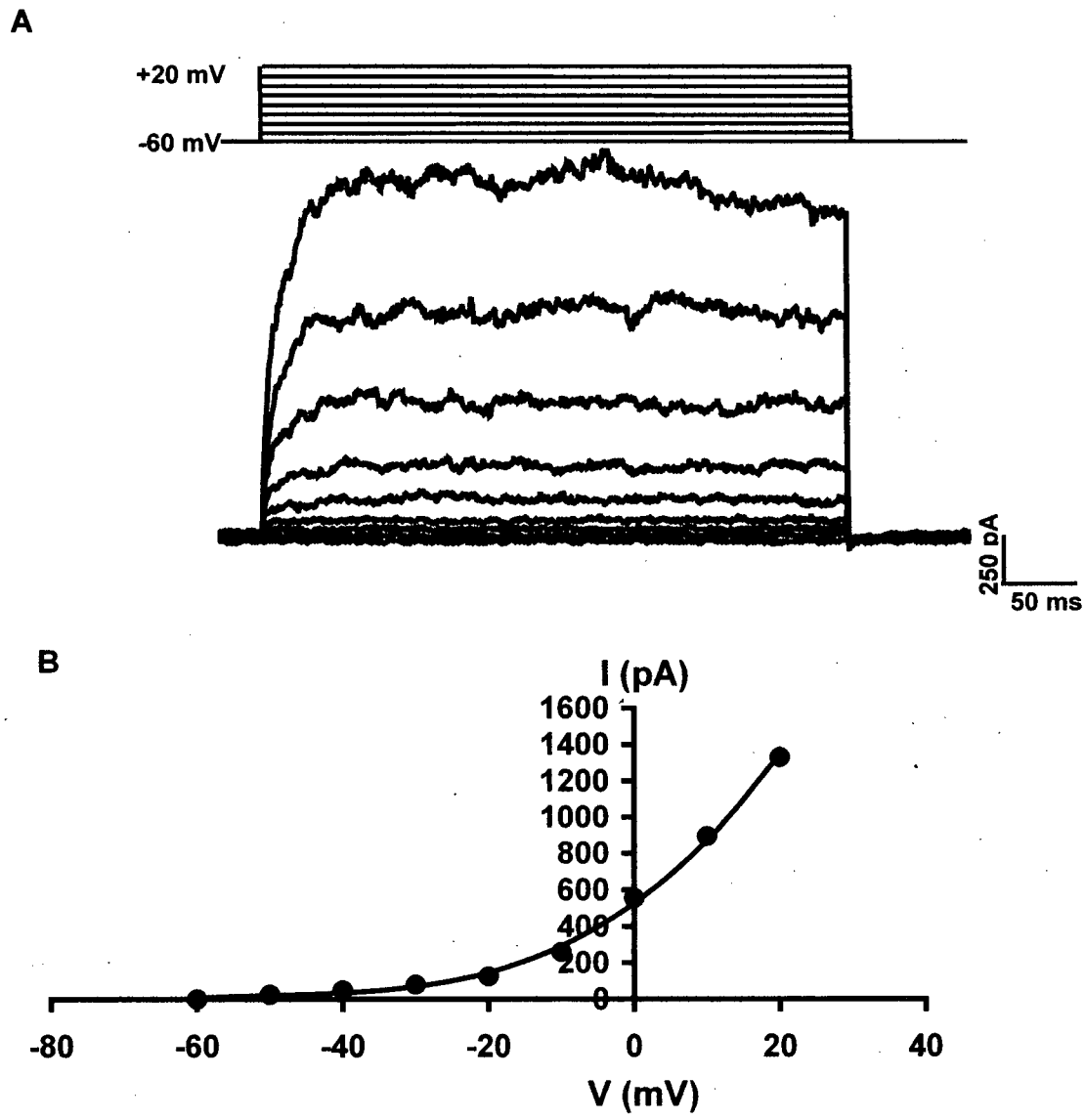

Figure 3-2. Voltage-dependent activation of $A\beta_{1-42}$-induced outward current.
A) Outward current, induced by $A\beta_{1-42}$ (5 μM), with a series of depolarizing steps in 10 mV increments (applied from −60 mV to a maximum level of +20 mV). B) The *I–V* relationship constructed from the pulse protocol shown in panel A. The outward current induced by acute $A\beta_{1-42}$ was outwardly rectifying with a threshold of –40 mV. The figure is a representative recording from one cell.

The reversal potential of the outward current was determined using a protocol consisting of a depolarizing step to +20 mV (applied from a holding potential of –60 mV) followed by varying the step potential from –100 to –20 mV (increments of 20 mV). The tail currents induced by varying the step potentials from (-100 to –20mV) are shown in Figure 3-3A. The arrows denote peak tail currents at the start of the different test potentials. These tail currents rapidly inactivated to baseline at the holding potential (V_h) of –60 mV. A typical plot of tail currents versus step potentials (-100 to –20mV) is shown in Figure 3-3B. Overall, the current's reversal potential was -76 ± 3.6 mV (n=6 cells) close to the equilibrium potential for K^+ (in this study E_{rev} for K^+ = -84 mV). Therefore, these results indicate that the $A\beta_{1-42}$-induced K^+ current was outward and due to K^+.

These results were confirmed since addition of the non-selective K^+ channel blocker 4-AP (2 mM) in the presence of $A\beta_{1-42}$ inhibited the current to 52% of control in response to a depolarizing step and the current in response to a depolarizing step recovered after wash-off of 4-AP (Figure 3-4). Overall, 4-AP at 2 and 5 mM in the maintained presence of $A\beta_{1-42}$ reduced the $A\beta_{1-42}$-induced K^+ current in response to a depolarizing step to 58 ± 7.3% (n=3 cells) and 38 ± 6.6% (n=3 cells). Tetraethylammonium (TEA) (10 mM), a non-selective K^+ channel blocker, inhibited the outward K^+ current to 28 ± 5.9% (n=3 cells) of the $A\beta_{1-42}$-induced current in response to a depolarizing step. Overall, the outward K^+ current elcited in response to a depolarizing step was induced by $A\beta_{1-42}$ in 40% of cells and was present for periods of up to 10-15 minutes.

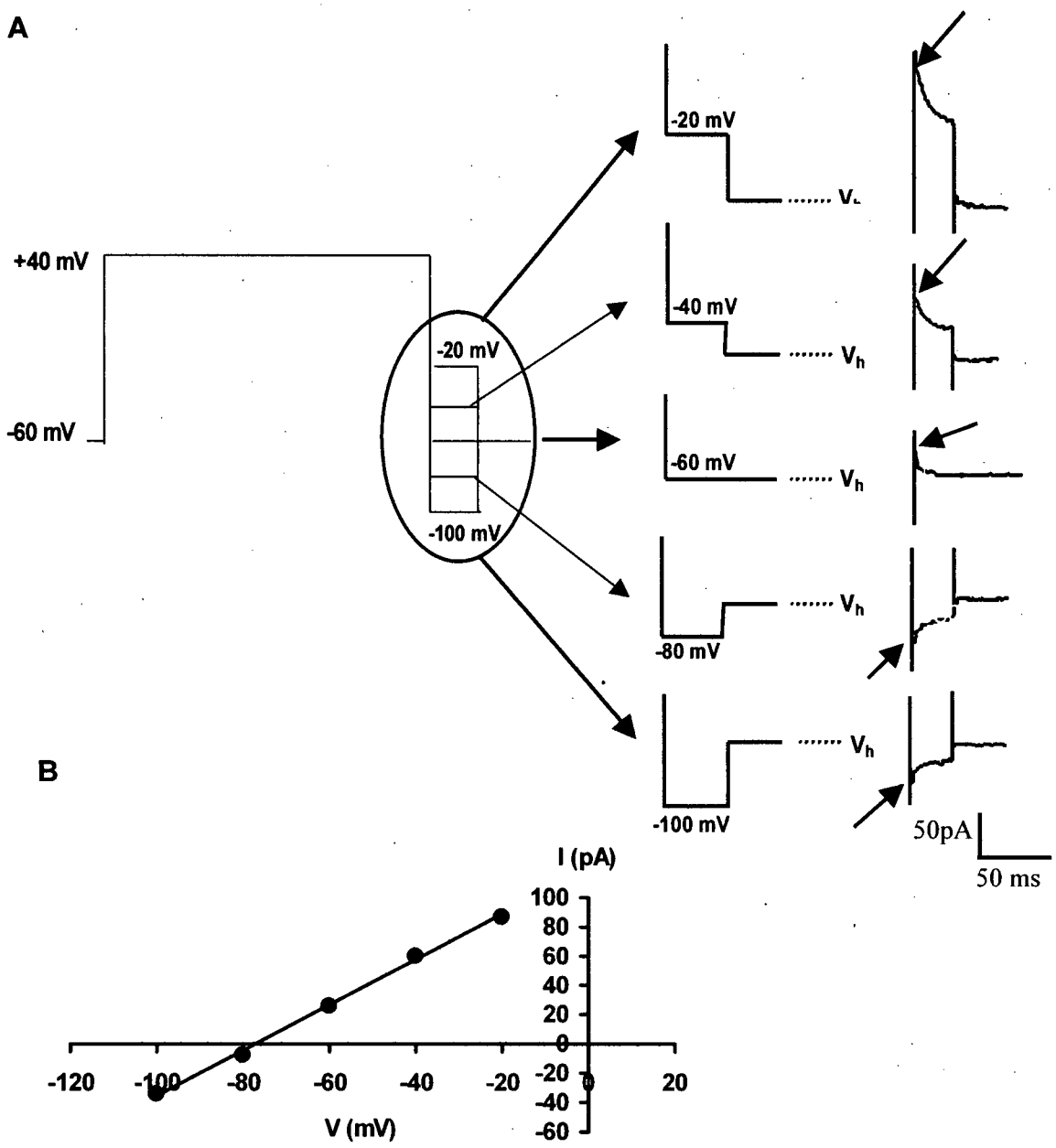

Figure 3-3. Determination of the $A\beta_{1-42}$-induced outward current's reversal potential via analysis of tail currents.

A depolarizing step from –60 mV to +20 mV was followed by a secondary step to potentials varying from –100 mV to –20 mV. The resulting tail currents elicited with the secondary steps from –100 mV to –20 mV in a representative experiment are shown in A. The arrows indicate the peak of the tail currents at the start of the different test potentials. A plot of tail current amplitudes versus step potential is shown in B. The current's reversal potential was determined as –78 mV which is close to the equilibrium potential for K^+.

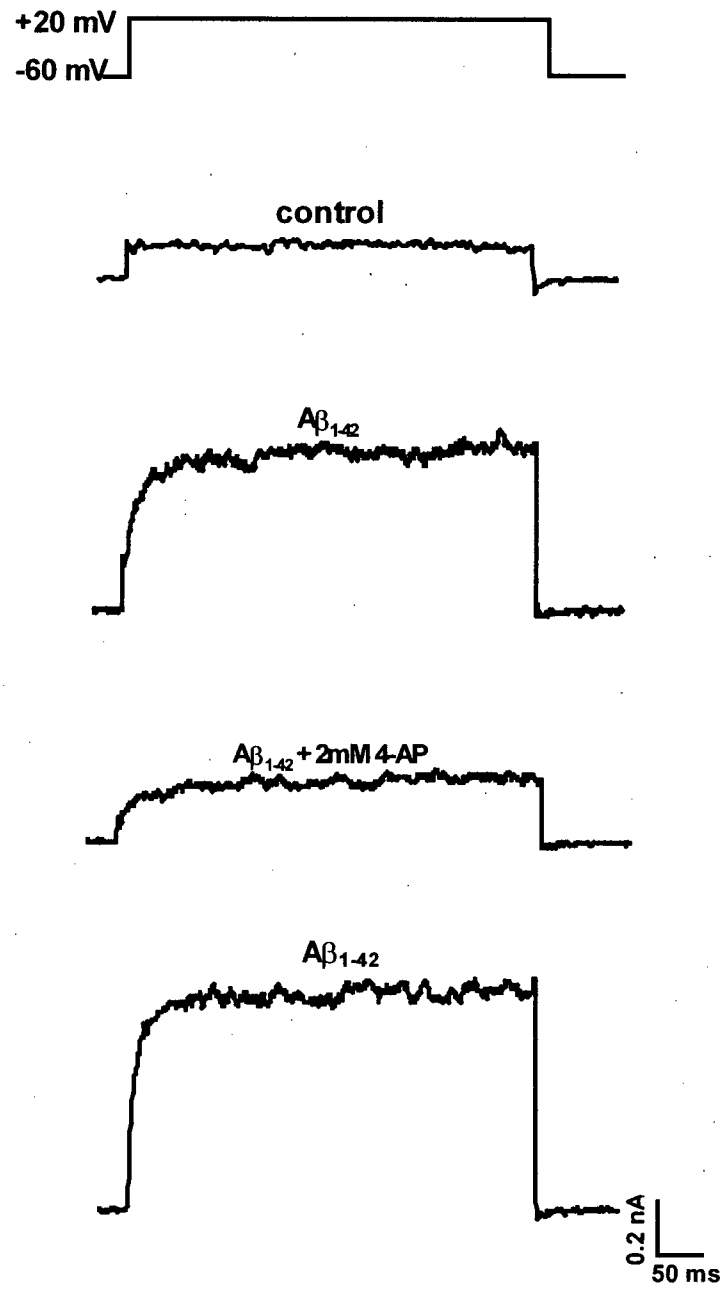

Figure 3-4. The $A\beta_{1-42}$-induced outward K^+ current is inhibited by the non-selective K^+ channel inhibitor 4-AP

A representative recording from one cell is shown. The first trace is a typical current evoked in control solution with a depolarization step to +20 mV. $A\beta_{1-42}$ (5 μM) elicited an outward K^+ current with the same step depolarization. Application of 4-AP (2 mM) with $A\beta_{1-42}$ reduced the outward K^+ current to 52% of control. The current recovered subsequent to wash off of 4-AP with $A\beta_{1-42}$ maintained in bath solution.

3.2.2 The Involvement of a G protein in the $A\beta_{1-42}$ Induction of an outward K^+ current

The possibility that a G protein may be involved in the induction of the outward K^+ current upregulated by $A\beta_{1-42}$ in human microglia was tested since a previous study indicated that intracellular application of the non-hydrolyzable analogue of GTP, GTPγS, upregulated an outward K^+ current in macrophages (McKinney and Gallin, 1993). The outward K^+ current in macrophages had similar properties to the outward K^+ current induced by $A\beta_{1-42}$ including rapid activation, non-inactivation during a depolarizing step and sensitivity to 4-AP. In the present study, GTPγS was added into the electrode solution and dialysed into the cell upon rupture of the seal in the whole-cell patch clamp mode. In these experiments, the extracellular bathing solution contained NPPB (500 μM) in order to inhibit any contribution of anion channels to outward currents induced by cell depolarization.

Intracellular application of GTPγS induced a rapidly activating outward K^+ current in response to a depolarizing step within minutes of forming a whole-cell seal, however, the current showed rundown within ten minutes of its upregulation. A typical profile of the outward current's amplitude over time is presented in Figure 3-5. Rundown of the GTPγS-induced current may be due to equilibration of GTPγS between the electrode and cell. Overall, upon formation of a whole-cell seal, the mean current amplitude in response to a depolarizing step was 147 ± 49 pA (n=5 cells). In response to a depolarizing step, intracellular GTPγS induced an outward K^+ current in 70% of cells with an average peak current amplitude of 1149 ± 113 pA (n=9 cells).

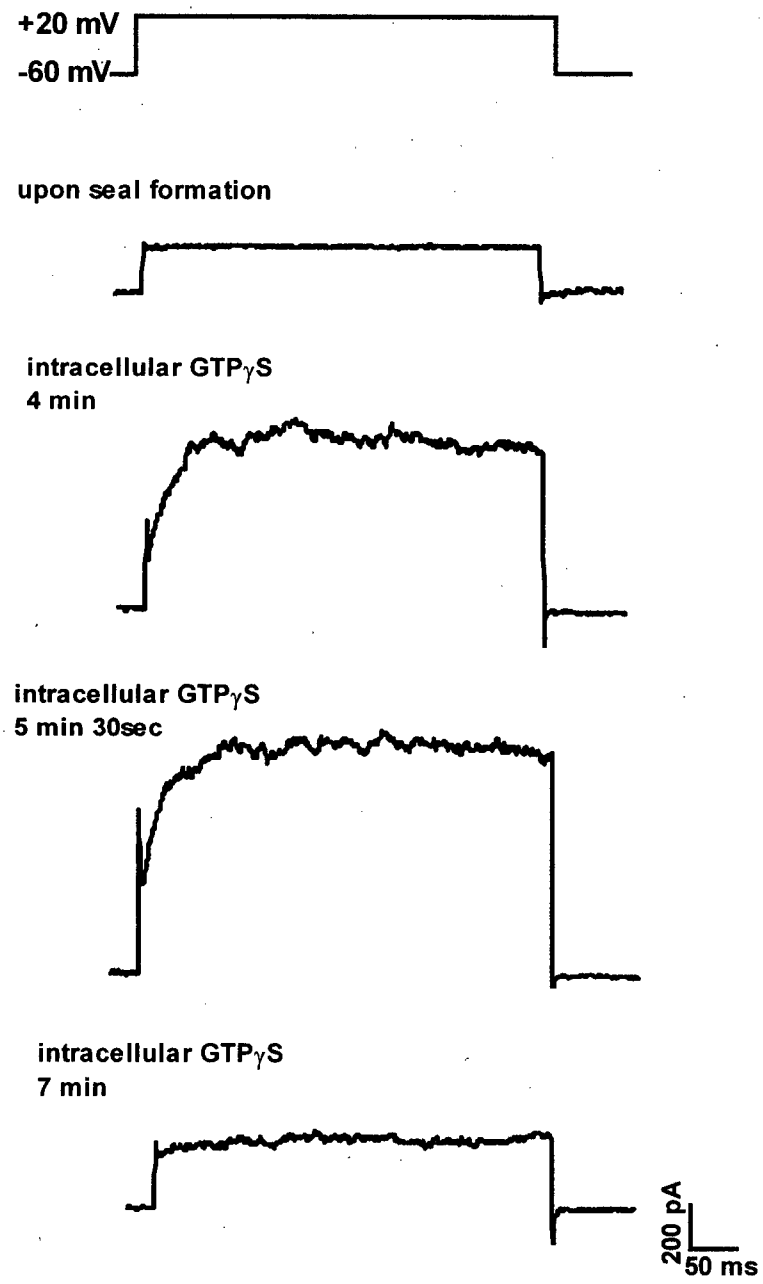

Figure 3-5. Typical profile of the intracellular GTPγS-induced outward current. The top trace represents the voltage step (-60 mV to +20 mV) applied. The bottom four traces are currents at the times indicated after dialysis of GTPγS in response to a depolarizing step. Intracellular application of GTPγS (10 μM) via the electrode induced an outward current within minutes of rupture of the cell membrane in the whole cell patch clamp mode. A representative experiment indicates that the current amplitude increased transiently and randown within 10 minutes of seal formation.

The current induced with intracellular GTPγS in response to a depolarizing step was similar in characteristics to the $A\beta_{1-42}$-induced K^+ current (Figure 3-6A,B). The GTPγS-induced K^+ current in response to a depolarizing step was rapidly activating, slow to plateau and did not inactivate with time typical of the $A\beta_{1-42}$-induced K^+ current. The peak amplitude in response to a depolarizing step to + 20 mV from a holding potential of –60 mV of the GTPγS-induced K^+ current (1149 ± 113 pA; n=9 cells) was not significantly different from the amplitude of the $A\beta_{1-42}$-induced K^+ current (850 ± 102 pA; n=7 cells) ($p>0.05$). Overall, the threshold for activation of the GTPγS- induced current was found to be –28 ± 1.7 mV (n=4 cells) which was not significantly different from the threshold for activation of the $A\beta_{1-42}$-induced K^+ current (–33.7 ± 2.4 mV; n=4 cells) (Figure 3-6A; $p > 0.05$). Tail current measurements of the GTPγS- induced outward current indicated that the current had an overall reversal potential of –68 ± 1.1 mV (n=4) which was not significantly different from the reversal potential of the $A\beta_{1-42}$-induced outward K^+ current (E_{rev} = -76 ± 3.6 mV; n=6 cells) (Figure 3-6B; $p > 0.05$). The difference in reversal potentials of the GTPγS induced outward current (-68 mV) and the calculated reversal potential for K^+ in these experiments (-84 mV) may be due to contribution of a leak current to the overall whole-cell current induced with intracellular GTPγS.

A representative result of the effect of 4-AP applied extracellularly on the GTPγS-induced K^+ current in response to a depolarizing step is shown in Figure 3-7. 4-AP (2 mM) reduced the GTPγS-induced K^+ current to 65% of control and the current recovered upon wash off of 4-AP similar to the 4-AP effect on the $A\beta_{1-42}$-induced K^+ current

(Figure 3-7). Overall, 4-AP (2 mM) reduced the GTPγS-induced outward K^+ current to 61 ± 9.5 % of control (n=6 cells); a similar inhibitory effect 4-AP was observed on the $A\beta_{1-42}$-induced K^+ current in response to a depolarizing step (to 58 ± 7.3% (n=3 cells); $p > 0.05$). Since properties of the GTPγS-induced outward K^+ current were similar to the K^+ current induced with acute application of $A\beta_{1-42}$ in response to a depolarizing step, the results would suggest that $A\beta_{1-42}$ induces an outward K^+ current via activation of a G protein in human microglia.

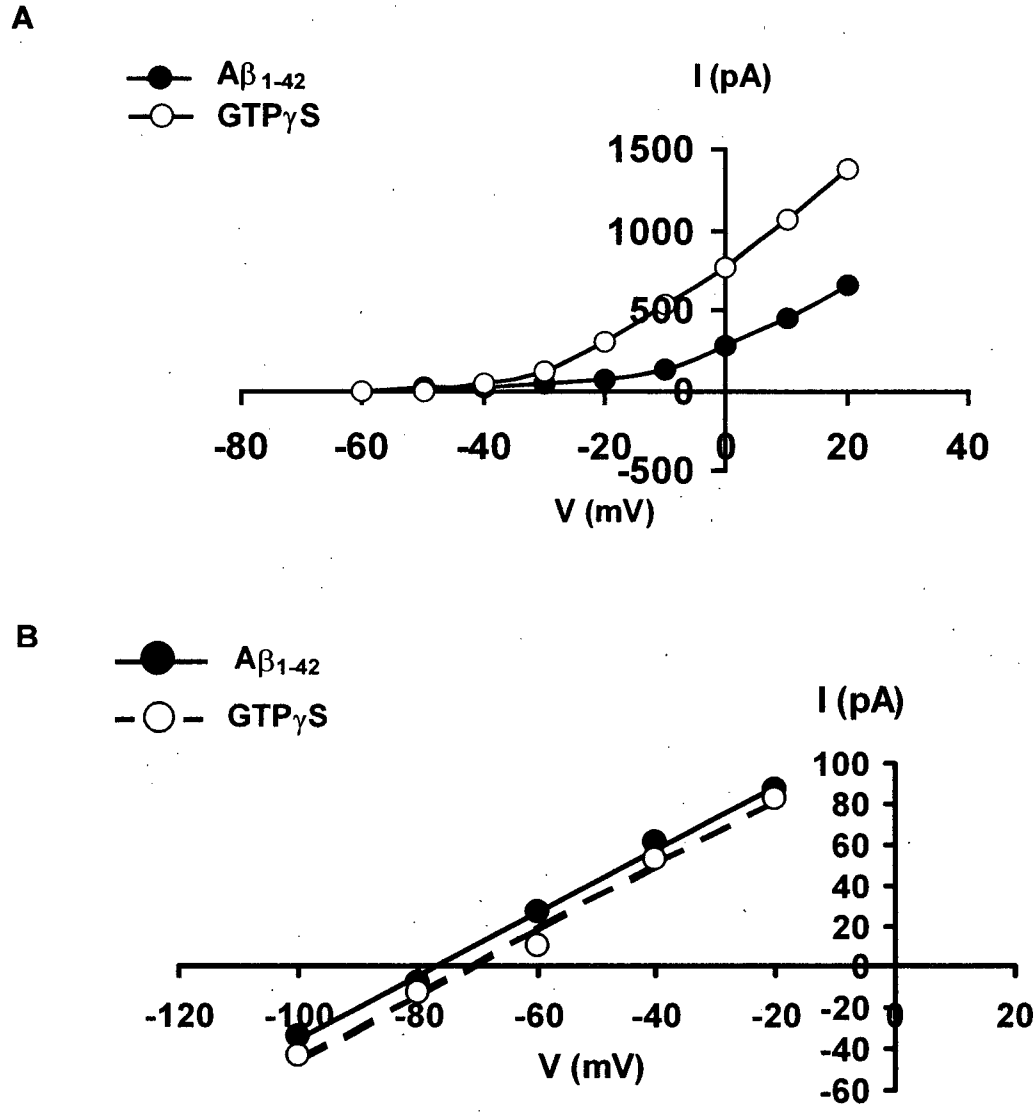

Figure 3-6. The current induced with intracellular GTPγS and the current induced with extracellular $A\beta_{1-42}$ had similar thresholds for activation and reversal potentials.

A) The threshold for activation was determined as described above (Section 3.2.1.1). An I/V plot for the threshold of activation is shown from a representative experiment for each of GTPγS and $A\beta_{1-42}$-induced outward currents. Results indicate that the threshold for activation for each of the currents is similar: GTPγS -31 mV; $A\beta_{1-42}$ -38 mV. B) The reversal potential for each of the currents was determined as described above (Section 3.2.1.1). An I/V plot of tail currents from a representative experiment for each of the GTPγS and $A\beta_{1-42}$ currents indicates that the reversal potentials are similar: GTPγS –70 mV; $A\beta_{1-42}$ –78 mV.

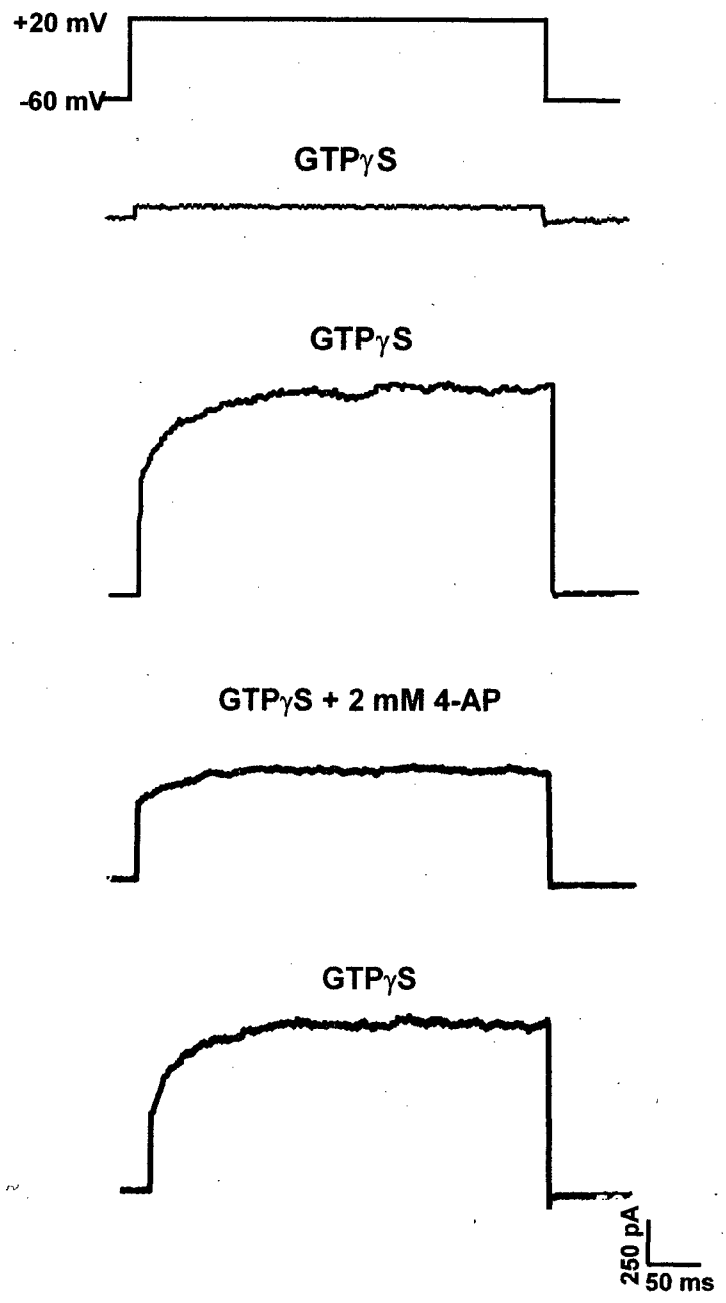

Figure 3-7. Effect of 4-AP (2 mM) on the intracellular GTPγS-induced outward K^+ current

Current evoked upon rupture of the cell membrane with GTPγS in the intracellular recording solution in response to a depolarizing step to +20 mV. The outward K^+ current elicited with intracellular GTPγS for the same step depolarization. Extracellular application of 4-AP (2 mM) reduced the outward K^+ current to 65% of the control. Wash-off of 4-AP allowed the current to recover.

3.2.2.1 Concentration-Dependent Inhibition of the GTPγS-Induced Outward K^+ Current by 4-AP and Several Other K^+ Channel Inhibitors

Since induction of an outward K^+ current was more common with intracellular application of GTPγS than with acute application of $A\beta_{1-42}$ and costs of peptide are high, I carried out further pharmacology on the outward K^+ current induced with intracellular GTPγS. Effects of 4-AP concentrations on the amplitude of the GTPγS-induced K^+ current were used to construct a concentration-response curve (Figure 3-8). A concentration dependent inhibition of the GTPγS-induced outward K^+ current with 4-AP was observed where C denotes control in the concentration-response plot. Overall, 4-AP was found to have an IC_{50} of 5 mM for inhibition of the outward K^+ current induced by intracellular application of GTPγS. TEA (10 mM) blocked the K^+ current induced with intracellular GTPγS to 23.8 ± 3.7% of control level (n=3 cells). The GTPγS-induced K^+ current was unaffected by the high conductance calcium-dependent K^+ channel (BK-type K_{Ca}) inhibitor iberiotoxin (50 nM) or by the small conductance calcium-dependent K^+ channel (SK-type K_{Ca}) inhibitor apamin (100 nM). These results indicate that upregulation of the $A\beta_{1-42}$-induced K^+ current is mediated through a G-protein and is not a BK- or SK-type K_{Ca} channel. An outwardly rectifying current with a similar threshold for activation of –30 mV and sensitivity to 4-AP and TEA has been reported in rat astrocytes and referred to as a delayed rectifier type of K^+ current (K_{DR}) (Bordey and Sontheimer, 1999).

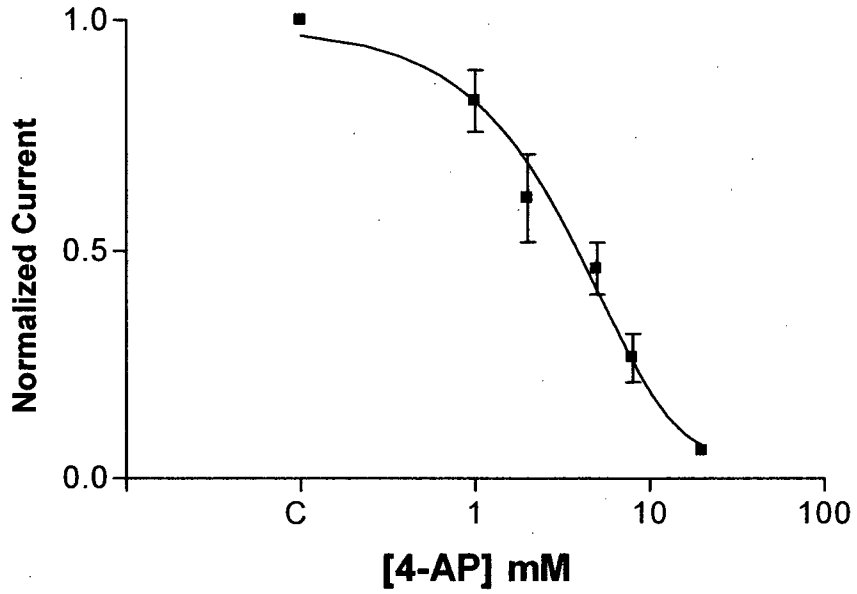

Figure 3-8. Concentration-dependent inhibition of the intracellular GTPγS-induced outward K^+ current by 4-AP.

4-AP was applied in the extracellular bath solution and amplitudes of the GTPγS-induced currents were measured in the presence of 4-AP and normalized to control amplitudes, C (current amplitude prior to 4-AP application). Results are a summary of: n=4 cells for 1 mM; n=6 cells for 2 mM; n=10 cells for 5 mM; n=4 cells for 10 mM and n=3 cells for 20 mM.

3.2.3 Molecular Biology Analysis of the Outward K^+ Current

Based on electrophysiological data, the channel induced with acute $A\beta_{1-42}$ displayed the profile of a delayed rectifier type of K^+ channel. The expression of a series of K_v channels (Kv1.1, 1.2, 1.3, 1.5, 1.6, 2.1, 3.1) with delayed rectifier K^+ channel properties reported previously in human cells (Jiang et al., 2002) were examined in unstimulated human microglia or cells incubated with $A\beta_{1-42}$ for 10 min, 0.5, 1 and 2 hrs using RT-PCR. Results from a representative experiment are shown in Figure 3-9A. Results indicate a time-dependent increase in Kv3.1. All other Kv channels showed basal expression in unstimulated microglia. An abundant increase in the expression of Kv3.1 was observed after 10 min incubation with $A\beta_{1-42}$. The expression of Kv3.1 peaked at 0.5 hr then decreased at 1 and 2 hrs. No expression of Kv3.1 channel was evident at 4hrs $A\beta_{1-42}$ exposure. The absence of Kv3.1 expression under control conditions and transient expression with $A\beta_{1-42}$ incubation is in agreement with the transient upregulation of an outward K^+ current induced with acute $A\beta_{1-42}$ in whole cell patch clamp experiments (Section 3.2.1). Kv1.1, 1.2, 1.3 and 2.1 remained unchanged with treatment with $A\beta_{1-42}$ whereas Kv1.5 and Kv1.6 decreased in expression from control levels as the treatment time with $A\beta_{1-42}$ increased. Incubation with $A\beta_{42-1}$ (5 μM) at 10 min, 0.5, 1 and 2 hr did not alter Kv3.1 channel expression from control.

Densitometry analysis of PCR product band intensities demonstrated the effect of $A\beta_{1-42}$ to time-dependently increase Kv3.1 channel expression with no effects of $A\beta_{1-42}$ to increase Kv1.1, 1.2, 1.3, 1.5, 1.6 and 2.1 channel expression. Results are summarized in Figure 3-9B. $A\beta_{1-42}$ significantly increased Kv3.1 channel expression at 10 min by 5.6

fold as compared to control ($p < 0.05$). Incubation with $A\beta_{1-42}$ for 30 min significantly increased Kv3.1 channel expression further by 13 fold as compared to Kv3.1 channel expression in control ($p < 0.001$). Kv3.1 channel expression increased at 1 hr by 3 fold as compared to Kv3.1 channel expression in control; this increase was not significant ($p > 0.05$). Similarly, Kv3.1 channel expression was increased at 2 hr $A\beta_{1-42}$ incubation by 3 fold relative to control, however the increase was not significant ($p > 0.05$). Furthermore, relative mRNA levels of Kv1.1, 1.2, 1.3, 1.5, 1.6 and 2.1 channels induced with different $A\beta_{1-42}$ treatment times were not significantly different from control ($p > 0.05$).

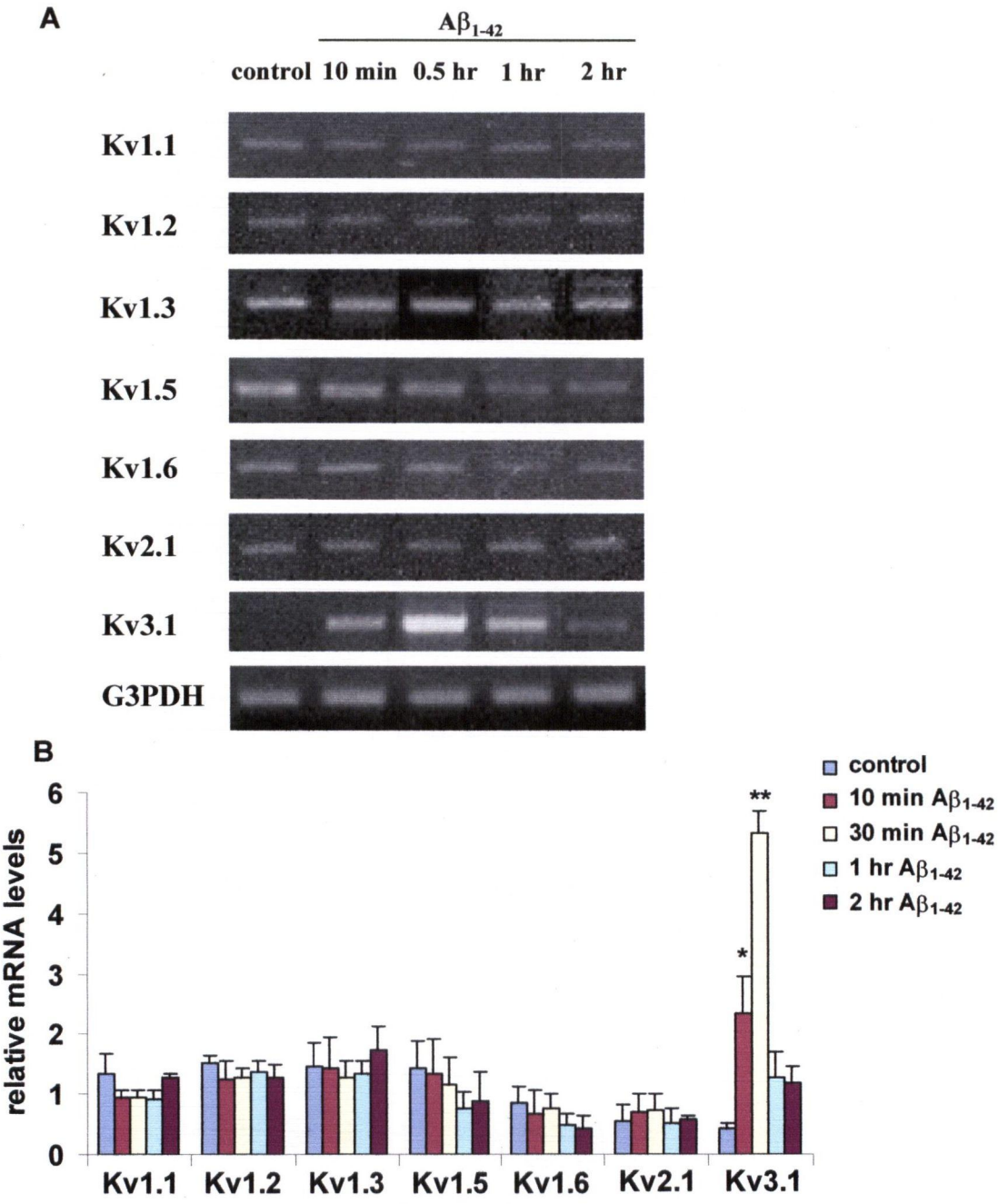

Figure 3-9. Effects of $A\beta_{1-42}$ on Kv expression in human microglia
A) A representative RT-PCR experiment of $A\beta_{1-42}$ (5 μM) treatment for 10 min, 0.5, 1 and 2 hr on Kv1.1, 1.2, 1.3, 1.5, 1.6, 2.1 and 3.1 channel expression from n=3 independent experiments. G3PDH served as a reaction standard. B) Summary of relative mRNA levels of Kv channels induced by $A\beta_{1-42}$. Results are expressed as mean ± SEM from n=3 independent experiments. One-way ANOVA and Newman-Keuls multiple comparison test was used to evaluate statistical significance (*indicates statistical significance from control $p < 0.05$; **indicates statistical significance from control $p < 0.001$).

3.2.4 Effect of $A\beta_{1-42}$ on Membrane Potential of Human Microglia; Current Clamp Studies

Overall, the average resting V_m of human microglia was –35.6 ± 2.2 mV (n=16 cells). Within seconds of acute application, $A\beta_{1-42}$ induced an immediate transient depolarization of microglia. A representative effect of $A\beta_{1-42}$ on V_m is presented in Figure 3-10A. $A\beta_{1-42}$ induced a transient change in V_m (ΔV_m) (peak-baseline) of +8.5 mV with a $t_{1/2}$ of 75 sec. $t_{1/2}$ was determined as the time between the half-maximal points of the transient V_m response. Overall, the ΔV_m from baseline induced by acute $A\beta_{1-42}$ was +8.9 ± 0.9 mV; $t_{1/2}$ of 73 ± 8 sec (n=5 cells).

Acute application of $A\beta_{42-1}$ had no effect on V_m (n=3 cells; $p > 0.05$). A representative experiment indicating no effect of $A\beta_{42-1}$ on membrane potential is shown in Figure 3-10B.

It was of interest to compare the effects of $A\beta_{1-42}$ with effects of high K^+ solution on depolarization of human microglia. A high K^+ solution has been shown previously to induce depolarization of human microglia (McLarnon et al., 1999). A representative result with application of high extracellular K^+ (40 mM) to human microglia is presented in Figure 3-10C. High K^+ induced a transient depolarization with a ΔV_m of +33.5 mV and $t_{1/2}$ of 33 sec. Overall, high K^+ induced a transient depolarization with a ΔV_m of +28.3 ± 6.2 mV and $t_{1/2}$ of 27 ± 7 sec (n=3 cells).

Furthermore, application of either vehicle or bath solution alone did not induce a significant change in V_m ($n = 3$ cells; $p > 0.05$). A representative trace indicating no effect of bath solution on membrane potential is shown in Figure 3-10D.

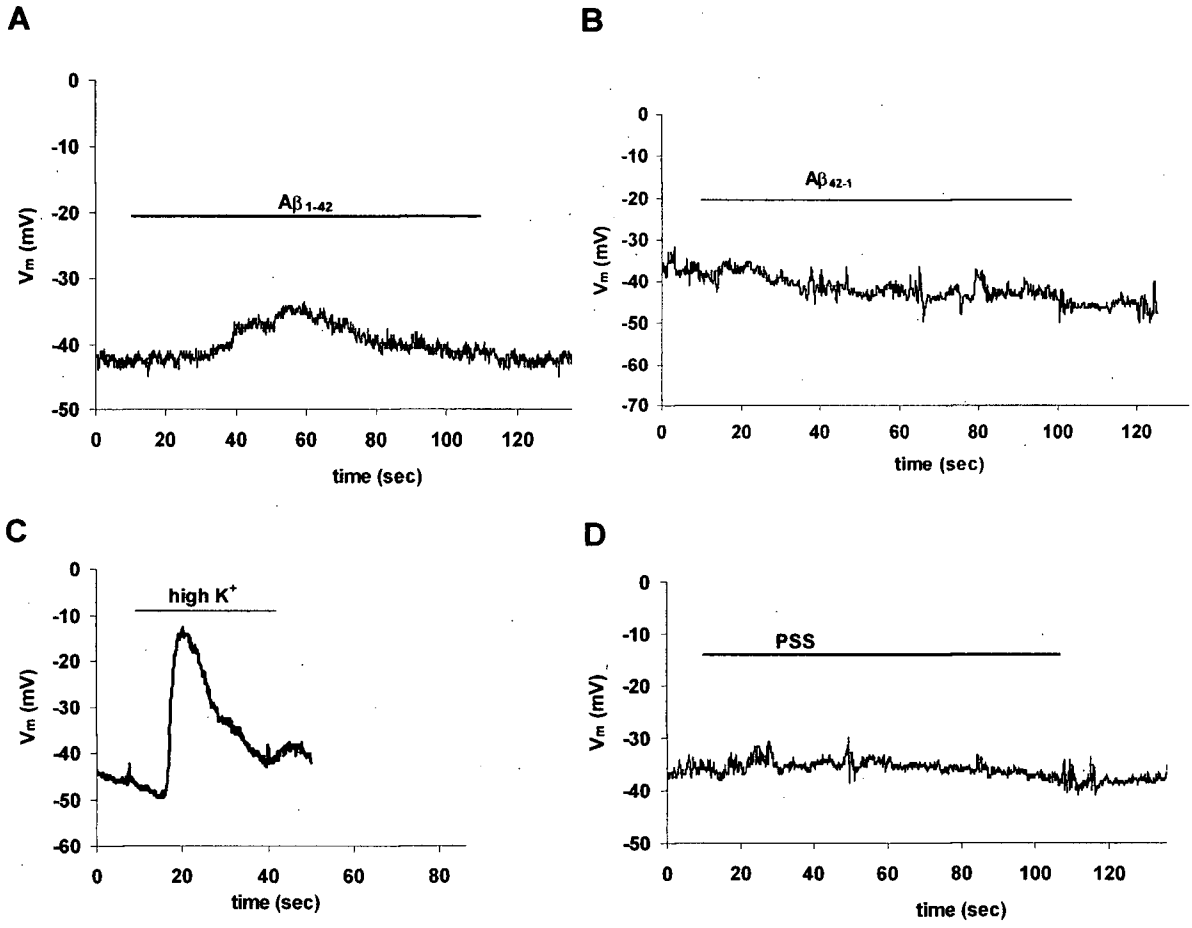

Figure 3-10. Effects of $A\beta_{1-42}$, $A\beta_{42-1}$, High K^+ and PSS on V_m of Human Microglia Changes in membrane potential (Vm) were recorded with acute application of A) $A\beta_{1-42}$ (5 μM); B) $A\beta_{42-1}$ (5 μM); C) 40 mM K^+ solution D) normal bath solution (PSS).

3.2.5 Effect of $A\beta_{1-42}$ on FcγRII Receptor Expression

Since acute $A\beta_{1-42}$ induces depolarization of human microglia (Section 3.2.4) and reports indicate that the Fcγ receptor is linked to a non-selective cationic channel which induces cellular depolarization when activated, the effect of $A\beta_{1-42}$ on Fcγ receptor expression and of Fcγ receptor inhibition on $A\beta_{1-42}$ induced depolarization was investigated.

After 30 min and 48 hr incubation of human microglia with $A\beta_{1-42}$ (5 μM), optical density of cell surface FcγRII was determined using a monoclonal antibody of FcγRII. The effect of $A\beta_{1-42}$ stimulation on FcγRII expression is presented in Figure 3-11. $A\beta_{1-42}$ significantly upregulated FcγRII expression for both treatment times: by 20% at 30 min ($n=6$; $p<0.01$) and by 36% at 48 hrs ($n=6$; $p< 0.001$).

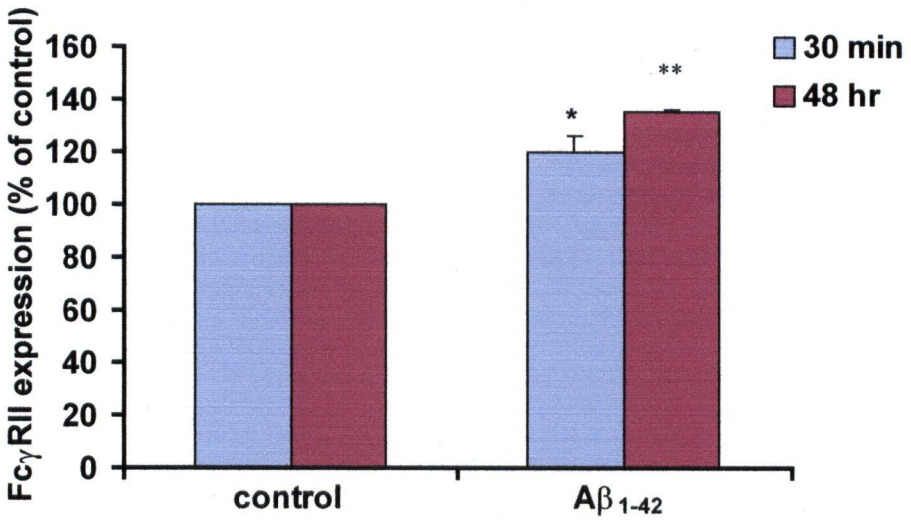

Figure 3-11. $A\beta_{1-42}$-induced expression of FcγRII
The effect 30 min and 48 hrs $A\beta_{1-42}$ treatment on FcγRII expression in human microglia was determined. The optical density of a monoclonal antibody to FcγRII after both 30 min and 48 hr treatment was measured and normalized to control. Results are a summary of n=4 independent experiments for 30 min treatment and n=6 independent experiments for 48 hr treatment. * indicates statistically significant from control ($p<0.01$); ** indicates statistically significant from control ($p<0.001$).

Voltage clamp studies were also carried out to determine the effects of acute $A\beta_{1-42}$ on inward currents in human microglia (personal communication with Dr. S. Jeong). Representative experiments of the effects of $A\beta_{1-42}$, $A\beta_{42-1}$ and vehicle on inward currents in human microglia are shown in Figure 3-12. Application of vehicle had no effects on inward currents in human microglia (Figure 3-12A). Acute application of $A\beta_{1-42}$ (5 μM) led to an immediate transient inward current (Figure 3-12B) which corresponded with the depolarization observed previously (Figure 3-10A). I next investigated the effect of FcγRII inhibition on $A\beta_{1-42}$ induced inward currents of human microglia. FcγRII receptors on microglia were inhibited by incubating microglia with FcγRII antibody (1:500) for 1hr at 37°C prior to treatment as has been described previously (Mitrasinovic and Murphy, 2003). Inhibition of the FcγRII antibody for 1 hr prior to $A\beta_{1-42}$ application, abrogated the induction of an inward current with acute $A\beta_{1-42}$ (Figure 3-12C). These results would indicate that the inward current induced with acute $A\beta_{1-42}$ was mediated by activation of FcγRII. Acute application of $A\beta_{42-1}$ (5 μM) had no effect on inward currents in human microglia (Figure 3-12D).

The effect of $A\beta_{1-42}$ to induce an inward current through FcγRII activation was confirmed using current clamp studies (personal communication with Dr. S. Jeong). Representative experiments are shown in Figure 3-13. Acute application of $A\beta_{1-42}$ (5 μM) led to an immediate transient depolarization (Figure 3-13A) as previously shown in Figure 3-10A followed by a small hyperpolarization indicative of K^+ channel activation. In a separate set of experiments, inhibition of FcγRII for 1 hr abrogated the effects of acute $A\beta_{1-42}$ (5 μM) to induce an immediate transient depolarization of human microglia

(Figure 3-13B). These results indicate that binding of $A\beta_{1-42}$ to FcγRII leads to an inward current, a corresponding cellular depolarization and subsequent K^+ channel activation.

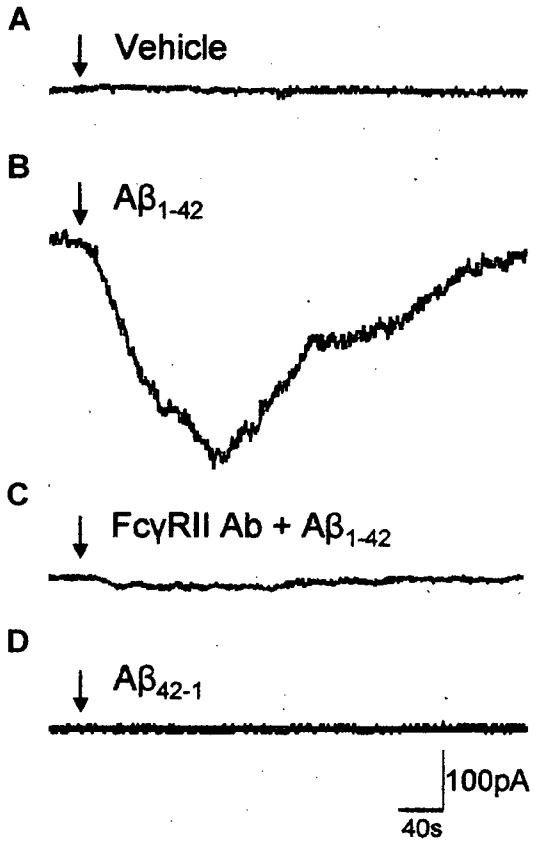

Figure 3-12. $A\beta_{1-42}$ induces an inward current in human microglia mediated by FcγRII activation.
Representative traces of the effects of acute A) vehicle B) 5 μM $A\beta_{1-42}$ C) 5 μM $A\beta_{1-42}$ in the presence of FcγRII inhibition D) 5 μM $A\beta_{42-1}$ on inward currents of human microglia. C) Inhibition of FcγRII inhibition with FcγRII antibody incubation for 1 hr prior to acute $A\beta_{1-42}$ (5 μM) application inhibited the $A\beta_{1-42}$ induced inward current (B). A)Vehicle and D) $A\beta_{42-1}$ had no effects on inward currents.

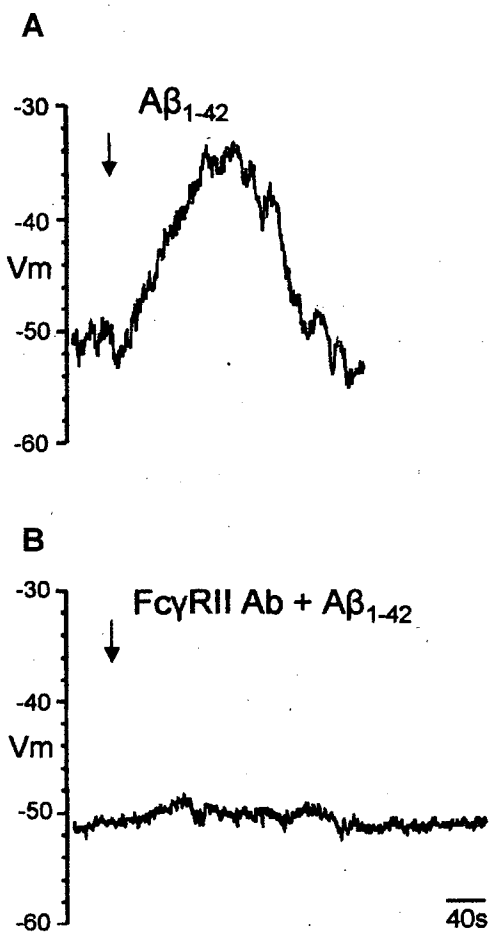

Figure 3-13. The $A\beta_{1-42}$-induced transient depolarization of human microglia is mediated by FcγRII activation
B) Inhibition of the FcγRII with an antibody for 1 hr prior to acute application of $A\beta_{1-42}$ (5 μM), prevented the transient depolarization induced with acute $A\beta_{1-42}$ (A). These results would indicate that the depolarization induced by acute $A\beta_{1-42}$ is mediated by FcγRII activation.

3.3 CONCLUSION

Results from this section show that acute application of $A\beta_{1-42}$ induces a non-inactivating outward K^+ current via a G- protein. This current is sensitive to 4-AP and TEA. Methods of molecular biology indicate that the channel activated by $A\beta_{1-42}$ is the delayed rectifier type Kv3.1. Results also indicate that acute application of $A\beta_{1-42}$ results in transient depolarization of microglia mediated via activation of FcγII receptors.

Under unstimulated conditions, rodent (Eder, 1998; Ilschner et al., 1995; Kettenmann et al., 1990) and human (McLarnon et al., 1999) microglia lack an outward K^+ current. This is the first study reporting the upregulation of a rapidly activating, non-inactivating, outwardly rectifying K^+ current in human microglia in response to acute $A\beta_{1-42}$ (Figure 3-1). This current differs from the transiently inactivating K^+ current attributed to activation of the "n-type" K^+ channel (Kv1.3) with LPS in rodent microglia (Norenberg et al., 1992; 1993). Expression of Kv1.3 requires cell exposure to LPS for periods in excess of 12 hrs. Effects of Aβ on membrane currents have been reported previously; however, treatments with Aβ were long-term and carried out using rat microglia (Chung et al., 2001). Incubation with $A\beta_{25-35}$ or $A\beta_{1-42}$ for 12-24 hours led to a hyperpolarization of cells attributed to upregulation of a Kv current (Kv1.3 and Kv1.5). Upregulation of K^+ channels in microglia are considered an indicator of cellular activation (Fischer et al., 1995) and may counteract the depolarizing effects of certain metabolites present during inflammation (Norenberg et al., 1992; Illes et al., 1996). However, the electrophysiological results in this study indicate that upregulation of a K^+

channel in response to acute $A\beta_{1-42}$ may serve as an early signaling event in the activation process of human microglia by the peptide.

Another novel finding from our patch clamp studies was that the intracellular application of the non-hydrolyzable GTP analogue, GTPγS, induced an outward K^+ current in human microglia (Figure 3-5) similar to the outward K^+ current upregulated by acute application of $A\beta_{1-42}$ (Figure 3-1). The GTPγS activated outward K^+ current displayed similar kinetics of activation (Figure 3-6A), ion selectivity (Figure 3-6B) and sensitivity to 4-AP (Figure 3-7) and TEA as the $A\beta_{1-42}$-induced outward K^+ current which indicated that $A\beta_{1-42}$ induces an outward K^+ current via activation of a G protein. Previous studies have indicated that Aβ mediates its effects on microglia by activating a pertussis toxin sensitive G protein, G_i (Lorton et al., 1997; Tiffany et al., 2001) as well as other second messengers (Combs et al., 1999). G protein-induced K^+ currents have been reported previously in other cell types including murine macrophages (McKinney and Gallin, 1992), T lymphocytes (Schumann and Gardner, 1989) and mast cells (McCloskey and Cahalan, 1990). Interestingly, the K^+ current induced by intracellular application of GTPγS in macrophages (McKinney and Gallin, 1992) was also sensitive to pertussis toxin and 4-AP but unaffected by both apamin, an inhibitor of small conductance calcium activated K^+ channels, and charybdotoxin, an inhibitor of large and intermediate conductance Ca^{2+}-activated K^+ channels similar to the GTPγS-induced K^+ current observed in this study in human microglia. These results could suggest a common G protein signaling pathway in the induction of an outward K^+ current in both human microglia and murine macrophages.

In this study, data from the kinetics of activation and rectification indicated that the channel was a delayed rectifier type of K^+ channel similar to an LPS-induced delayed rectifier K^+ channel reported previously in rat astrocytes (Bordey and Sontheimer, 1999). The transient expression of Kv3.1 channels in response to short incubations with $A\beta_{1-42}$ (10 min to 0.5 hr) (Figure 3-9) is in agreement with the results from patch clamp studies indicating upregulation of an outward K^+ current within minutes of acute $A\beta_{1-42}$ application followed by its rundown within 10-15 minutes (Figure 3-1). Pharmacological characterization of the $A\beta_{1-42}$-induced outward K^+ current indicated sensitivity to both 4-AP (2 mM) (Figure 3-4) and to TEA (10 mM). Previous work has demonstrated Kv3.1 sensitivity to 4-AP and TEA (Grissmer et al., 1994). Previous reports have also indicated that Kv3.1 mRNA is regulated by basic fibroblast growth factor, depolarization, Ca^{2+} and cAMP levels (Perney et al., 1992; Gan et al., 1996) and also that increased expression of Kv3.1 was observed in a subset of proliferating T lymphocytes (Kv3.1 referred to as Type *l* K^+ channel in T lymphocytes; Grissmer et al., 1992) in autoimmune diseases including experimental allergic encephalomyelitis (EAE) (Chandy et al., 1990) and arthritis (Grissmer et al., 1990). These reports support the involvement of second messengers such as a G protein in coupling $A\beta_{1-42}$ to an outward K^+ current and of the Kv3.1 channel in mediating this current in human microglia. Furthermore, the immediate depolarization and progressive increase in $[Ca^{2+}]_i$ with acute $A\beta_{1-42}$ application in this study could act as precedents for the rapid induction of Kv3.1 mRNA observed in microglia.

It is also possible that the rapid induction in Kv3.1 expression within minutes of incubation with $A\beta_{1-42}$ and the upregulation of the outward K^+ current observed within

minutes of acute application of $A\beta_{1-42}$ in whole-cell patch clamp experiments are independent events in microglia. The earliest time point reported for Kv channel protein translation to occur was 15 minutes (Deutsch, 2002) which is a longer time point than that required for the outward current to be induced upon acute application of $A\beta_{1-42}$ (within 3-4 minutes) in whole-cell patch clamp experiments. The induction of the outward K^+ current upon acute application of $A\beta_{1-42}$ within minutes would indicate that the channel protein is either rapidly inserted into the plasma membrane from intracellular vesicles or that the channel is constitutively located in the plasma membrane and is non-functional but requires phosphorylation by second messengers to become activated. The rapid transcription of Kv3.1 could be a compensatory mechanism to replenish the intracellular Kv3.1 protein pool needed for induction of an outward K^+ current induced with acute $A\beta_{1-42}$.

Molecular biology studies indicated basal expression in human microglia of all delayed rectifiers (Kv1.1, 1.2, 1.3, 1.5, 1.6, 2.1) with the exception of Kv3.1. Treatment with $A\beta_{1-42}$ did not alter levels of Kv1.1, 1.2, 1.3, 2.1 whereas Kv1.5 and 1.6 decreased as the incubation period with $A\beta_{1-42}$ increased. No corresponding delayed rectifier currents, i.e. transient inactivating K^+ current typical of Kv1.3 upregulation, were observed in unstimulated conditions nor after $A\beta_{1-42}$ application. This discrepancy between the presence of K_v mRNA under unstimulated and stimulated conditions and the lack of corresponding current has been reported previously in microglia (Norenberg et al., 1993; Khanna et al., 2001). This discrepancy was attributed to constitutive transcription and possible stimulus control of post-translational synthesis of the channel protein, translocation or insertion of the channel protein into the plasma membrane. In our study,

microglia expressed mRNA for Kv1.5 and 1.6, however, expression decreased as $A\beta_{1-42}$ incubation time increased. Previous reports have also reported a decrease in Kv1.5 current expression as microglia transform from a resting to activated state and proliferate (Kotecha and Schlichter, 1999).

Results from this study also indicate that the mechanism through which $A\beta_{1-42}$ induces depolarization in microglia (Figure 3-10) is via activation of the FcγRII since $A\beta_{1-42}$ time-dependently increased FcγRII expression (Figure 3-11) and inhibition of FcγRII completely abolished the depolarization induced with $A\beta_{1-42}$ (Figure 3-13). This was confirmed since acute application of $A\beta_{1-42}$ induced an inward current which was inhibited by incubation of microglia with an FcγRII antibody (Figure 3-12). The mean resting membrane potential of microglia in our study was approximately –36 mV which agrees with previous reports of resting membrane potentials in rodent microglia (Chung et al., 1998; 2001). This immediate depolarization induced by acute $A\beta_{1-42}$ through the FcγRII receptor could in turn activate Kv3.1 since the immediate transient depolarization induced with acute application of $A\beta_{1-42}$ led to an immediate small hyperpolarization of microglia (Figure 3-13). Kv3.1 has been shown previously to be regulated by cellular depolarization (Perney et al., 1992). A role of FcγRII receptor activation in the upregulation of Kv3.1 is supported from previous studies indicating that FcγRII activation leads to pertussis toxin sensitive G protein activation and subsequent inflammatory mediator production in macrophages (DuBourdieu and Morgan, 1990; Bronner et al., 1990). This is also the first study to report the acute action of $A\beta_{1-42}$ to induce a transient depolarization of microglia.

Chapter 4: MODULATION OF $A\beta_{1-42}$-INDUCED INTRACELLULAR SIGNALING AND FUNCTIONAL RESPONSES OF MICROGLIA BY 4-AMINOPYRIDINE: *IN VITRO* AND *IN VIVO*

4.1 RATIONALE

The molecular mechanisms by which microglia become activated and then mediate inflammatory responses in the CNS remain elusive. The activation process in microglia has been associated with specific cell signaling pathways and factors including membrane K^+ current expression (Norenberg et al., 1992; Fischer et al., 1995), altered calcium homeostasis (Vehratsky et al., 1998), activation of p38 MAP kinase (McDonald et al., 1998; Pyo et al., 1998) and activation of the transcription factor NFκB (Combs et al., 2001; Bonaiuto et al., 1997). K^+ channel activation has been shown to have wide spread effects on microglial cell function including proliferation (Schlichter et al., 1996; Kotecha and Schlichter, 1999), migration (Schilling et al., 2004) and respiratory burst activity (Khanna et al., 2001). Modulation of the intracellular signaling pathways associated with microglial activation in response to inflammatory stimuli such as Aβ could serve as a rational approach to inhibit inflammation in AD.

4-aminopyridine (4-AP) has been used as a potential therapeutic in AD due to its ability to prolong the nerve action potential, enhance calcium influx and consequently increasing neurotransmitter release (Glover, 1982). AD pathophysiology is associated with deficits in neurotransmitter systems including the cholinergic, noradrenergic, serotonergic, dopaminegic, GABAergic and glutamatergic systems (Nordberg, 1992) and 4-AP has been reported to increase levels of these neurotransmitters in the brain (Tapia and Sitges, 1982; Dolezal and Tucek, 1983; Hu and Fredholm, 1991; Boireau et al., 1991; Scheer and Lavoie, 1991; Tapia et al., 1985; Tibbs et al., 1989). 4-AP also possesses

favourable properties as an AD therapeutic as compared to other non-selective potassium channel inhibitors such as tetraethylammonium (TEA) since 4-AP has a rapid onset of action and the capability to cross the blood brain barrier (Soni and Kam, 1982). Results from two clinical trials using 4-AP as a putative potentiator of cognitive function in patients with AD have been published (Wesseling et al., 1984; Davidson et al., 1988). One study (Wesseling et al., 1984) reported reduced mental deterioration in patients receiving 4-AP whereas a subsequent study (Davidson et al., 1988) reported no significant difference between patients receiving 4-AP and those receiving placebo. These discrepancies as well as the differences in study design between the two trials precluded a direct comparison and it was concluded that 4-AP warranted further evaluation as a potential AD therapeutic (Wiseman and Jarvik, 1991).

As results from Chapter 3 indicate, 4-AP inhibits the upregulation of an outward K^+ current induced by acute $A\beta_{1-42}$. Molecular biology analysis indicated that the underlying channel is likely Kv3.1. Thus, I have investigated further the potential of 4-AP as a modulator of $A\beta_{1-42}$-induced intracellular signaling pathways and functional responses of human microglia. *In vitro,* I have studied the effects of $A\beta_{1-42}$ on intracellular calcium $[Ca^{2+}]_i$ and modulation by 4-AP of $A\beta_{1-42}$-induced $[Ca^{2+}]_i$ signaling, p38 MAPK and NFκB activation, the expression and production of pro-inflammatory mediators and potential neurotoxicity *in vitro*. *In vivo,* I have investigated the effects of 4-AP on $A\beta_{1-42}$-induced microglial activation and neurotoxicity. The results from this study indicate that 4-AP exhibits wide spectrum anti-inflammatory activity both *in vitro* and *in vivo*.

4.2 RESULTS

4.2.1 Effect of $A\beta_{1-42}$ on Intracellular Calcium of Human Microglia; Inhibition by 4-aminopyridine

4.2.1.1 Effects of $A\beta_{1-42}$ on $[Ca^{2+}]_i$

Calcium spectrofluorometry was used to investigate the effects of $A\beta_{1-42}$ and of 4-AP on $A\beta_{1-42}$-induced intracellular calcium responses in human microglia. In these experiments, levels of $[Ca^{2+}]_i$ are specified as ratios of 340/380. Baseline calcium levels ranged from 0.2 to 0.3. In standard physiological solution (PSS), $A\beta_{1-42}$ (5 μM) acutely applied induced a slow progressive increase in $[Ca^{2+}]_i$, to a plateau; a representative result is shown in Figure 4-1. A peak increase in $[Ca^{2+}]_i$ of 0.07 was attained within 380 sec (n = 39 cells) (Figure 4-1). Overall, the mean amplitude of the response was 0.11 ± 0.01 (n = 98 cells). As shown in Figure 4-1, the replacement of Ca^{2+}-PSS with $A\beta_{1-42}$ in Ca^{2+}-free-PSS following the plateau inhibited this increase by 96%. Overall, in Ca^{2+}-free PSS the $A\beta_{1-42}$-induced response was decreased by 93 ± 5 % (n=98 cells). Washout of $A\beta_{1-42}$ with Ca^{2+}-PSS had no effect to alter the plateau level of $[Ca^{2+}]_i$ induced by $A\beta_{1-42}$ indicative that the responses induced by $A\beta_{1-42}$ were not reversible. Furthermore, acute application of $A\beta_{42-1}$ (5 μM) (n= 68 cells) or vehicle (n=83 cells) had no effect on $[Ca^{2+}]_i$.

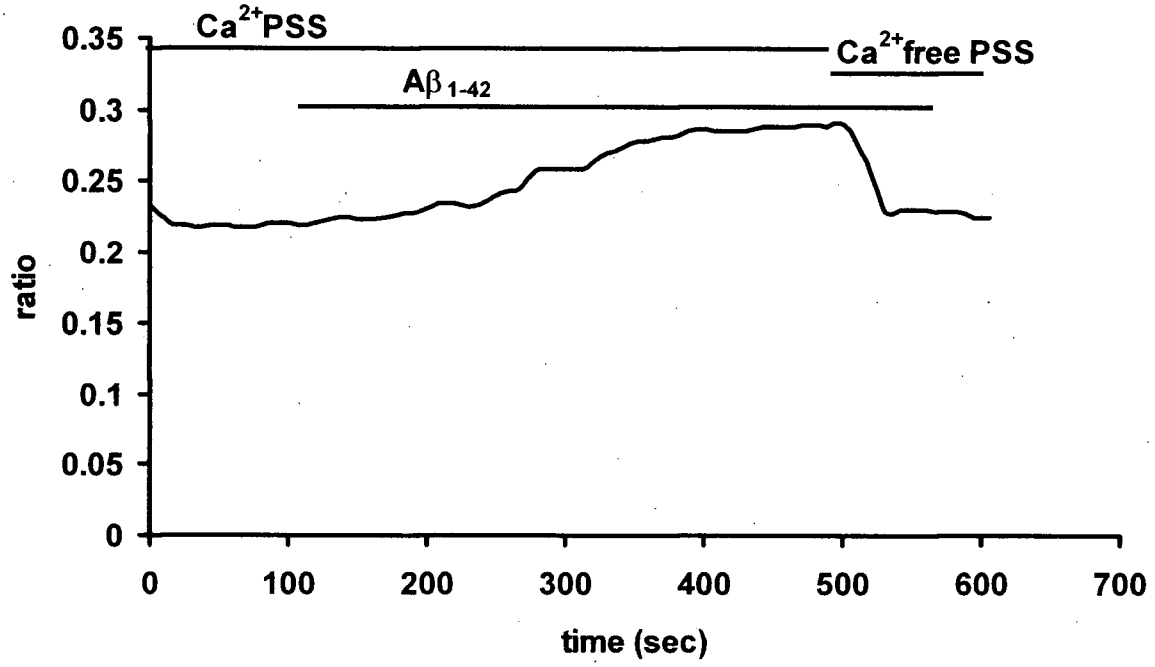

Figure 4-1. Acute application of $A\beta_{1-42}$ induces a slow, progressive increase in $[Ca^{2+}]_i$ Representative trace of the increase in $[Ca^{2+}]_i$ induced by $A\beta_{1-42}$ (5 μM) (n=21 cells). Subsequent application of Ca^{2+} free PSS with $A\beta_{1-42}$ maintained in solution decreased $[Ca^{2+}]_i$ to baseline levels.

The decrease in $[Ca^{2+}]_i$ induced with $A\beta_{1-42}$ in Ca^{2+}-free solution suggests that an influx pathway mediates the increase in $[Ca^{2+}]_i$ with $A\beta_{1-42}$. This point was further tested with application of $A\beta_{1-42}$ in Ca^{2+}-free PSS. As shown in Figure 4-2 (n= 23 cells), $A\beta_{1-42}$ induced a negligible transient increase in $[Ca^{2+}]_i$ with a peak amplitude of 0.03 and a $t_{1/2}$ of 185 sec. Overall, in Ca^{2+} free PSS, $A\beta_{1-42}$ induced a mean increase in $[Ca^{2+}]_i$ of 0.02 ± 0.01 and mean $t_{1/2}$ of 170 ± 15 sec (n=38 cells). These results indicate that $A\beta_{1-42}$ induces an increase in $[Ca^{2+}]_i$ primarily through a Ca^{2+} influx pathway.

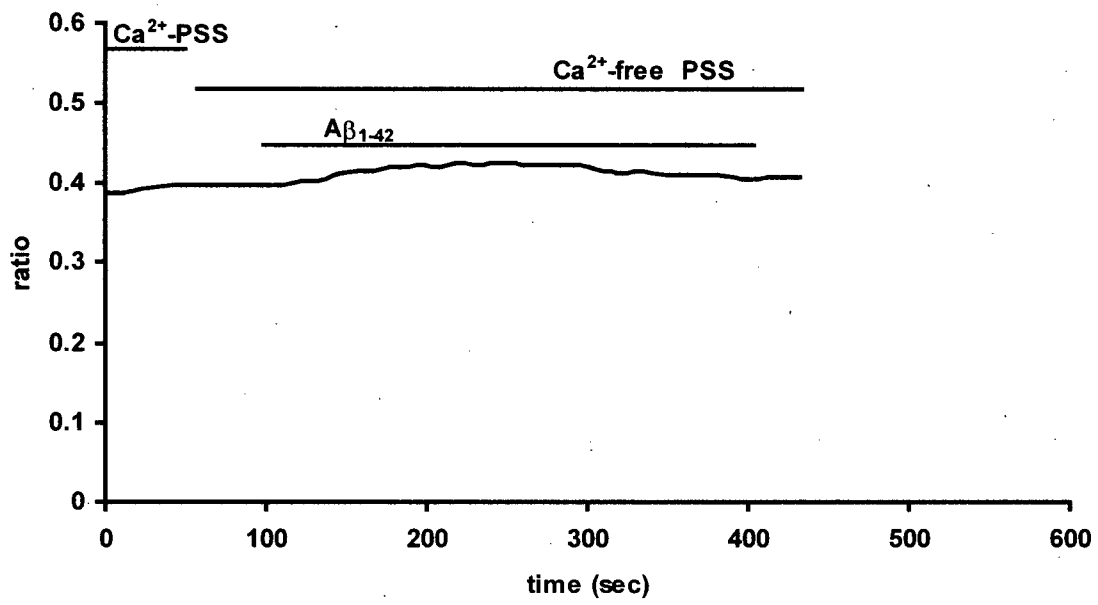

Figure 4-2. In Ca^{2+}-free solution, $A\beta_{1-42}$ induces a small increase in $[Ca^{2+}]_i$.
The standard PSS solution was first exchanged for Ca^{2+}-free PSS. A negligible increase in $[Ca^{2+}]_i$ was induced by acute application of $A\beta_{1-42}$ (5 μM) in Ca^{2+}-free PSS (n=23 cells). This representative trace indicates that the increase in $[Ca^{2+}]_i$ induced with $A\beta_{1-42}$ is primarily due to influx of Ca^{2+}.

Previous work from this laboratory has reported that microglia do not possess voltage-gated Ca^{2+} channels (McLarnon et al., 1999) and that the major Ca^{2+} influx pathway in human microglia is store-operated channel (SOC). I investigated the possibility that SOC could mediate the entry of Ca^{2+} induced by $A\beta_{1-42}$ by using SKF96365, an agent which inhibits SOC in human microglia (Choi et al., 2003) and in other cells (Li et al., 1999). Upon attainment of a plateau in $[Ca^{2+}]_i$ with $A\beta_{1-42}$, application of SKF96365 (50 μM) did not alter $[Ca^{2+}]_i$ (Figure 4-3; n=23 cells). The subsequent exchange of bath solution to Ca^{2+}-free PSS caused an immediate decrease of $[Ca^{2+}]_i$ to baseline levels. Overall, SKF96365, applied at the peak of the response, did not significantly alter the increase in $[Ca^{2+}]_i$ induced by acute $A\beta_{1-42}$ (n=81 cells). This result would indicate that SOC does not contribute to the influx pathway induced by acute $A\beta_{1-42}$ in human microglia.

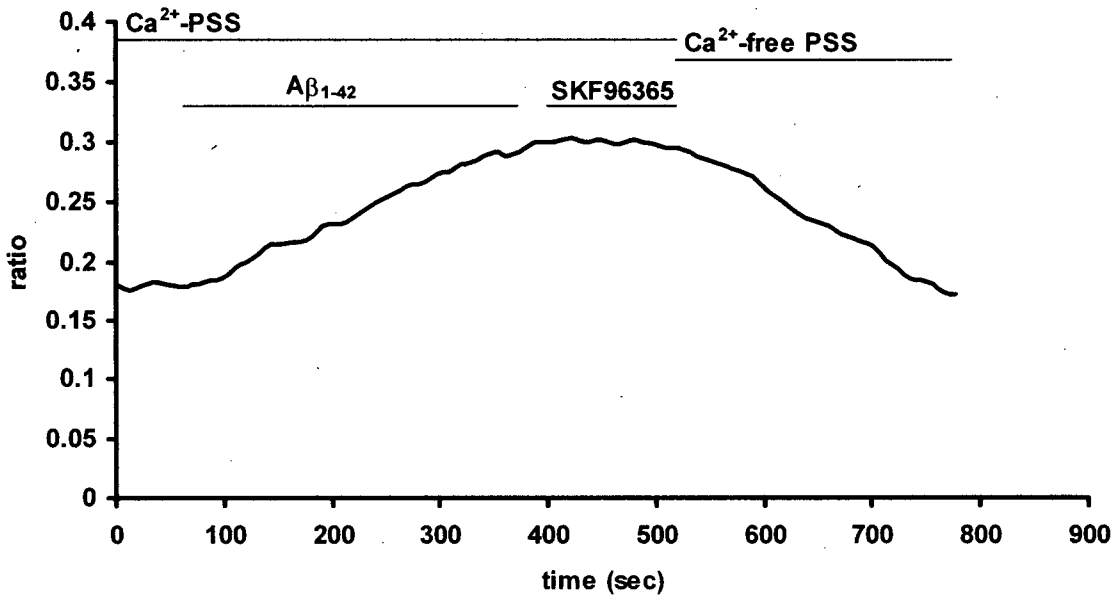

Figure 4-3. SKF96365, an inhibitor of SOC, had no effect on the increase in $[Ca^{2+}]_i$. A representative trace of the effect of SKF96365 on the increase in $[Ca^{2+}]_i$ induced by $A\beta_{1-42}$ (n=21 cells). Subsequent to the plateau in $[Ca^{2+}]_i$ induced by acute $A\beta_{1-42}$ (5 μM) in Ca^{2+} PSS, application of SKF96365 (50 μM) had no effect to alter the increase in $[Ca^{2+}]_i$ induced by $A\beta_{1-42}$. Application of Ca^{2+} free PSS reduced $[Ca^{2+}]_i$ to baseline. This result would indicate that the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ was not due to SOC. Note that wash-out of $A\beta_{1-42}$ did not reduce $[Ca^{2+}]_i$.

4.2.1.2 Effects of 4-AP on $A\beta_{1-42}$-induced Ca^{2+} influx pathway

A representative figure (Figure 4-4) demonstrates the effects of 4-AP on the $A\beta_{1-42}$-induced $[Ca^{2+}]_i$ increase. $A\beta_{1-42}$ induced a progressive increase in $[Ca^{2+}]_i$ with an amplitude of 0.06 (mean, n=26 cells). Application of 4-AP (2 mM) near the peak of the response decreased $[Ca^{2+}]_i$ to baseline levels (Figure 4-4). The effect of 4-AP to decrease $[Ca^{2+}]_i$ was rapid. Overall, 4-AP inhibited the $A\beta_{1-42}$-induced increase in $[Ca^{2+}]_i$ by 96 ± 0.53 % (n = 149 cells). These results may indicate that cellular depolarization mediated by 4-AP inhibition of voltage-dependent K^+ channels inhibits influx of Ca^{2+} induced by acute $A\beta_{1-42}$.

As shown in Figure 4-5, La^{3+} (50 μM), an inhibitor of Ca^{2+} permeable channels, applied at the peak of the $A\beta_{1-42}$ response inhibited completely the $A\beta_{1-42}$-induced Ca^{2+} increase (n=12 cells). Overall, La^{3+} inhibited the $A\beta_{1-42}$-induced increase in $[Ca^{2+}]_i$ to baseline levels (n=44 cells). These results would indicate that $A\beta_{1-42}$ induces Ca^{2+} influx through a cationic permeable channel which is sensitive to depolarization by agents such as 4-AP.

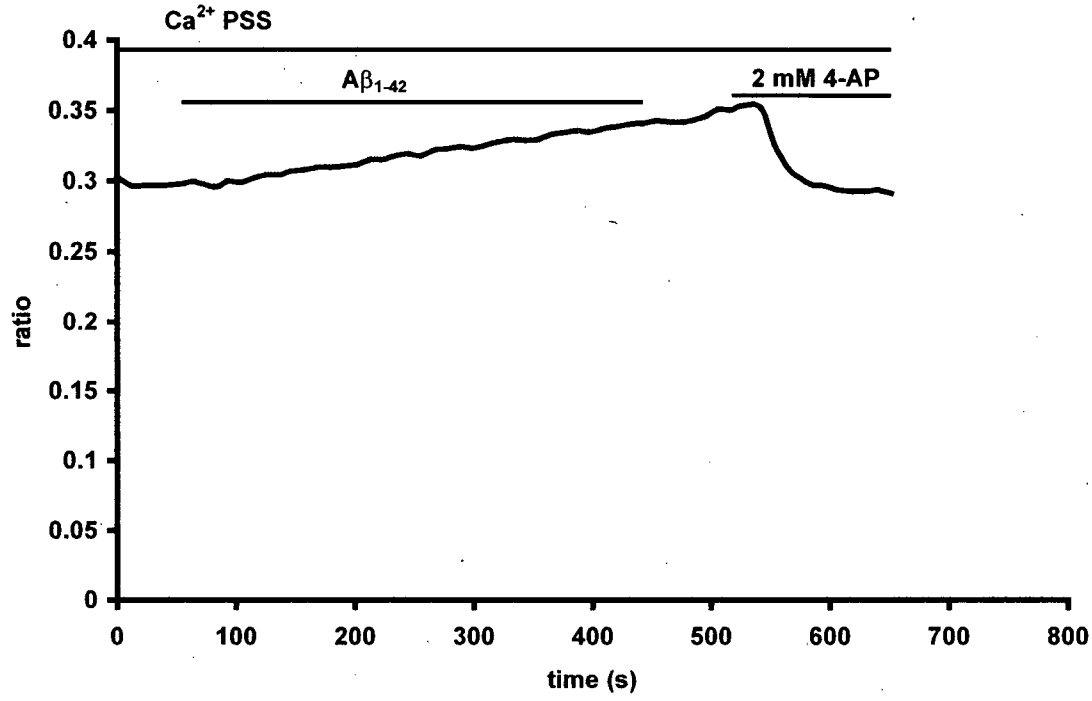

Figure 4-4. The non-selective K^+ channel inhibitor, 4-AP, inhibits the $A\beta_{1-42}$-induced increase in $[Ca^{2+}]_i$.
A representative trace of the effect of 4-AP on the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ (n=26 cells). Subsequent to the slow progressive increase in $[Ca^{2+}]_i$ induced by acute $A\beta_{1-42}$ (5 μM) in Ca^{2+} PSS, application of 4-AP (2 mM) rapidly decreased $[Ca^{2+}]_i$ to baseline levels. This result would indicate that the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ is sensitive to depolarization induced by 4-AP.

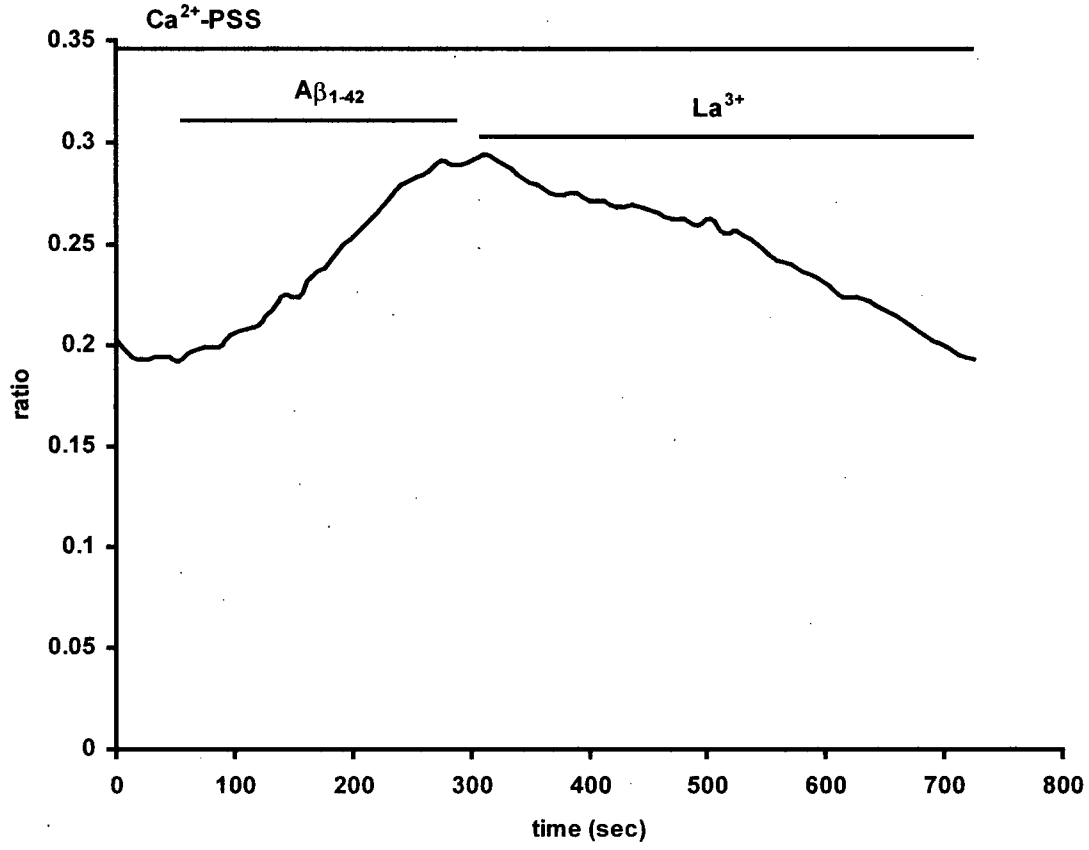

Figure 4-5. La^{3+}, an inhibitor of Ca^{2+} permeable channels, inhibits the $A\beta_{1-42}$-induced increase in $[Ca^{2+}]_i$.
A representative trace of the effect of La^{3+} on the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ (n=12 cells). Subsequent to the slow progressive increase in $[Ca^{2+}]_i$ induced by acute $A\beta_{1-42}$ (5 μM) in Ca^{2+} PSS, application of La^{3+} (50 μM) decreased $[Ca^{2+}]_i$ to baseline levels. This result would indicate that the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ is mediated through a cationic channel.

4.2.2 Effects of $A\beta_{1-42}$ on p38 MAP Kinase and NFκB Activation in Human Microglia; Attenuation by 4-aminopyridine

Effects of 4-AP on $A\beta_{1-42}$-induced p38 MAP kinase activation in human microglia

p38 MAP kinase and NFκB activation have been implicated in linking of inflammatory stimuli to functional responses of microglia. Stimuli include LPS and Aβ which induce secretion of pro-inflammatory factors (Lee et al., 2000; McDonald et al., 1998; Combs et al., 2001; Bonaiuto et al., 1997). Increased NFκB and p38 MAPK are observed in AD brain (Terai et al., 1996; Hensley et al., 1999). I have investigated the effects of 4-AP on p38 MAP kinsase and NFκB using immunocytochemical procedures (see methods section 2.4.1).

The effects of 4-AP on $A\beta_{1-42}$-induced p38 MAP kinase activation in human microglia are presented in Figure 4-6A. Under basal conditions, low numbers of positively stained cells for phospho-p38 MAP kinase were detected. Stimulation with $A\beta_{1-42}$ (5 μM for 30 min) induced an increased expression of phospho-p38 MAP kinase in microglia (green), which was blocked if 4-AP (2 mM) was included with $A\beta_{1-42}$ treatment. 4-AP itself showed no effect to alter phospho-p38 MAP kinase from control. Overall, p38 MAP kinase activation was analyzed from n=4 independent experiments. The results (Figure 4-6A, B) indicate that $A\beta_{1-42}$ significantly increases (by 371%) the number of cells expressing activated p38 MAP kinase in human microglia ($p < 0.001$). 4-AP (2 mM), in the maintained presence of $A\beta_{1-42}$, resulted in a significant reduction (by 58%) in the number of cells expressing phospho-p38 MAP kinase as compared to stimulation with $A\beta_{1-42}$ alone ($p < 0.001$). 4-AP (2 mM) and $A\beta_{42-1}$ (5 μM) did not significantly alter the basal level of phospho-p38 MAP kinase stained cells ($p > 0.05$).

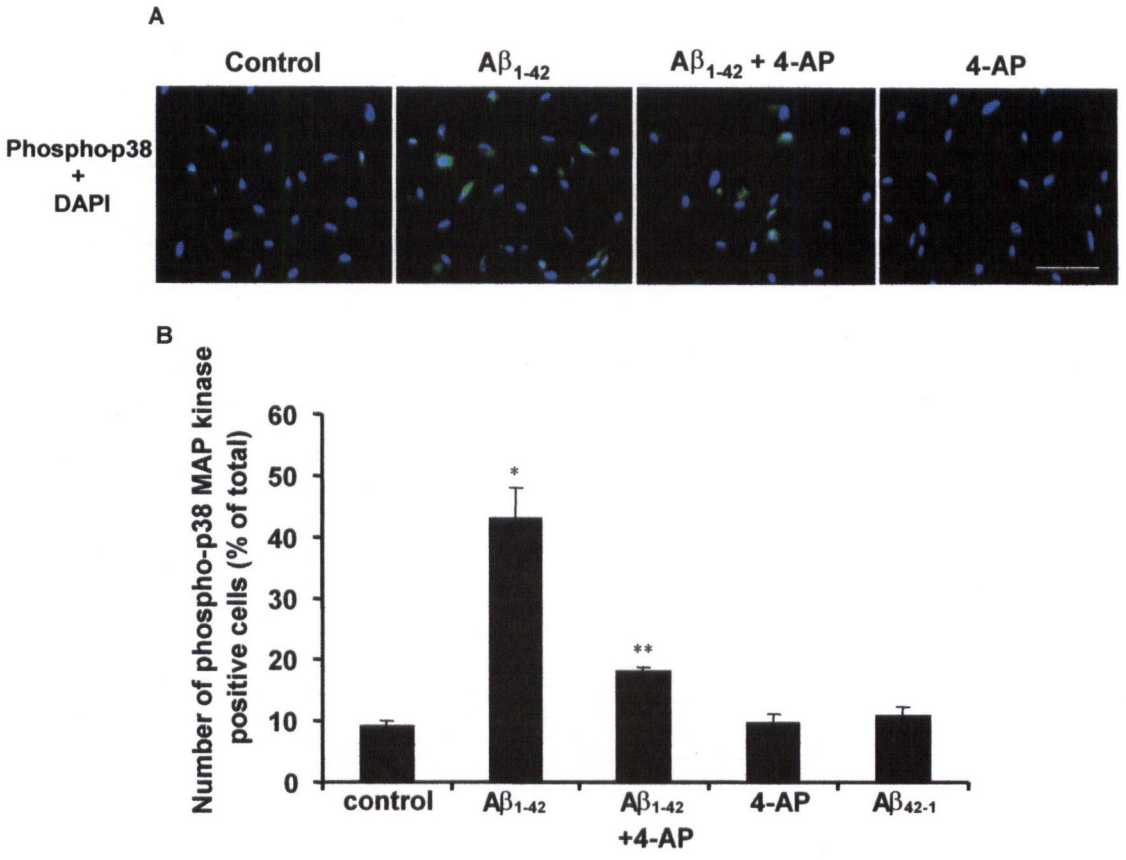

Figure 4-6. Effects of 4-AP on $A\beta_{1-42}$-induced p38 MAP kinase activation
A) Representative photomicrographs of phosphorylated p38 (phospho-p38) stained microglia. The green and blue indicate staining for phospho-p38 and DAPI positive nuclei, respectively. Under control conditions, little or no phospho-p38 expression was observed. $A\beta_{1-42}$ (5 μM) treatment of microglia for 30 min induced an intense expression of phospho-p38. $A\beta_{1-42}$ in the maintained presence of 4-AP (2 mM) inhibited expression of phospho-p38. Application of 4-AP (2 mM) alone had no effect on phospho-p38 expression. B) The percentage of phospho-p38 positive microglia relative to total cells are shown. Data are means ± SEM from four independent experiments. *indicates significance compared with control $p < 0.001$ and **indicates significance compared with $A\beta_{1-42}$ $p < 0.001$. Scale bar = 50 μm.

In positive control experiments, LPS stimulation of microglia (for 30 min) resulted in a significant increase in p38 MAPK positive microglia (to 73.7 ± 5.8%) from the number of p38 MAPK positive cells in untreated conditions (control: 3.7 ± 1.3%; $p < 0.001$).

Effects of 4-AP on $A\beta_{1-42}$-induced NF-κB activation in human microglia

The modulatory actions of 4-AP were also investigated on $A\beta_{1-42}$-induced activation of the transcription factor, NF-κB. The presence of the active subunit of NF-κB, p65, was determined using immunocytochemistry. As shown in Figure 4-7A, a low number of cells express p65 under basal conditions (green). Stimulation with $A\beta_{1-42}$ (5 μM for 8 hr) increased the expression of p65 in microglia which was blocked if 4-AP (2mM) was included with $A\beta_{1-42}$ treatment. 4-AP itself showed no effect to alter p65 levels from control.

Overall, p65 levels were analyzed from n=5 independent experiments. The results (Figure 4-7A, B) indicate that $A\beta_{1-42}$ induces a significant increase (by 493%) in p65 expressing cells ($p < 0.01$). 4-AP in the maintained presence of $A\beta_{1-42}$ significantly decreased (by 60%) the number of cells expressing p65 ($p < 0.01$). 4-AP (2 mM) alone induced an increase in p65 positive cells, however, the increase was not significant ($p > 0.05$). $A\beta_{42-1}$ did not alter basal levels of p65 expressing cells ($p > 0.05$).

In positive control experiments, LPS stimulation of microglia (12 hrs) resulted in a significant increase in p65 positive microglia (to 27 ± 2.9%) from the number of p65 positive cells in untreated conditions (control: 0.3 ± 0.1%; $p < 0.001$).

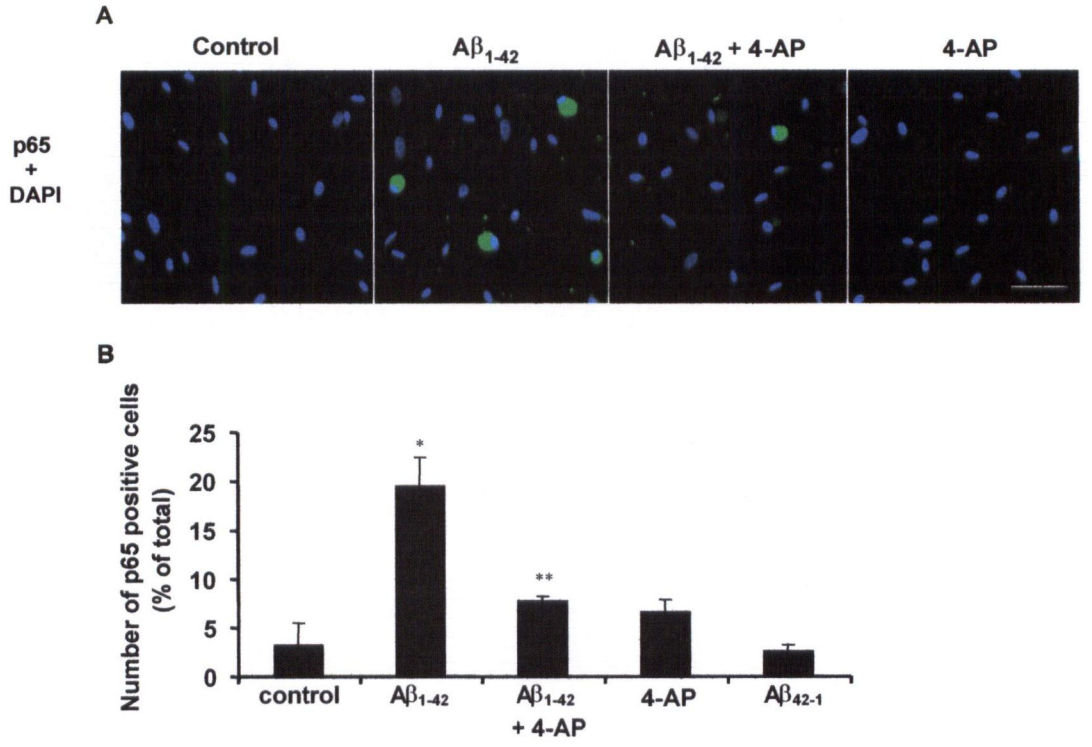

Figure 4-7. Effects of 4-AP on $A\beta_{1-42}$-induced NFκB activation
A) Representative photomicrographs of p65 (the active subunit of NFκB) stained microglia. The green and blue indicate staining for p65 and DAPI positive nuclei, respectively. Under control conditions, little or no p65 expression was observed. $A\beta_{1-42}$ (5 μM) treatment of microglia for 8 hrs induced an intense expression of p65. $A\beta_{1-42}$ in the maintained presence of 4-AP (2 mM) inhibited expression of p65. Application of 4-AP (2 mM) alone had no effect on p65 expression. B) The percentage of p65 positive microglia relative to total cells are shown. Data are means ± SEM from five independent experiments. *indicates significance compared with control and **indicates significance compared with $A\beta_{1-42}$; $p < 0.01$. Scale bar = 50 μm

4.2.3 Effects of $A\beta_{1-42}$ on Expression of Pro-Inflammatory Mediators; Attenuation by 4-aminopyridine

Microglia are a major source of pro-inflammatory mediators. In this study, I examined the effects of 4-AP on $A\beta_{1-42}$-induced expression of the pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), chemokine (CXCL8 (IL-8)) and inducible enzyme COX-2. Results from a representative experiment are shown in Figure 4-8A. Overall, microglia express CXCL8 (IL-8) constitutively under unstimulated conditions, whereas IL-1β, IL-6, TNF-α and COX-2 were not expressed. After 8 hr incubation with 5 μM $A\beta_{1-42}$, increased expression of all pro-inflammatory mediators was observed. In the presence of $A\beta_{1-42}$, 4-AP (2 mM) decreased the expression of all pro-inflammatory mediators. 4-AP alone had little or no effect on expressions of pro-inflammatory mediators. $A\beta_{42-1}$ (5 μM) was ineffective to alter expression of pro-inflammatory mediators from control.

Densitometry analysis of PCR product band intensities showed that 4-AP reduces $A\beta_{1-42}$-induced pro-inflammatory mediator expression. A summary of relative mRNA levels of inflammatory mediators induced with $A\beta_{1-42}$, 4-AP and of $A\beta_{1-42}$ + 4-AP is shown in Figure 4-8B (n=7 experiments). Overall, $A\beta_{1-42}$ (5 μM) significantly increased relative mRNA levels of all pro-inflammatory mediators (* indicates $p < 0.05$). $A\beta_{1-42}$, in the presence of 4-AP led to a significant decrease in pro-inflammatory mediator expression relative to $A\beta_{1-42}$ stimulated levels (** indicates $p < 0.05$). Stimulation with 4-AP alone led to a small increase in levels of pro-inflammatory mediators relative to control, however, the increases were not significant ($p > 0.05$). Fold increases in relative pro-inflammatory mediator mRNA induced by $A\beta_{1-42}$, 4-AP, $A\beta_{1-42}$ + 4-AP, and $A\beta_{42-1}$

compared to control are summarized in Table 4-1. Overall, $A\beta_{1-42}$ increased the expression of pro-inflammatory mediators (relative to control) by (x fold increase): IL-1β: 3.8; IL-6: 6.8; CXCL8 (IL-8): 1.8; TNF-α: 3.6; COX-2: 2.9. 4-AP alone increased the expression of pro-inflammatory mediators (relative to control) by (x fold increase): IL-1β: 1.3; IL-6: 1.5; CXCL8 (IL-8): 1.1; TNF-α: 1.5; COX-2: 1.5. Inhibition of $A\beta_{1-42}$-induced expression of pro-inflammatory mediators by 4-AP was (x fold decrease): IL-1β: 0.56; IL-6: 0.60; CXCL8 (IL-8): 0.65; TNF-α: 0.61; COX-2: 0.51. $A\beta_{42-1}$ did not alter relative mRNA levels of pro-inflammatory mediators from control ($p > 0.05$).

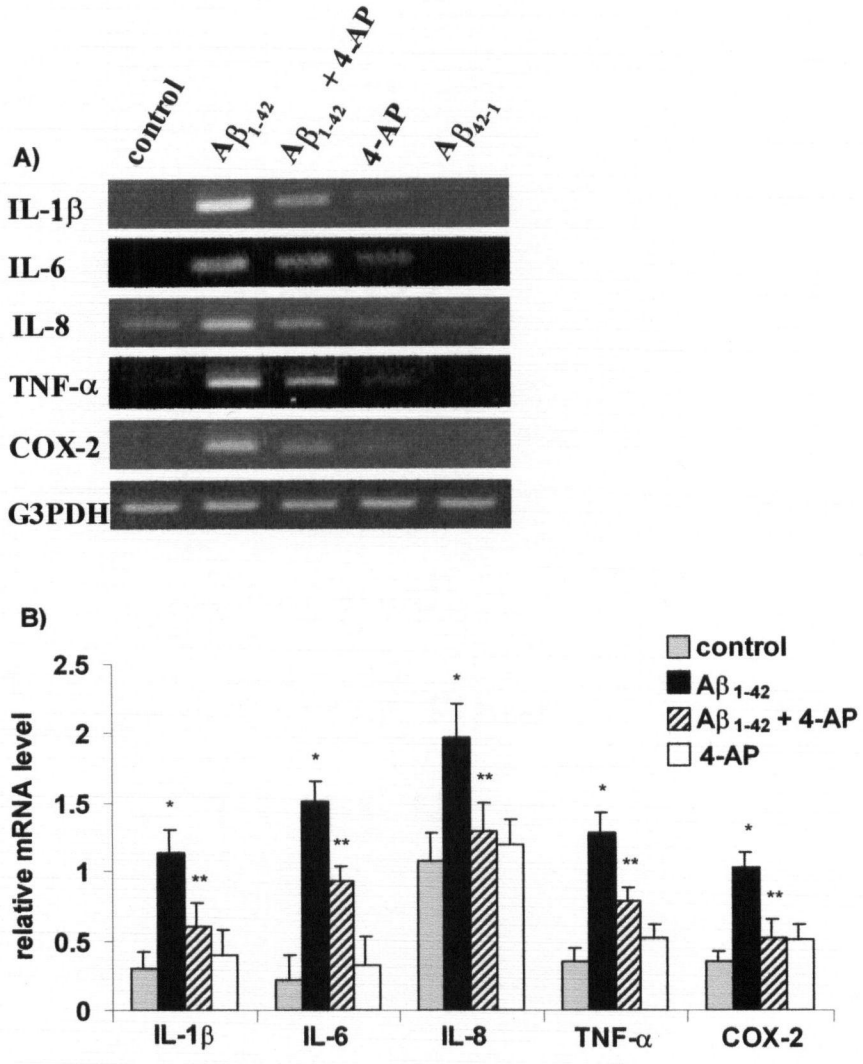

Figure 4-8. Effects of 4-AP on $A\beta_{1-42}$-induced expression of pro-inflammatory mediators

Effects of $A\beta_{1-42}$ and of $A\beta_{1-42}$ in the presence of 4-AP on the expression of pro-inflammatory mediators by human microglia using semiquantitative RT-PCR. Expression of (A) TNF-α, IL-6, IL-1β, CXCL8 (IL-8) and COX-2 were examined in microglia incubated for 8 hrs with $A\beta_{1-42}$, 4-AP, $A\beta_{1-42}$ in the combined presence of 4-AP, or with medium alone. Stimulation of microglia with $A\beta_{42-1}$ (5 μM) alone served as a control experiment. The results shown are a representative of seven independent experiments. The expression of G3PDH served as a reaction standard. B) Summary of relative mRNA levels of inflammatory mediators induced by $A\beta_{1-42}$, 4-AP and combined $A\beta_{1-42}$ and 4-AP. Results are expressed as mean ± SEM from n=7 independent experiments. One-way ANOVA and Newman-Keuls multiple comparison post-test was performed to evaluate statistical significance ($p < 0.05$) (*indicates statistically significant from control; ** indicates statistically significant from $A\beta_{1-42}$ stimulated levels).

Table 4-1. Fold increases in relative pro-inflammatory mediator mRNA induced by $A\beta_{1-42}$, 4-AP, $A\beta_{1-42}$ + 4-AP, and $A\beta_{42-1}$ compared to relative mRNA in control using semiquantitative RT-PCR

	$A\beta_{1-42}$	$A\beta_{1-42}$ + 4-AP	4-AP	$A\beta_{42-1}$
IL-1β	3.8*	**2.0**	**1.3**	**1.2**
IL-6	6.8**	**4.2****	**1.5**	**1.4**
CXCL8 (IL-8)	1.8*	**1.2**	**1.1**	**1.1**
TNF-α	3.6*	**2.2***	**1.5**	**1.4**
COX-2	2.9*	**1.5**	**1.5**	**1.3**

* $p < 0.05$; **$p < 0.01$

4.2.4 Effects of $A\beta_{1-42}$ on Production of Pro-Inflammatory Mediators; Attenuation by 4-aminopyridine

I next investigated the effects of $A\beta_{1-42}$ exposure on microglial production of inflammatory factors and actions of 4-AP to modulate the effects of Aβ on production of these inflammatory factors. The production of TNF-α, IL-6, IL-1β and CXCL8 (IL-8) were investigated after 48 hrs stimulation with $A\beta_{1-42}$ in the presence and absence of 4-AP (2 mM) using ELISA. 48 hrs was chosen as the optimum time point to determine the modulatory actions of 4-AP on cytokine production using ELISA since protein levels continued to accumulate through 48 hrs and incubations with $A\beta_{1-42}$ for periods longer than 24hrs could induce both direct and indirect effects of the peptide (Walker et al., 2001). A summary of results is presented in Figure 4-9. $A\beta_{1-42}$ increased secretion of TNF-α (by 2.3 fold; n=4 independent experiments) (Figure 4-9A); IL-6 (by 46 fold; n=3 independent experiments) (Figure 4-9B); IL-1β (by 1.9 fold; n=6 independent experiments) (Figure 4-9C); CXCL8 (IL-8) (by 4.5 fold; n=4 independent experiments) (Figure 4-9D) and compared to basal levels in human microglia; all increases were significant ($p < 0.001$).

$A\beta_{1-42}$ in the maintained presence of 4-AP (2 mM) decreased levels of TNF-α (by 46%); IL-6 (by 73%); IL-1β (by 26%); CXCL8 (IL-8) (by 47%) as compared to $A\beta_{1-42}$ alone; all decreases were significant ($p < 0.001$). The changes in levels of pro-inflammatory mediators induced with 4-AP and $A\beta_{42-1}$ each alone were not significantly different from basal levels ($p > 0.05$). A summary of fold increases in pro-inflammatory mediator production induced by $A\beta_{1-42}$, 4-AP and $A\beta_{1-42}$ in the present of 4-AP as compared to levels in control is presented in Table 4-2.

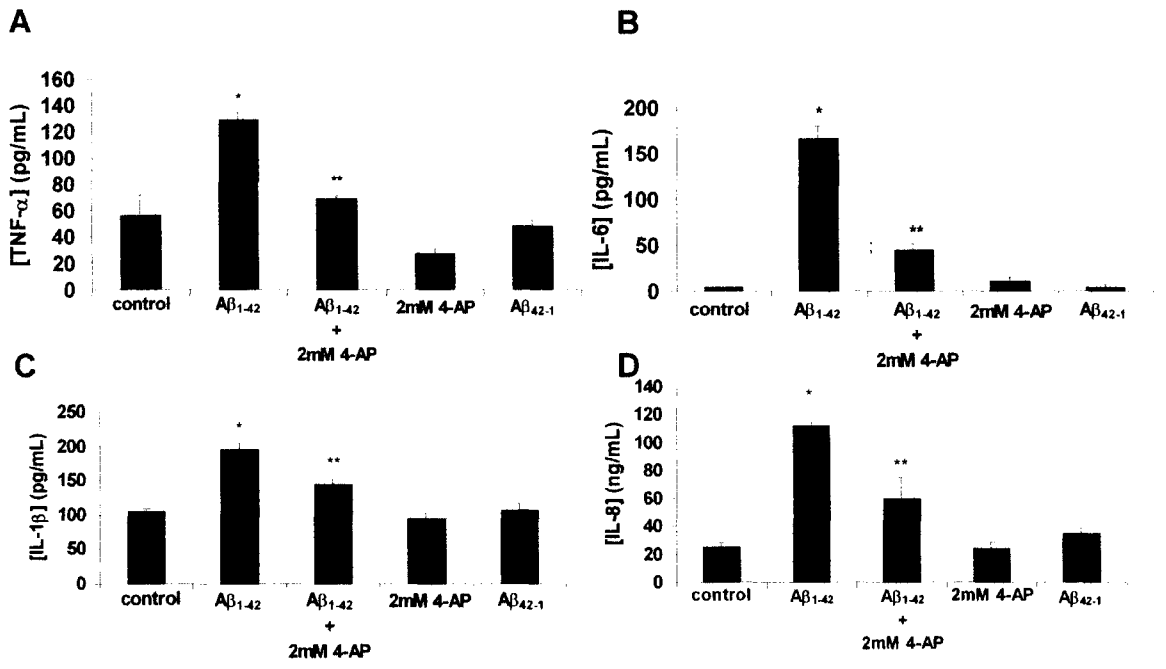

Figure 4-9. Effects of 4-AP on $A\beta_{1-42}$-induced pro-inflammatory mediator production.

Effects of $A\beta_{1-42}$, 4-AP and $A\beta_{1-42}$ in the maintained presence of 4-AP on pro-inflammatory cytokine secretion by human microglia using ELISA. Data are mean ± SEM of four independent experiments for (A) TNF-α; three independent experiments for (B) IL-6; six independent experiments for (C) IL-1β and four independent experiments for (D) CXCL8 (IL-8), each performed in duplicate. Human microglia were exposed to either medium alone, $A\beta_{1-42}$ (5 μM), 4-AP (2 mM), $A\beta_{1-42}$ in the presence of 4-AP or $A\beta_{42-1}$ for 48 hrs. One-way ANOVA and Newman-Keuls multiple comparison post-test was performed to evaluate statistical significance ($p < 0.001$) (*indicates statistical significance from control; **indicates statistical significance from $A\beta_{1-42}$ stimulated levels).

Table 4-2. Fold increases in pro-inflammatory mediator production induced by $A\beta_{1-42}$, 4-AP, $A\beta_{1-42}$ + 4-AP and $A\beta_{42-1}$compared to levels in control

	$A\beta_{1-42}$	4-AP	$A\beta_{1-42}$+4-AP	$A\beta_{42-1}$
IL-1β	1.9***	0.90	1.4**	1.0
IL-6	46***	2.9	13**	1.2
CXCL8 (IL-8)	4.5***	0.95	2.4	1.4
TNF-α	2.3***	0.48	1.2	0.86

***p < 0.05; **p < 0.01; ***p < 0.001**

The production of COX-2 was determined using immunocytochemistry and representative results are presented in Figure 4-10A. Stimulation with $A\beta_{1-42}$ (5 μM for 24 hrs) induced an increase in the number of COX-2 positive microglia (green) from control which was blocked with 4-AP (2 mM) in the maintained presence of $A\beta_{1-42}$. 4-AP and $A\beta_{42-1}$ each alone had no effect to alter COX-2 levels from control.

Overall, results of the effects of $A\beta_{1-42}$ and of $A\beta_{1-42}$ on COX-2 expression were determined from n=6 independent experiments. $A\beta_{1-42}$ significantly increased the percentage of microglia expressing COX-2 by 5.1 fold from control levels ($p < 0.001$) (Fig. 4B). 4-AP (2 mM) in the maintained presence of $A\beta_{1-42}$ significantly decreased (by 0.43 fold) the percentage of COX-2 expressing cells ($p < 0.01$). 4-AP (2 mM) and $A\beta_{42-1}$ (5 μM) each alone induced an increase in the percentage of COX-2 positive microglia compared to unstimulated conditions, however, the increases were not significant ($p > 0.05$).

In positive control experiments, stimulation of microglia with LPS (for 12 hr) resulted in a significant increase (by 54 fold) in COX-2 positive cells from untreated conditions (n=3 independent experiments; $p < 0.001$).

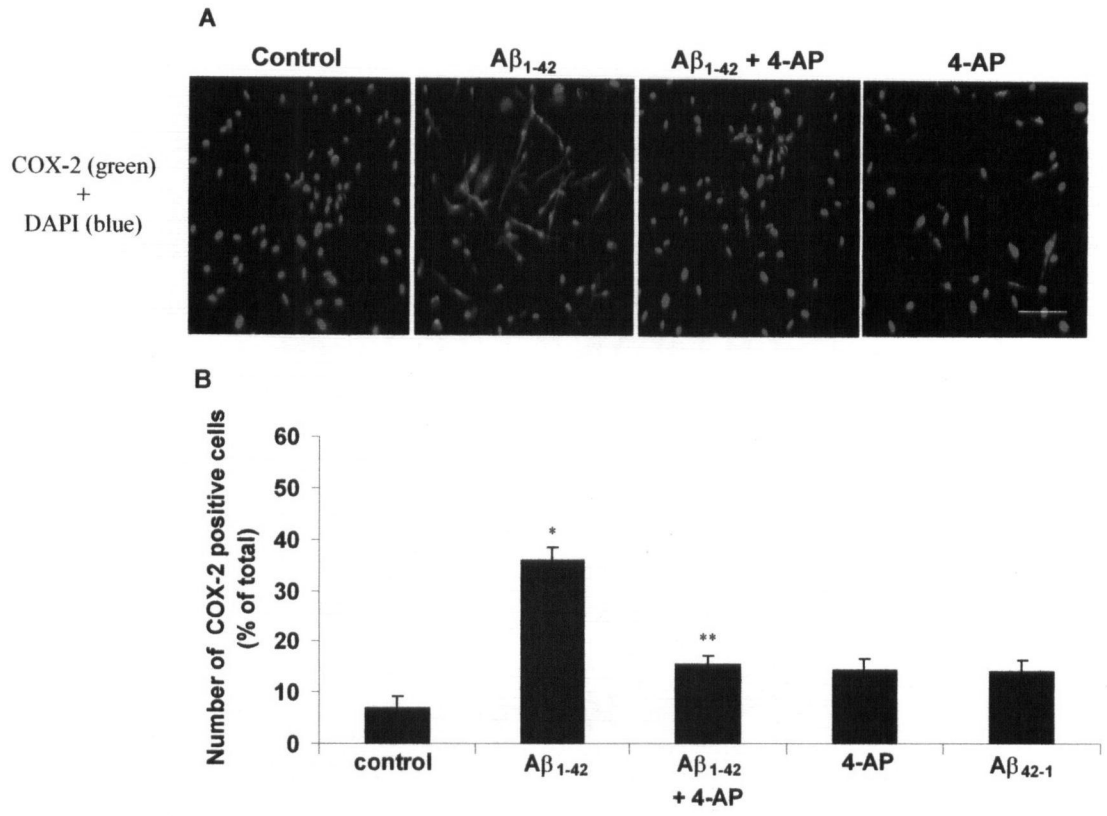

Figure 4-10. Effects of 4-AP on $A\beta_{1-42}$-induced COX-2 expressing microglia
A) Representative photomicrographs of COX-2 positively stained microglia. The green and blue indicate staining for COX-2 and DAPI positive nuclei, respectively. Under control conditions, little or no COX-2 expression was evident. Treatment of microglia for 24 hrs with $A\beta_{1-42}$ (5 μM) induced an intense expression of COX-2. $A\beta_{1-42}$ in the presence of 4-AP treatment inhibited production of COX-2. 4-AP alone had no effect on basal levels of COX-2 production. B) The percentage of COX-2 positive microglia relative to total cells are shown under the different experimental conditions. Data are means ± SEM from six independent experiments. * indicates significance compared to control ($p < 0.001$) and **denotes significance compared to $A\beta_{1-42}$ ($p < 0.01$). Scale bar = 50 μm.

4.2.5 Effects of $A\beta_{1-42}$ on microglial mediated primary hippocampal neuronal toxicity; inhibition by 4-aminiopyridine

An important study in my research program was to investigate $A\beta_{1-42}$ stimulated microglia as a possible source of neurotoxicity both in the presence and absence of 4-AP. The strategy used to investigate microglial mediated neurotoxicity has been described in Chapter 2 (Section 2.9) and is presented in Figure 4-11A. Briefly, the supernatant of human microglia stimulated with $A\beta_{1-42}$, in the presence and absence of 4-AP (2 mM), for 48 hr (conditioned medium) was transferred to primary rat hippocampal neuronal cultures. Following 16 hr incubation with microglial conditioned media, neurons were stained with DAPI and the number of neurons with condensed nuclei were counted. Controls consisted of stimulating neurons with medium lacking microglia (unconditioned medium). As shown in Figure 4-11B, incubation of neurons with medium from $A\beta_{1-42}$ stimulated microglia resulted in increased numbers of neurons with condensed nuclei (bright fluorescent nuclei) and 4-AP (2 mM) in the maintained presence of $A\beta_{1-42}$ decreased microglial mediated neurotoxicity. 4-AP had no effect to alter basal levels of neuronal death or with $A\beta_{42-1}$. Stimuli alone (unconditioned medium) had no effect on neuronal viability.

Overall, neuronal toxicity from microglial conditioned medium and unconditioned medium was obtained from n=5 independent experiments. The percentage neurotoxicity induced by microglial conditioned medium (from microglia stimulated for 48 hrs with $A\beta_{1-42}$, 4-AP, $A\beta_{42-1}$, and $A\beta_{1-42}$ in the presence of 4-AP) as well as with unconditioned medium have been summarized in Figure 4-11C. Incubation of neurons with the supernatant of $A\beta_{1-42}$-stimulated microglia induced a significant increase (by

232%) in the number of neurons with condensed nuclei as compared to levels induced by unstimulated microglia ($p < 0.001$). 4-AP significantly reduced the amount of $A\beta_{1-42}$-induced microglial neurotoxicity by 54% ($p < 0.001$). Conditioned medium from microglia treated with 4-AP and $A\beta_{42-1}$ each separately increased the level of neuronal death by 9% and 14% respectively as compared to basal levels, however, the increases were not significant ($p > 0.05$). Treatment of neurons with medium from unstimulated microglia did not alter the basal level of neuronal damage ($p > 0.05$).

Incubation of neurons for 16 hrs with unconditioned medium containing $A\beta_{1-42}$ (5 μM) alone and in the presence of 4-AP (2 mM), 4-AP alone and $A\beta_{42-1}$ (5 μM) did not alter the basal percentage of neuronal damage ($p > 0.05$).

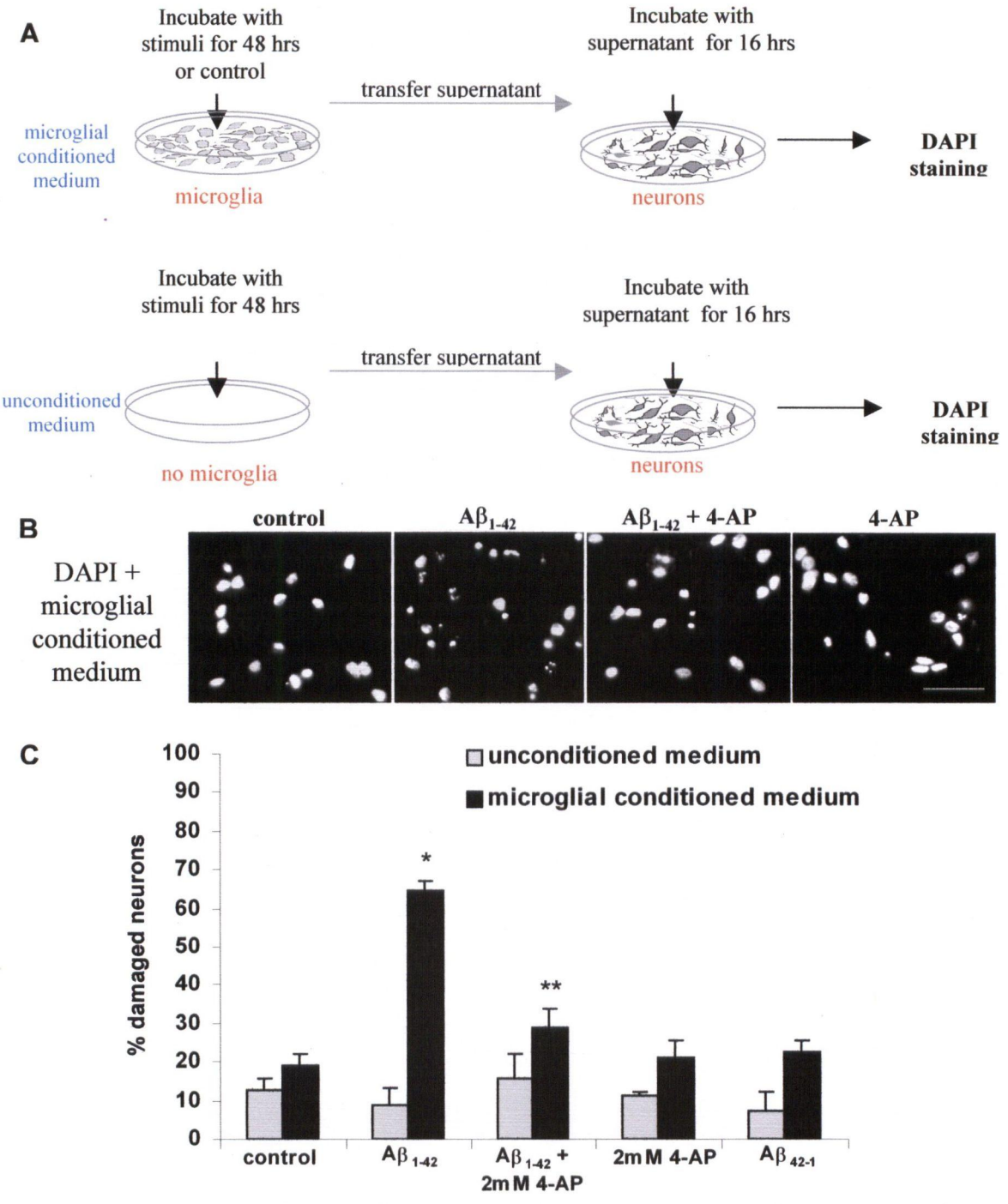

Figure 4-11. Effects of microglial conditioned medium from microglia stimulated with $A\beta_{1-42}$, 4-AP each alone or in combination on neuronal survival.
A) Strategy used to investigate microglial mediated neurotoxicity (taken from Figure 2-4) B) Representative photomicrographs of neurons treated with microglial conditioned medium. Condensed and fragmeneted nuclei were considered damaged neurons. Scale bar = 50 μm C) Summary of microglial mediated neurotoxicity results from n=5 independent experiments. * indicates statistically significant from medium of unstimulated microglia ($p<0.001$); ** indicates statistically significant from conditioned medium of $A\beta_{1-42}$ stimulated microglia ($p<0.001$).

4.2.6 *In vivo* effect of 4-aminopyridine on $A\beta_{1-42}$-induced neurotoxicity and microglial activation

An important aspect of my work was to determine the anti-inflammatory potential and neuroprotective actions of 4-AP *in vivo*. This was done by microinjection of $A\beta_{1-42}$ (1 nmol) into the dentate gyrus of rat hippocampus and at 7 days post-injection, both microglial activation and neuronal toxicity was determined (refer to Methods section 2.10). The effects of 4-AP were investigated in rats administered 4-AP (1 mg/kg) i.p. daily for 7 days.

4.2.6.1 4-AP reduces $A\beta_{1-42}$-induced neurotoxicity *in vivo*

I examined the effect of 4-AP on $A\beta_{1-42}$–induced neuronal toxicity *in vivo* using NeuN, a marker of viable neurons. Representative NeuN positive staining results from $A\beta_{1-42}$ injection into the dentate granule cell layer of rat hippocampus, in the absence and presence of 1 mg/kg 4-AP, are presented in Figure 4-12A-D.

Loss of NeuN positive granule neurons was evident at 7 days post $A\beta_{1-42}$ injection (Figure 4-12B) compared to vehicle (Figure 4-12A). As shown in Figure 4-12C, 4-AP was effective in protecting neurons from $A\beta_{1-42}$-induced damage. Little or no loss of neurons was observed with 4-AP alone (Figure 4-12D) or with $A\beta_{42-1}$ alone.

Overall, at 7 days after $A\beta_{1-42}$ injection, the number of NeuN positive neurons in the superior blade of dentate granule cell layer decreased by 18% compared to vehicle (Figure 4-12E; $p < 0.05$). Treatment with 4-AP reduced the neurotoxic effect of $A\beta_{1-42}$ since the number of NeuN positive cells was significantly increased by 16 % in $A\beta_{1-42}$ injected brain administered 4-AP relative to the number of NeuN positive cells in

peptide-injected brain ($p < 0.05$). No significant neuronal loss was observed in $A\beta_{42\text{-}1}$-injected or 4-AP treated rat brain (Figure 4-12E; $p>0.05$).

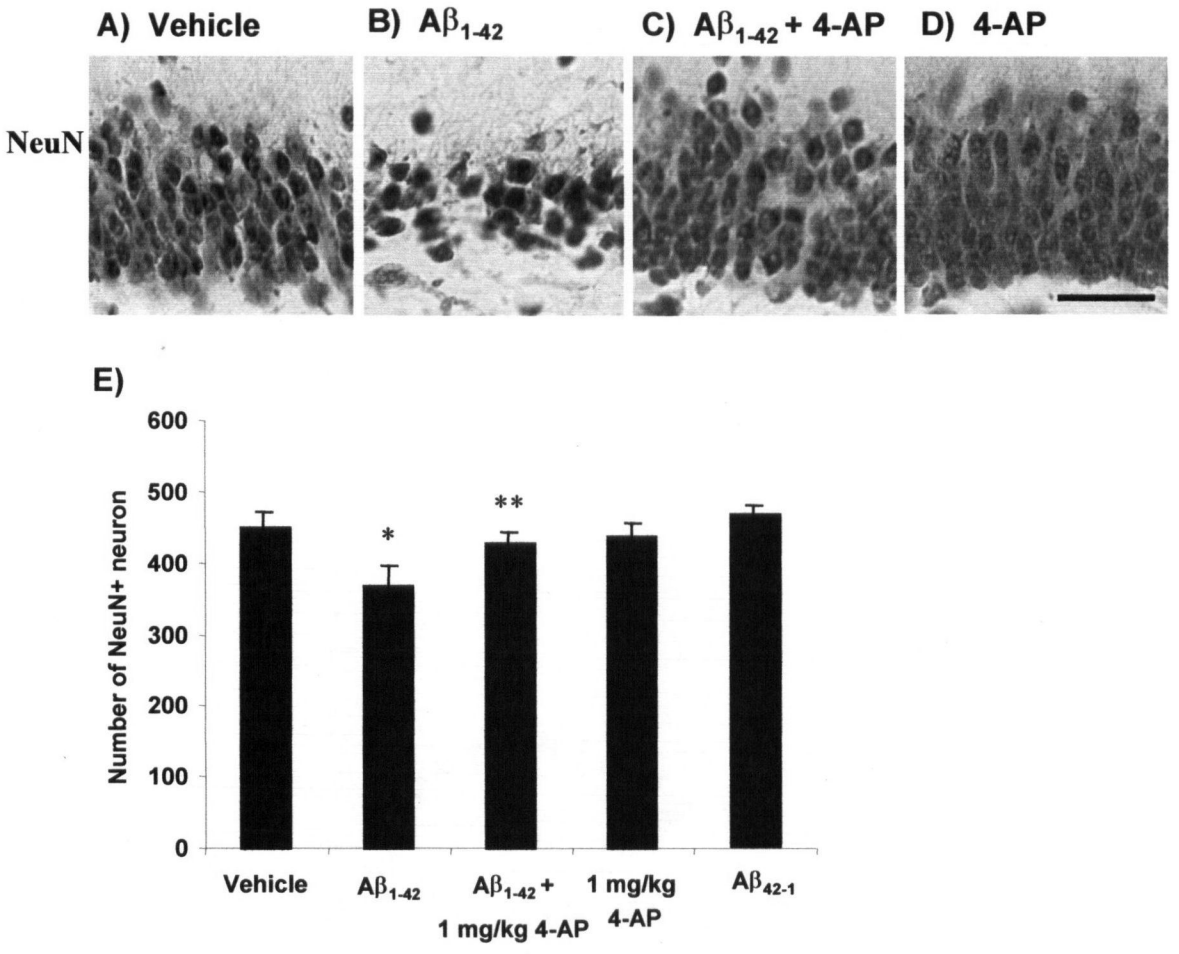

Figure 4-12. Effects of 4-AP on $A\beta_{1-42}$-induced hippocampal neuron degeneration *in vivo*

Representative photographs of tissue sections stained with NeuN antibody from superior blade of dentate granule cell layer taken from A) vehicle injected rats and rats treated with B) $A\beta_{1-42}$ (1 nmol), C) $A\beta_{1-42}$ plus 4-AP (1 mg/kg i.p.) and (D) 4-AP seven days after injection. (E) Quantification of the effects of $A\beta_{1-42}$ and of $A\beta_{1-42}$ in the presence of 4-AP on NeuN positive neurons. Data are mean ± SEM (n = 4/group). * $p < 0.05$ vs control; ** $p < 0.05$ vs $A\beta_{1-42}$. Scale bar = 50 µm.

4.2.6.2 4-AP reduces $A\beta_{1-42}$-induced microglial activation *in vivo*

I next examined the effect of 4-AP on $A\beta_{1-42}$–induced microglial responses *in vivo* using ED1, a marker of microglial activation. Representative ED1 positive staining results from $A\beta_{1-42}$ injection into the dentate granule cell layer of rat hippocampus, in the absence and presence of 1 mg/kg 4-AP, are presented in Figure 4-13A-D.

Numbers of ED1 positive microglia (Figure 4-13B) were considerably increased with peptide relative to vehicle (Figure 4-13A). 4-AP treatment attenuated the number of activated microglia in $A\beta_{1-42}$ injected rat brain (Fig. 4-13C). Injection of 4-AP alone had no effect on numbers of activated microglia (Fig. 4-13D). $A\beta_{42-1}$ had a small effect to increase the numbers of ED1 positive cells (Fig. 4-13E).

Overall, in $A\beta_{1-42}$-injected brain, the numbers of microglia were significantly increased by 18-fold compared to vehicle (Fig. 4-13F; $p < 0.05$). In $A\beta_{1-42}$-injected brain, administration of 4-AP resulted in a significant reduction in the number of ED1 positive microglia (by 68%) relative to numbers of microglia with $A\beta_{1-42}$ (Fig. 4-13F; $p < 0.05$). No significant increase in the number of ED1 positive microglia was found in 4-AP treated brain compared to vehicle (Fig. 4-13F; $p > 0.05$). $A\beta_{42-1}$ injection resulted in increased ED1 positive microglia compared to vehicle, however, the increase was not significant (Fig. 4-13F; $p > 0.05$).

The above results *in vivo* indicate that 4-AP is neuroprotective in $A\beta_{1-42}$-injected brain and reduces the number of activated microglia induced by $A\beta_{1-42}$.

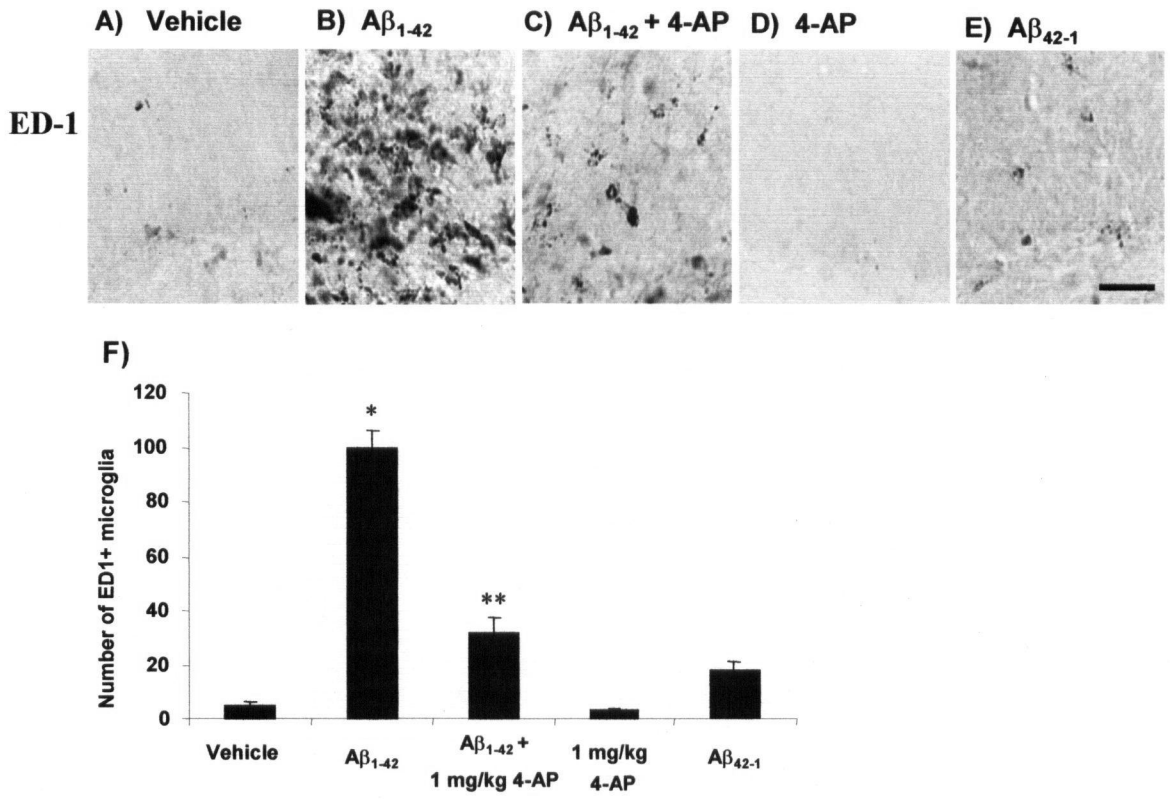

Figure 4-13. ***In Vivo*** **effects of 4-AP on $A\beta_{1-42}$-induced microglial activation Representative photographs of tissue sections stained with ED1 from superior blade of dentate granule cell layer taken from A) vehicle injected rats and rats treated with (B) $A\beta_{1-42}$ (1 nmol) (C) $A\beta_{1-42}$ plus 4-AP (1 mg/kg), (D) 4-AP or E) $A\beta_{42-1}$ (1 nmol) at seven days post-injection. F) Quantification of the effects of $A\beta_{1-42}$, 4-AP and of $A\beta_{1-42}$ in the presence of 4-AP on ED1 positive microglia. Data are mean ± SEM (n = 4/group). * $p < 0.05$ vs control. ** $p < 0.05$ vs $A\beta_{1-42}$. Scale bar = 50 µm**

4.3 CONCLUSION

My results show, 4-AP in both *in vivo* and *in vitro* assays, modulates $A\beta_{1-42}$-induced microglial activation and subsequent functional responses including neuronal toxicity. Administration of 4-AP reduced $A\beta_{1-42}$ mediated neurotoxicity and microglial activation *in vivo* (Figure 4-12; 4-13). *In vitro*, the results suggest that a possible mechanism through which 4-AP reduces neuronal toxicity is via effects on $A\beta_{1-42}$-stimulated microglia (Figure 4-11). Acute application of $A\beta_{1-42}$ induced an increase in intracellular calcium $[Ca^{2+}]_i$ through a Ca^{2+} influx pathway which was inhibited by 4-AP (Figure 4-4). In longer-term studies, 4-AP also inhibited $A\beta_{1-42}$-induced activation of p38 MAP kinase (Figure 4-6) and NFκB activation (Figure 4-7) in human microglia. Furthermore, 4-AP inhibited $A\beta_{1-42}$-induced functional responses of human microglia including the expression (Figure 4-8) and production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), the chemokine CXCL8 (IL-8) (Figure 4-9) and inducible enzyme COX-2 (Figure 4-10). A summary of the effects of 4-AP on $A\beta_{1-42}$ induced intracellular signaling and functional responses in human microglia is shown in Figure 4-14.

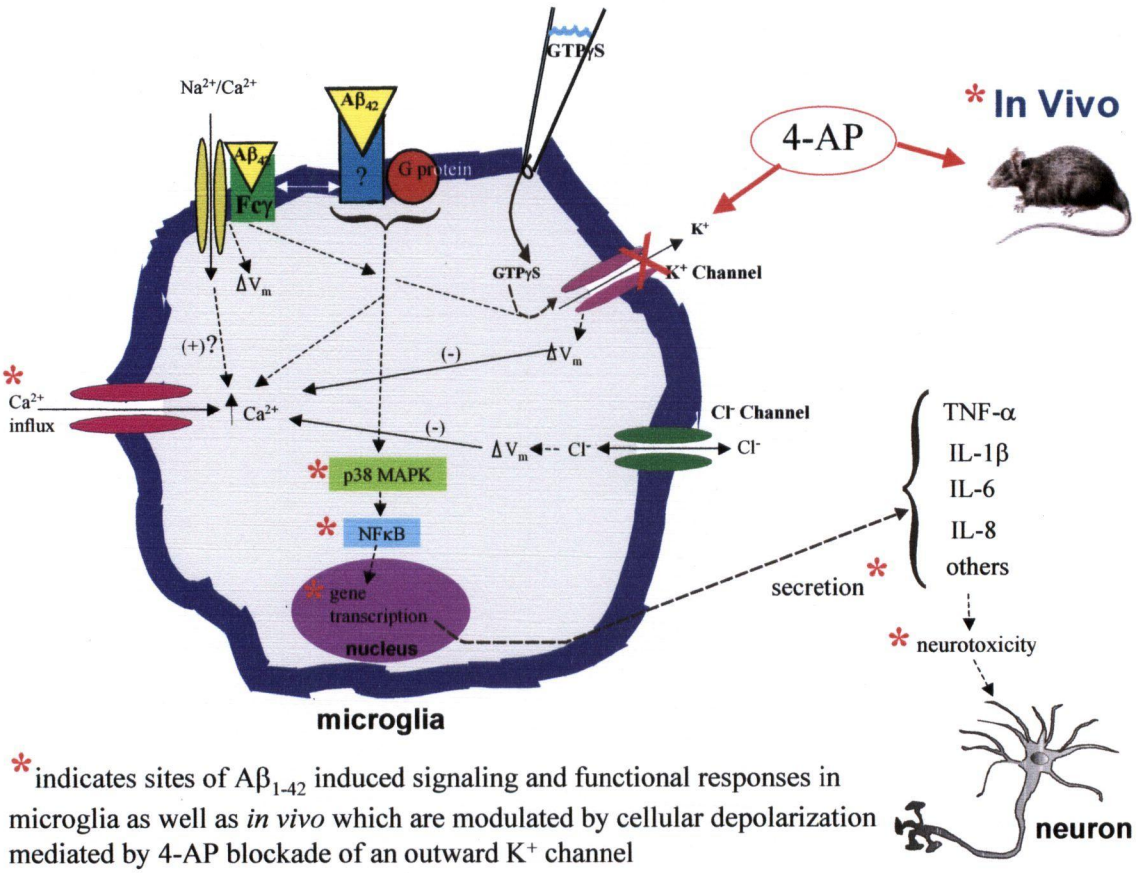

Figure 4-14. Summary of the effects of 4-AP on $A\beta_{1-42}$-induced intracellular signaling and functional responses in human microglia in this study.

Administration of 4-AP markedly decreased $A\beta_{1-42}$-induced hippocampal neuronal cell death *in vivo* (Figure 4-12). To my knowledge this study is the first to report therapeutic effects of 4-AP towards $A\beta_{1-42}$-induced neurotoxicity *in vivo*. My results also show significant effects of 4-AP to reduce microgliosis in peptide-injected rat brain (Figure 4-13). The *in vitro* neuroprotection study (Figure 4-11) provided a clear indication that the presence of microglia is essential for $A\beta_{1-42}$-induced neurotoxicity since $A\beta_{1-42}$ directly did not induce killing of neurons. Rather, the addition of conditioned medium from $A\beta_{1-42}$-stimulated microglia induced significant killing of neurons (Figure 4-11) and further, the neuroprotective effects of 4-AP were clearly induced by the inhibition of $A\beta_{1-42}$-stimulated microglia. These results support the findings that $A\beta_{1-42}$ alone is not neurotoxic but rather that microglia may play a role in inflammation-mediated neurodegeneration in AD (McDonald et al., 1997; Minghetti et al., 1998). Specifically, microglial activation has been associated with neurodegeneration through the production of neurotoxic factors such as pro-inflammatory cytokines, NO, superoxide anion (McDonald et al., 1997; Griffin et al., 1998; Combs et al., 1999, 2000) and an unidentified neurotoxin (Giulian et al., 1995). In this study, $A\beta_{1-42}$ caused microglial activation both *in vivo* (Figure 4-13) and *in vitro* (Figure 4-1; 4-6; 4-7) and also caused a significant increase in the production of pro-inflammatory mediators (Figure 4-9; 4-10); 4-AP inhibited these stimulatory effects of $A\beta_{1-42}$ in human microglia. These results would suggest that 4-AP is exerting its neuroprotective effects by deactivating microglia and subsequently inhibiting $A\beta_{1-42}$-induced production of pro-inflammatory mediators (Figure 4-9; 4-10) and other potentially unknown neurotoxic factors thereby reducing neurotoxicity (Figure 4-11).

Ca^{2+} ions play a central role in several cellular functions including regulation of cell volume, cell motility and serves as a signal transduction element to activate other membrane ion channels (e.g., Ca^{2+}-activated K^+ and Cl^- channels) and transporters. The inhibitory effects of 4-AP on the $A\beta_{1-42}$-induced Ca^{2+} influx pathway indicates that this influx pathway is sensitive to changes in membrane potential (Figure 4-4). Time frames for acute effects of $A\beta_{1-42}$ on membrane currents (Chapter 3) and intracellular Ca^{2+} should not be taken as written. The sequence of events occurring in these studies upon binding of $A\beta_{1-42}$ to a receptor, likely FcγRII, is depolarization, subsequent activation of a K^+ channel, likely Kv3.1 (as described in Chapter 3) which in turn increases the driving force for Ca^{2+} influx leading to the subsequent slow progressive increase in $[Ca^{2+}]_i$. Inhibition of this K^+ channel with 4-AP reduces this driving force for Ca^{2+} to enter the cell leading to a subsequent decrease in $[Ca^{2+}]_i$. The major influx pathway in microglia is store-operated (SOC) since microglia do not possess voltage-gated Ca^{2+} channels (McLarnon et al., 1999). Since the SOC inhibitor SKF96365 had no effect on the $A\beta_{1-42}$-induced increase in $[Ca^{2+}]_i$, these results would indicate that the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ in human microglia was not SOC mediated. The inhibition of the $A\beta_{1-42}$-induced increase in $[Ca^{2+}]_i$ by La^{3+} would suggest that the $A\beta_{1-42}$ effect was mediated through a non-specific cation permeable channel however, the specific identity of the pathway remains unknown. The Ca^{2+} increase could be mediated by FcγRII receptor activation since the FcγRII receptor is coupled to a non-selective cationic channel which would allow Ca^{2+} into the cell once activated. Aβ has been shown to activate a G protein-coupled extracellular calcium-sensing receptor (CaR) in neurons which stimulates a Ca^{2+}-permeable, non-selective cation channel leading to increasing $[Ca^{2+}]_i$ (Ye et al.,

1997). A CaR has been identified in rat microglia (Chattopadhyay et al., 1999) and thus, $A\beta_{1-42}$ may be acting on a CaR in human microglia resulting in an increase in $[Ca^{2+}]_i$. Interestingly, a similar unidentified Ca^{2+} influx pathway to that induced with $A\beta_{1-42}$ in this study has been reported previously in human microglia in response to acute IFN-γ (Franciosi et al., 2002). La^{3+} had a similar slow inhibitory effect on the increase in $[Ca^{2+}]_i$ with acute IFN-γ as it did on the $[Ca^{2+}]_i$ increase with acute $A\beta_{1-42}$ reported in this study. The immediate inhibition of the $A\beta_{1-42}$-induced Ca^{2+} entry pathway by 4-AP (Figure 4-4) indicates that membrane depolarization inhibits this influx pathway. It could also indicate that 4-AP is exerting effects other than non-selectively blocking K^+ channels such as inhibiting non-specific cation permeable channels or activating Ca^{2+} extrusion mechanisms such as the PMCA. In contrast to our findings, 4-AP has been shown to increase $[Ca^{2+}]_i$ in other cells including astrocytes, (Grimaldi et al., 2001) and monocytes (Lajdova et al., 2004) which would indicate that the effect of 4-AP on $[Ca^{2+}]_i$ differs between cell types studied. Aβ-induced increases in $[Ca^{2+}]_i$ have been reported previously in microglia (Silei et al., 1999; Korotzer et al., 1995).

4-AP also inhibited $A\beta_{1-42}$ activation of the SER-kinase, p38 MAP kinase, in human microglia (Figure 4-6). Aβ has been shown previously to induce pro-inflammatory effects in microglia via activation of p38 MAP kinase (McDonald et al., 1998; Pyo et al., 1998; Kim et al., 2004) and inhibition of p38 MAP kinase resulted in decreased microglial inflammatory reactions and subsequent neurodegeneration (Giovannini et al., 2002) similar to the results of this study. The activation of p38 MAP kinase leads to the phosphorylation of a multitude of downstream kinases and transcription factors, regulates mRNA stability of pro-inflammatory cytokines such as

TNF-α, and influences chromatin accessibility to transcription factors such as NF-κB (Koistinaho and Koistinaho, 2002). Therefore, altered p38 MAP kinase activation may provoke altered signaling pathways downstream of the kinase. Previous work has shown that delayed rectifier K^+ currents are inhibited by p38 MAP kinase in microglia and attributed this inhibition by p38 MAP kinase as the mechanism by which delayed rectifier currents are time-limited (Schilling and Eder, 2003). Therefore, the increase in phosphorylated p38 MAP kinase observed with $A\beta_{1-42}$ may play a role in the downregulating the outward K^+ current induced with $A\beta_{1-42}$ in human microglia (Chapter 3) and a similar time course of p38 MAP kinase activation by $A\beta_{1-42}$ (10-30 minutes) and downregulation of the outward K^+ current (Chapter 3) was observed in this study (within 10-15 minutes). In a separate study, inhibition of stress-induced K^+ channel activity by 4-AP in a myelocytic leukemic cell line resulted in decreased p38 MAPK phosphorylation (Gao et al., 2004). It was suggested that inhibition of K^+ channel activity by 4-AP resulted in decreased cell volume and subsequent inhibition of MAPK translocation at the centrosome, the major microtubule organization center of the cell. This reduction in K^+ channel activity, alteration in cytoskeletal proteins and subsequent inhibition of p38 MAP kinase translocation may be the mechanism through which 4-AP inhibits p38 MAP kinase activation in human microglia. The inhibition of the $A\beta_{1-42}$-induced increase in phosphorylated p38 MAP kinase by 4-AP may also be due to the reduction by 4-AP in $[Ca^{2+}]_i$ levels induced by $A\beta_{1-42}$, however, studies have indicated that calcium is not a requirement for phosphorylation of p38 but rather plays a role downstream of MAP kinases in mediating pro-inflammatory mediator release (Hide et al., 2000).

The transcription factor NF-κB is one of the key molecules in the process of microglial activation on which several signaling pathways elicited by pro-inflammatory stimuli, converge (O'Neill and Kaltschmidt, 1997). NF-κB has been implicated in the transcription of numerous genes in microglia including the pro-inflammatory factors expressed by $A\beta_{1-42}$ stimulated microglia in this study: TNF-α, IL-1β, IL-6 (Combs et al., 2001; Kang et al., 2001; Heyen et al., 2000), the chemokine CXCL8 (IL-8) (Ehrlich et al., 1998) and COX-2 (Egger et al., 2003). In unstimulated cells, NF-κB exists in a latent form complexed with an inhibitory protein of the IκB family. Upon activation, IκB proteins are targeted for degradation, thus allowing the translocation of the active subunit of NF-κB (p65/p50) to the nucleus and the transcription of target genes. Our results indicate that $A\beta_{1-42}$ increases the percentage of microglia expressing the p65 subunit of NFκB and that 4-AP inhibits this effect of $A\beta_{1-42}$ on NFκB (Figure 4-7). $A\beta_{1-42}$ has been shown previously to activate NFκB in microglia (Bonaiuto et al., 1997). This is the first study demonstrating the modulatory effects of 4-AP on NF-κB activation. It has been shown previously that NFκB activation is involved in the upregulation of an outward K^+ current in microglia since addition of a selective NF-κB inhibitor in the presence of a viral protein resulted in complete inhibition of the outward K^+ current (Visentin et al., 2001). A possible mechanism for 4-AP mediated inhibition of $A\beta_{1-42}$-induced NF-κB activation is through the reduction in the upstream signaling factor p38 MAPK (Figure 4-6) since p38 MAPK may control NF-κB transactivation (Vanden Berghe et al., 1998; Madrid et al., 2001).

As expected, the inhibitory effects of 4-AP on $A\beta_{1-42}$-induced NF-κB activation had subsequent inhibitory effects downstream to NF-κB in the $A\beta_{1-42}$ signaling pathway in human microglia. The $A\beta_{1-42}$-induced gene expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), chemokine CXCL8 (IL-8) and inducible enzyme COX-2 (Figure 4-8) and production (Figure 4-9; 4-10) of these factors were inhibited by 4-AP. $A\beta_{1-42}$ has been shown previously to stimulate the expression of pro-inflammatory mediators in human microglia (Lue et al., 2001a). A previous report indicated that 4-AP inhibited LPS-induced production of IL-1β in rat microglia (Caggiano and Kraig, 1998). The inhibitory effect of 4-AP was attributed to the inability of the cell to compensate the LPS-induced cellular depolarization in the presence of 4-AP. The neuroprotective properties of 4-AP *in vitro* (Figure 4-11) are consistent with actions to inhibit $A\beta_{1-42}$-induced expression and production of pro-inflammatory mediators by microglia. Since 4-AP was also able to reduce production of pro-inflammatory cytokines, CXCL8 (IL-8) and COX-2 (Figure 4-9; 10), it is possible that 4-AP blocks other microglial secretory products involved in neurodegeneration.

Interestingly, our results *in vivo* indicated that the administration of 1.0 mg/kg 4-AP was neuroprotective in $A\beta_{1-42}$ injected rat brain (Figure 4-12). In a previous study examining the effects of 4-AP on memory performance in rats, lower concentrations of 4-AP (0.10-1.0 mg/kg) led to enhanced performance (Haroutunian et al., 1985). Administration of 1 mg/kg 4-AP is estimated to give a cerebrospinal fluid concentration of ~2-20 μM, falling to ~1 μM after 10-30 minutes (Lemeignan et al., 1984; Jankowska et al., 1982). The concentration of 4-AP which was effective *in vitro* in blocking microglial-induced intracellular signaling and functional responses including

neurotoxicity in this study was in the mM range (~1000 fold higher than that used *in vivo*). This discrepancy between the concentration used *in vitro* and dose administered *in vivo* is due to the narrow therapeutic window of 4-AP. The maximum tolerable CSF concentration in humans is ~5 μM (Felts and Smith, 1994). The difficulty in administering higher concentrations of 4-AP *in vivo* is due its side effects since numerous types of K^+ channels are present in the CNS, each with differing sensitivities to 4-AP.

The inhibition of microglial mediated neurotoxicity by 4-AP would indicate that the K^+ channel Kv3.1 induced by $A\beta_{1-42}$ in human microglia (Chapter 3) is involved in mediating microglial neurotoxicity. K^+ channels in microglia are believed to participate in K^+ homeostasis and may play a role in cell proliferation/cell differentiation. In particular, 4-AP was shown to inhibit microglial proliferation (Kotecha and Schlichter, 1999). 4-AP non-selectively inhibits different voltage-gated K^+ channels directly regulating membrane potential and cell volume. But 4-AP may also act indirectly by affecting ion transport through other channels, exchangers, and pumps. Our results demonstrate that K^+ channels, $[Ca^{2+}]_i$, p38 MAPK and NF-κB are pivotal signaling proteins/molecules to transduce $A\beta_{1-42}$ actions in microglia. As the results from this study indicate, 4-AP not only inhibits the K^+ channel induced by $A\beta_{1-42}$ (Chapter 3) but as a consequence of this change in membrane potential, alters the phosphorylation of kinases and factors involved in gene transcription, subsequent gene expression and production of inflammatory products in human microglia. These results would indicate that these signaling molecules are somewhat integrated with each other. Moreover, the reciprocal cross-amplification or inhibition between mediators also implies the physiological significance of these signaling factors in the progress of inflammation.

Our principal result in this work is the finding that block of a K^+ channel (likely Kv3.1) is a putative strategy to reduce Aβ-induced inflammation. The present data suggests some degree of clinical efficacy for 4-AP in AD since the agent is permeable to the BBB. However, a major problem in the use of this agent clinically is that it has non-selective actions on other K^+ channels which can cause unwanted side effects. Development of a compound with specificity to block Kv3.1 could serve as a rational strategy to reduce inflammation and slow the progression of the pathology in AD.

Chapter 5: CXCL8 (IL-8) POTENTIATION OF $A\beta_{1-42}$-INDUCED FUNCTIONAL RESPONSES OF HUMAN MICROGLIA

5.1 RATIONALE

The chemokine CXCL8 (IL-8) plays an important role in inflammation. CXCL8 (IL-8) is an autocrine agent for microglia inducing chemotaxis of these cells to sites of injury (Cross and Woodroofe, 1999). This chemokine is also released by microglia (Ehrlich et al., 1998) activated by stimuli such as Aβ (Nagai et al., 2001). Importantly, elevated levels of CXCL8 (IL-8) (Galimberti et al., 2003) and CXCL8 (IL-8) receptors (Xia et al., 1997) have been detected in AD brain. In a detailed analysis of gene expression profiling of $A\beta_{1-42}$ stimulated post-mortem adult human microglia isolated from non-demented individuals, CXCL8 (IL-8) was observed to be strongly upregulated (Walker et al., 2001). These results suggest that CXCL8 (IL-8) may play a role in the network of inflammatory responses which contribute to the pathogenesis of AD.

In the present study, I have investigated the effects of CXCL8 (IL-8) on $A\beta_{1-42}$-induced expression of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, the inflammatory enzyme COX-2 and the anti-inflammatory cytokines IL-10, $TGF\beta_1$. In addition, in cases where expression were altered, I then examined CXCL8 (IL-8) as a modulator of $A\beta_{1-42}$ actions to alter production of the agents.

5.2 RESULTS

5.2.1 Effect of $A\beta_{1-42}$, CXCL8 (IL-8) Alone and Combined on Morphology of Human Microglia

Morphologically, unstimulated microglia are typically ramified indicative of a resting state (Ling and Wong, 1993) (Figure 5-1A). Stimulation with CXCL8 (IL-8) for 24 hrs had little or no effect on cell morphology as indicated in Figure 5-1B with a majority of cells ramified in morphology. After 24 hrs incubation with aggregated $A\beta_{1-42}$ (5 μM) (Figure 5-1C), microglia showed an ameboid (activated) phenotype characterized by attenuated processes, swelling and presence of intracellular vacuoles (Walker et al., 2001). A similar activated morphology was observed after 24 hrs incubation with $A\beta_{1-42}$ and CXCL8 (IL-8) (100 ng/mL) combined (Figure 5-1D). Morphology of microglia stimulated for 24 hrs with reverse peptide $A\beta_{42-1}$ (5 μM) was similar to that of unstimulated microglia (Figure 5-1E). Addition of $A\beta_{42-1}$ in combination with CXCL8 (IL-8) had little or no effect to alter the ramified morphology of microglia (Figure 5-1F) as was observed with CXCL8 (IL-8) stimulation alone (Figure 5-1B).

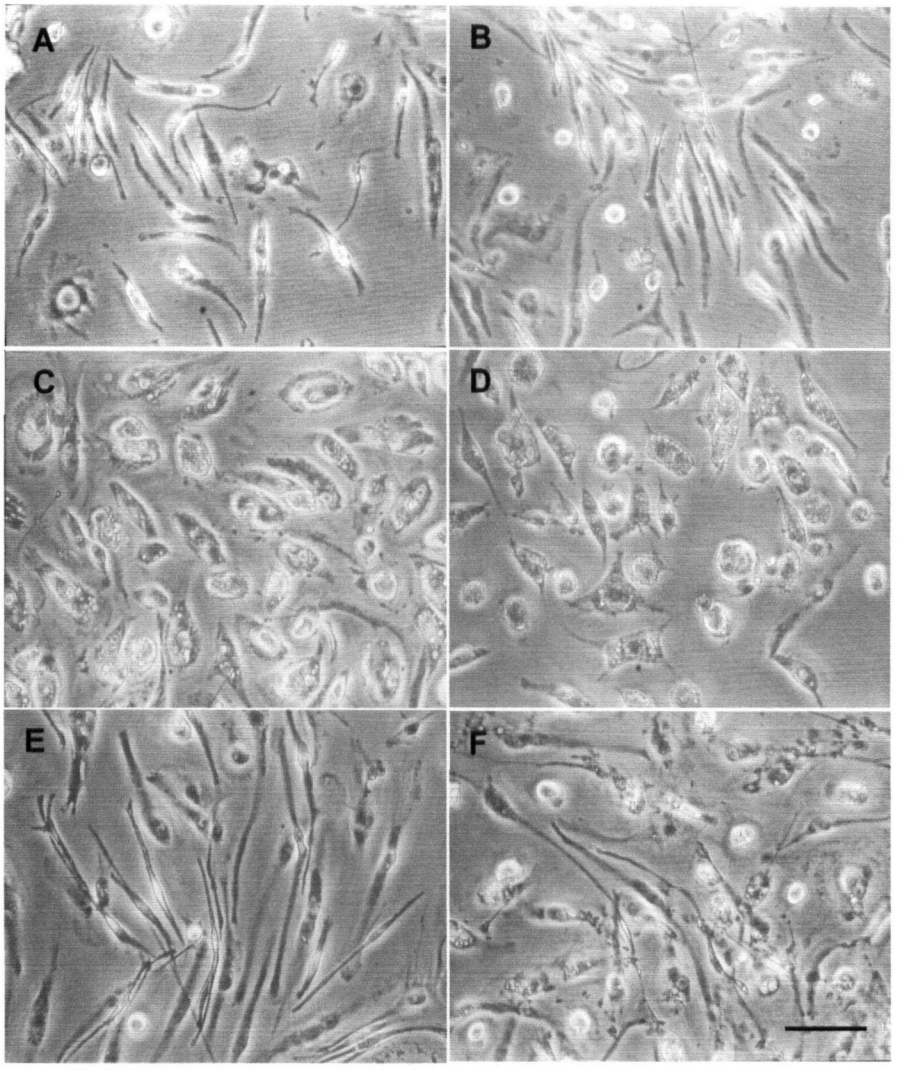

Figure 5-1. Effects of $A\beta_{1-42}$, CXCL8 (IL-8) each alone or in combination on morphology of human microglia

Phase contrast images of human microglia after 24 hrs in (A) unstimulated conditions (B) CXCL8 (IL-8) (100 ng/mL) (C) $A\beta_{1-42}$ (5 μM) (D) $A\beta_{1-42}$ and CXCL8 (IL-8) combined (E) $A\beta_{42-1}$ and (F) $A\beta_{42-1}$ and CXCL8 (IL-8) combined. (x100 magnification; scale bar = 50 μM in A-F).

5.2.2 Effects of CXCL8 (IL-8) on $A\beta_{1-42}$-Induced Expression of Pro-inflammatory Mediators

I next investigated the expression of pro- and anti-inflammatory cytokines, CXCL8 (IL-8) and COX-2 in microglia stimulated for 8 hrs with $A\beta_{1-42}$ or CXCL8 (IL-8) separately or both in combination. A single time point of 8 hrs was chosen for RT-PCR analysis since at this time point, optimal expression of inflammatory mediators was induced by $A\beta_{1-42}$ in human microglia. Representative results with $A\beta_{1-42}$, CXCL8 (IL-8) and combined $A\beta_{1-42}$ and CXCL8 (IL-8) on inflammatory mediator expression are shown in Figure 5-2. Unstimulated human microglia did not express IL-1β, IL-6, TNF-α, or COX-2 constitutively, however, CXCL8 (IL-8) was expressed under basal conditions (Figure 5-2A). Stimulation with $A\beta_{1-42}$ or CXCL8 (IL-8) for 8 hrs induced the expression of IL-1β, IL-6, CXCL8 (IL-8), TNF-α and COX-2. Incubation of human microglia with $A\beta_{1-42}$ in the presence of CXCL8 (IL-8) led to enhanced expression of all pro-inflammatory factors as compared to stimulation with either $A\beta_{1-42}$ or CXCL8 (IL-8). Treatment with reverse peptide $A\beta_{42-1}$ alone (5 μM) had no effect on expression of pro-inflammatory factors compared to control. Furthermore, $A\beta_{42-1}$ did not alter CXCL8 (IL-8) induced expression of pro-inflammatory mediators when $A\beta_{42-1}$ was co-applied with CXCL8 (IL-8).

Effects of $A\beta_{1-42}$, CXCL8 (IL-8) and of $A\beta_{1-42}$ and CXCL8 (IL-8) combined on anti-inflammatory cytokine expression were also examined (Figure 5-2B). The anti-inflammatory cytokines IL-10 and $TGF\beta_1$ were constitutively expressed under unstimulated conditions and were unaffected by $A\beta_{1-42}$ or CXCL8 (IL-8) alone, $A\beta_{1-42}$

and CXCL8 (IL-8) combined, $A\beta_{42\text{-}1}$ alone or $A\beta_{42\text{-}1}$ in combination with CXCL8 (IL-8). G3PDH served as a reaction standard (Figure 5-2C).

Densitometry analysis of PCR product band intensities indicate a similar effect of CXCL8 (IL-8) to enhance $A\beta_{1\text{-}42}$ effects to increase pro-inflammatory mediator expression with no effects on anti-inflammatory cytokine expression. Results are summarized in Figure 5-2D. $A\beta_{1\text{-}42}$ and CXCL8 (IL-8) each alone significantly increased relative mRNA levels of the pro-inflammatory mediators as compared to control ($p < 0.05$). CXCL8 (IL-8) in the combined presence of $A\beta_{1\text{-}42}$ significantly increased relative mRNA levels of all pro-inflammatory mediators as compared to $A\beta_{1\text{-}42}$ stimulated levels ($p < 0.05$). Fold increases in relative pro-inflammatory mediator mRNA as a result of $A\beta_{1\text{-}42}$, CXCL8 (IL-8) and combined $A\beta_{1\text{-}42}$ and CXCL8 (IL-8) stimulation compared to levels in control is summarized in Table 5.1. Overall, the fold increases in pro-inflammatory mediators induced by $A\beta_{1\text{-}42}$ and CXCL8 (IL-8) each alone compared to control were: IL-1β: 3.4, 2.7; IL-6: 6.9, 12.2; CXL8 (IL-8): 1.9, 1.3; TNF-α: 3.2, 1.3; COX-2: 3.0, 2.3. The fold increase in pro-inflammatory mediators induced by $A\beta_{1\text{-}42}$ and CXCL8 (IL-8) combined compared to $A\beta_{1\text{-}42}$ alone were: IL-1β: 1.7; IL-6: 2.4; CXCL8 (IL-8): 1.4; TNF-α: 1.8; COX-2: 2.4. Application of $A\beta_{42\text{-}1}$ had no effect to alter relative mRNA levels of pro-inflammatory mediators and did not alter relative mRNA levels induced with CXCL8 (IL-8) when applied in combination (Figure 5-2A). Relative mRNA levels of the anti-inflammatory cytokines IL-10 and $TGF\beta_1$ were unchanged from basal levels despite treatment with stimuli (Figure 5-2D).

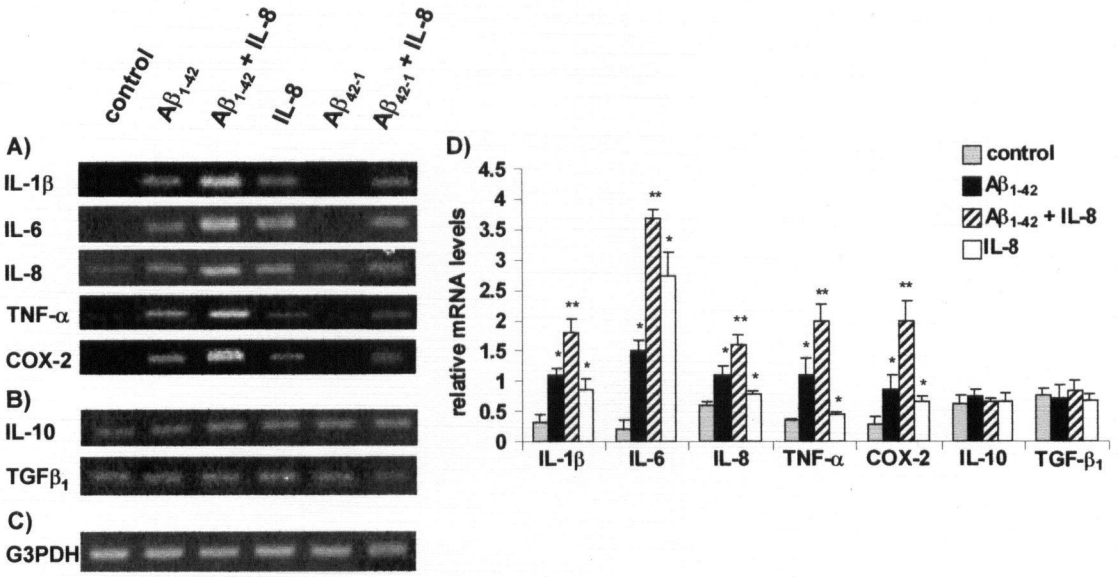

Figure 5-2. Effects of CXCL8 (IL-8) on $A\beta_{1-42}$-induced pro inflammatory mediator and anti-inflammatory cytokine expression in human microglia

Expression of (A) TNF-α, IL-6, IL-1β, CXCL8 (IL-8), COX-2 and (B) anti-inflammatory cytokines were examined in microglia incubated for 8 hrs with $A\beta_{1-42}$, CXCL8 (IL-8), $A\beta_{1-42}$ in the combined presence of CXCL8 (IL-8), or with medium alone. Stimulation of microglia with $A\beta_{42-1}$ (5 μM) alone or in combination with CXCL8 (IL-8) served as control experiments. The results shown are a representative of five independent experiments. (C) The expression of G3PDH served as a reaction standard. (D) Summary of relative mRNA levels of inflammatory mediators induced by $A\beta_{1-42}$, CXCL8 (IL-8) and combined $A\beta_{1-42}$ and CXCL8 (IL-8). Results are expressed as mean ± SEM from n=5 independent experiments. One-way ANOVA and Newman-Keuls multiple comparison post-test was performed to evaluate statistical significance ($p < 0.05$) (* indicates statistically significant from control; ** indicates statistically significant from $A\beta_{1-42}$ stimulated levels).

Table 5-1. Fold increases in relative pro-inflammatory mediator mRNA induced by $A\beta_{1-42}$, CXCL8 (IL-8), $A\beta_{1-42}$ + CXCL8 (IL-8) compared to relative mRNA in control

	$A\beta_{1-42}$	IL-8	$A\beta_{1-42}$+ IL-8
IL-1β	3.4*	2.7*	5.6***
IL-6	6.9**	12.2***	16.8***
IL-8	1.9*	1.3*	2.7***
TNF-α	3.2*	1.3*	5.7***
COX-2	3.0*	2.3*	6.9***

* $p< 0.05$; **$p<0.01$; ***$p<0.001$

5.2.3 Effects of CXCL8 (IL-8) on $A\beta_{1-42}$-Induced Production of Pro-inflammatory Mediators

I further investigated whether pro-inflammatory factors with increased expression were also increased at the protein level. The production of TNF-α, IL-6, IL-1β and CXCL8 (IL-8) were investigated after 24 hrs stimulation with $A\beta_{1-42}$ in the presence and absence of CXCL8 (IL-8) using ELISA. The time point 24 hrs was chosen since preliminary findings indicated $A\beta_{1-42}$ induced optimal production of cytokines at this time point. Incubations with $A\beta_{1-42}$ for periods longer than 24hrs could induce both direct and indirect effects of the peptide in human microglia (Walker et al., 2001). A summary of results is presented in Figure 5-3.

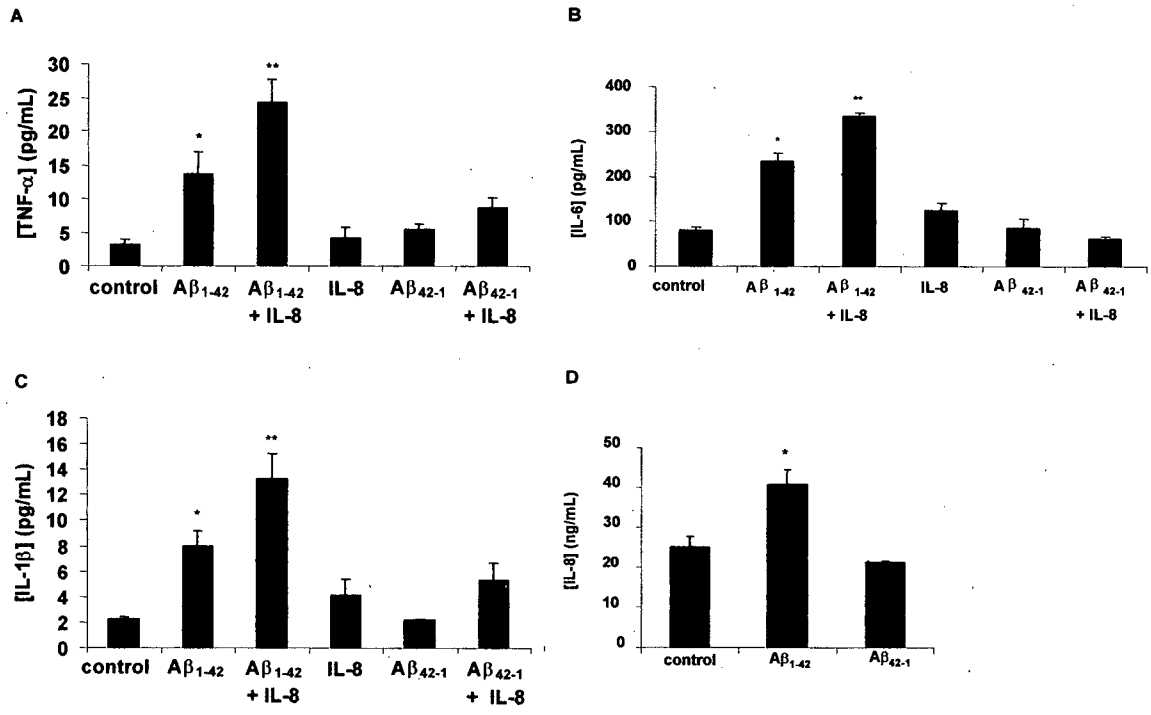

Figure 5-3. Effects of CXCL8 (IL-8), $A\beta_{1-42}$ each alone or in combination on pro-inflammatory mediator production

Effects of $A\beta_{1-42}$, CXCL8 (IL-8) and combined $A\beta_{1-42}$ and CXCL8 (IL-8) on pro-inflammatory cytokine secretion by human microglia using ELISA. Data are mean ± SEM of four independent experiments for (A) TNF-α and three independent experiments for (B) IL-6 (C) IL-1β and (D) CXCL8 (IL-8) each performed in duplicate. Human microglia were exposed to either medium alone, $A\beta_{1-42}$ (5 μM), CXCL8 (IL-8) (100 ng/mL), $A\beta_{1-42}$ in combination with CXCL8 (IL-8), $A\beta_{42-1}$ or to $A\beta_{42-1}$ in combination with CXCL8 (IL-8) for 24 hrs. One-way ANOVA and Newman-Keuls multiple comparison post-test was performed to evaluate statistical significance ($p < 0.05$) (* indicates statistically significant from control; ** indicates statistically significant from $A\beta_{1-42}$ stimulated levels).

Low levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) near the detection limits of the assay were produced by human microglia in serum-free medium under basal conditions. The higher level of IL-6 production as compared to TNF-α and IL-1β by microglia under basal conditions was attributed to the use of low serum (1%) in the IL-6 assay whereas serum-free medium was used in TNF-α and IL-1β assays. Fold increases in pro-inflammatory mediator production with $A\beta_{1-42}$, CXCL8 (IL-8) and of combined $A\beta_{1-42}$ and CXCL8 (IL-8) stimulation compared to control are summarized in Table 5-2. Overall, $A\beta_{1-42}$ (5 μM) alone significantly increased secretion of TNF-α (by 328%) (Figure 5-3A), IL-6 (by 191%) (Figure 5-3B), IL-1β (by 250%) (Figure 5-3C) and CXCL8 (IL-8) (by 60%) (Figure 5-3D) all values $p < 0.01$. CXCL8 (IL-8) in the combined presence of $A\beta_{1-42}$ significantly enhanced additively the levels of TNF-α (by 79%), IL-6 (by 43%) and IL-1β (by 66%) as compared to $A\beta_{1-42}$ stimulated levels ($p < 0.05$). CXCL8 (IL-8) (100 ng/mL) alone also increased IL-1β, TNF-α, and IL-6 as compared to control, however, the increases were not significant ($p > 0.05$). $A\beta_{42-1}$ (5 μM) had no effect to increase secreted levels of IL-1β, TNF-α, IL-6 or CXCL8 (IL-8) compared to unstimulated conditions ($p > 0.05$). Addition of $A\beta_{42-1}$ in combination with CXCL8 (IL-8) did not significantly increase levels of IL-1β, IL-6 and TNF-α as compared to levels induced with CXCL8 (IL-8) alone ($p > 0.05$). Pro-inflammatory cytokine production with $A\beta_{1-42}$ and CXCL8 (IL-8) co-stimulation was additive since the sum of pro-inflammatory cytokine production induced with $A\beta_{1-42}$ and CXCL8 (IL-8) each alone was equivalent to the effects of $A\beta_{1-42}$ and CXCL8 (IL-8) applied in combination.

Table 5-2. Fold increases in pro-inflammatory mediator production induced by $A\beta_{1-42}$, CXCL8 (IL-8), $A\beta_{1-42}$ + CXCL8 (IL-8) compared to levels in control

	$A\beta_{1-42}$	IL-8	$A\beta_{1-42}$+IL-8
IL-1β	3.5*	2.0	5.8**
IL-6	2.9*	1.6	4.2**
IL-8	1.7*		
TNF-α	4.3*	1.3	7.7**

***p < 0.01; **p < 0.001**

The production of COX-2 after stimulation with $A\beta_{1-42}$, CXCL8 (IL-8) or $A\beta_{1-42}$ and CXCL8 (IL-8) combined was determined using immunocytochemistry (Figure 5-4). Over the number of COX-2 positive microglia was determined from four representative fields in three independent experiments. $A\beta_{1-42}$ significantly increased the percentage of microglia expressing COX-2 by 230% from control levels ($p < 0.001$) (Figure 5-4B). CXCL8 (IL-8) increased COX-2 production (Figure 5-4A) compared to unstimulated cells but the increases were not significant ($p > 0.05$). Co-addition of CXCL8 (IL-8) in the presence of $A\beta_{1-42}$ enhanced additively the percentage of COX-2 positive microglia (by 71%) as compared to $A\beta_{1-42}$ levels and the effect was significant ($p < 0.001$). $A\beta_{42-1}$ also increased the percentage of COX-2 positive microglia compared to unstimulated levels but this effect was not significant ($p > 0.05$). Reverse peptide, $A\beta_{42-1}$ applied in combination with CXCL8 (IL-8) increased COX-2 production as compared to levels induced with CXCL8 (IL-8) alone but the increase was not significant ($p > 0.05$).

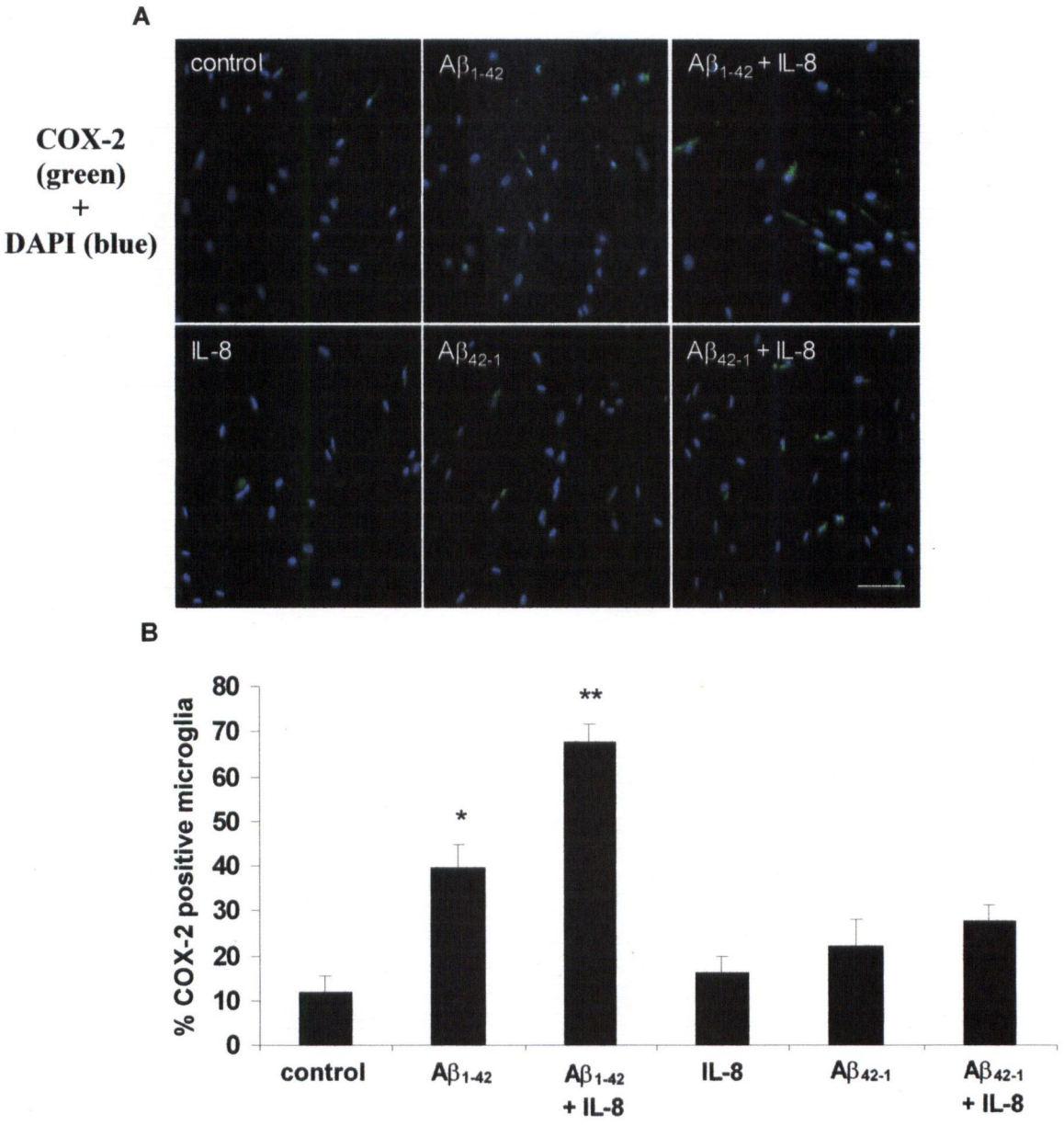

Figure 5-4. Effects of $A\beta_{1-42}$, CXCL8 (IL-8) each alone or in combination on COX-2 expression in human microglia

(A) Representative figures of COX-2 positively stained microglia (green) and nuclei of cells (blue). (B) The percentage of COX-2 positive microglia were measured in microglia after 24 hr incubation with 5 μM $A\beta_{1-42}$, 100 ng/mL CXCL8 (IL-8) alone or in combination with $A\beta_{1-42}$, $A\beta_{42-1}$ alone or in combination with CXCL8 (IL-8) or with medium alone and data presented as mean ± SEM. Significance was determined using one-way ANOVA and Newman-Keuls multiple comparison post-test ($p < 0.05$) (* indicates statistically significant from control; ** indicates statistically significant from $A\beta_{1-42}$ stimulated levels). Scale bar = 50 μm.

5.3 CONCLUSION

The principle novel finding of this aspect of my study is that CXCL8 (IL-8) enhances $A\beta_{1-42}$-induced human microglial expression and secretion of pro-inflammatory mediators. Stimulation with $A\beta_{1-42}$ and CXCL8 (IL-8) together led to significantly enhanced expression (Figure 5-2A) and additive effects to increase production (Figure 5-3, 5-4) of IL-6, IL-1β, TNF-α and COX-2 as compared to levels with $A\beta_{1-42}$ applied alone. Importantly, expression of the anti-inflammatory cytokines IL-10 and $TGF\beta_1$ were unchanged by microglial treatment with peptide or peptide plus CXCL8 (IL-8) (Figure 5-2B). Overall, the results suggest that CXCL8 (IL-8) chemokine activity could enhances additively $A\beta_{1-42}$-induced pro-inflammatory responses mediated by activated microglia in AD brain (see below).

Overall, the results from semi-quantitative RT-PCR analysis for expression (Figure 5-2) and ELISA analysis for production (Figure 5-3) showed reasonable agreement for CXCL8 (IL-8) enhancement of $A\beta_{1-42}$ stimulation of the inflammatory mediators. However, the results in Fig. 5-2A also show CXCL8 (IL-8) alone could induce expression of IL-6, TNF-α, IL-1β, CXCL8 (IL-8) and COX-2. Interestingly, the changes in expression of the pro-inflammatory cytokines and COX-2 by CXCL8 (IL-8) alone were not accompanied by increased production of the agents (Figure 5-3A-C; 5-4). On the basis of preliminary findings, optimal expression of the pro-inflammatory cytokines were evident with 8 hrs stimulation of microglia, however, at this time point little or no production of cytokines were observed. For production of cytokines, microglia were stimulated for 24 hrs in accord with previous studies using human microglia (Lee et al., 1993). Differences between CXCL8 (IL-8) effects on expression

and production could also reflect tight control of post-transcriptional events in microglia since previous work has shown similar behaviour for this chemokine applied to human neutrophils (Martinez et al., 2004).

A previous study has reported that amyloid peptide elicited functional responses in monocytes via a distinct signaling pathway from that induced by chemokines (Badolato et al., 1995). Our results are consistent with separate pathways for CXCL8 (IL-8) and $A\beta_{1-42}$-induced stimulation of human microglia. As shown in Figure 5-3A-C (for cytokines) and Figure 5-4 (for COX-2), CXCL8 (IL-8) applied in combination with $A\beta_{1-42}$ produced additive responses. An interesting possibility is that an autocrine feedback system may play a role in CXCL8 (IL-8) potentiation of $A\beta_{1-42}$ effects. In this case, $A\beta_{1-42}$ mediated release of CXCL8 (IL-8) (Fig. 5-3D) could stimulate microglia since receptors for this chemokine have been reported in these cells (Lee et al., 2002).

As evident in Figure 5-1A, human microglia exposed to serum-free medium exhibit a general profile of ramified morphology. After exposure of human microglia to $A\beta_{1-42}$, cells show a trend to ameboid morphology (Figure 5-1C). This result is consistent with results from previous work where treatment of microglia with $A\beta_{1-42}$ caused a shift from ramified to ameboid shape (Walker et al., 2001). Interestingly, in the present study I observed no changes in cell morphology after exposure to CXCL8 (IL-8) treatment where cells exhibited a predominant ramified shape similar to unstimulated conditions (Figure 5-1B). Since CXCL8 (IL-8) induced the expression of cytokines, our results suggest some dissociation between morphology and priming effects of CXCL8 (IL-8). This point is important since ramified and ameboid morphologies have been considered as indicative of resting and activated states of microglia (Ling and Wong, 1993).

Previous work has demonstrated $A\beta_{1-42}$-induced microglial expression of pro-inflammatory cytokines (Lue et al., 2001a; Walker et al., 1995; 2001) and COX-2 (Hoozemans et al., 2001; 2002b). Reports by Lee et al., 2002 and Nagai et al., 2001 indicate that unstimulated human microglia in a high serum (5 % horse serum) containing medium express basal levels of IL-1β, IL-6 and TNF-α and a prominent ameboid morphology. Since I found no basal expression of pro-inflammatory cytokines and a ramified morphology in control, it seems likely that differences could reflect our use of serum-free medium. The lack of effect of stimuli including amyloid peptide on expression of the anti-inflammatory cytokines IL-10 and $TGF\beta_1$ in microglia (Figure 5-2B) in the present study are in agreement with previous reports (Meda et al., 1999; Walker et al., 2001). These results would indicate that although microglia exhibit a pro-inflammatory profile as a result of amyloid peptide stimulation in the presence or absence of CXCL8 (IL-8), their anti-inflammatory properties are preserved. Enhancement of Aβ responses has also been reported previously with the agents interferon-γ (Meda et al., 1996), macrophage colony stimulating factor (M-CSF) (Murphy et al., 1998), C1q and serum amyloid P (Veerhuis et al., 2003). However, available evidence indicates that microglia produce little or no amounts of these factors (McGeer and McGeer, 1995; Yasojima et al., 2000; Lue et al., 2001a).

Our results have particular significance to inflammatory responses mediated by microglia in AD. CXCL8 (IL-8) has been observed as the most prominent factor expressed by adult human microglia stimulated with $A\beta_{1-42}$ (Walker et al., 2001) and CXCL8 (IL-8) is highly elevated in AD brain (Galimberti et al., 2003). Taken together, these findings suggest that autocrine release of CXCL8 (IL-8) from microglia enhances

$A\beta_{1-42}$ stimulation of cells and that this cytokine could function to exacerbate inflammatory responses in AD brain. The inhibition of CXCL8 (IL-8) actions could constitute an effective strategy for therapeutic intervention in slowing the progression of AD.

Chapter 6: DISCUSSION OF THESIS RESEARCH AND FUTURE DIRECTIONS

The studies presented in this thesis focused on determining the effects of acute and chronic treatment with full length, $A\beta_{1-42}$, on intracellular signaling pathways and functional responses of microglia using electrophysiology, calcium spectrofluorometry, RT-PCR, ELISA, immunocytochemistry and immunohistochemistry techniques. The hypothesis of my research was that inhibition of $A\beta_{1-42}$-induced microglial mediated intracellular signaling pathways would downregulate inflammatory functional responses of microglia which could serve as a therapeutic strategy for AD. The results presented in this thesis support this hypothesis.

I first investigated the effects of $A\beta_{1-42}$ on human microglial membrane potential and membrane current expression using electrophysiology. $A\beta_{1-42}$ acutely applied to human microglia induced the expression of a novel outward K^+ current which was sensitive to the non-selective K^+ channel blocker 4-aminopyridine (4-AP). A current similar in properties was observed with intracellular application of the non-hydrolyzable analogue of GTP, GTPγS. This would suggest that the outward K^+ current induced by acute $A\beta_{1-42}$ was induced via a G protein. Molecular biology studies indicated that the K^+ channel induced by $A\beta_{1-42}$ was likely due to Kv3.1. Also, $A\beta_{1-42}$ increased the expression of the FcγII receptor. Other studies using electrophysiology indicated that acute application of $A\beta_{1-42}$ induced a transient depolarization in human microglia; inhibition of the FcγII receptor inhibited this depolarization suggesting a link between the FcγII receptor and $A\beta_{1-42}$ effects on cell potential. From these studies, I established that $A\beta_{1-42}$

alters membrane current expressions in microglia and that 4-AP could serve as a potential modulator of $A\beta_{1-42}$-induced microglial inflammatory and neurotoxic responses.

I then examined the effects of 4-AP on $A\beta_{1-42}$ induced Ca^{2+} responses in human microglia using the calcium spectrofluorometry technique. I was able to show that $A\beta_{1-42}$ induces an increase in $[Ca^{2+}]_i$ through an unidentified influx pathway. 4-AP inhibited this Ca^{2+} influx pathway activated by acute $A\beta_{1-42}$. These result would indicate that the unidentified Ca^{2+} influx pathway induced by acute $A\beta_{1-42}$ in human microglia is sensitive to membrane potential.

Using a series of *in vitro* (RT-PCR, ELISA, immunocytochemistry) and *in vivo* assays (immunohistochemistry), the effects of $A\beta_{1-42}$ on functional responses of human microglia including potential neurotoxicity was investigated. *In vitro*, I was able to show that 4-AP inhibited $A\beta_{1-42}$ effects to activate p38 MAP kinase, NFκB, the expression and production of pro-inflammatory cytokines IL-1β, IL-6, TNF-α, the chemokine CXCL8 (IL-8) and inducible enzyme COX-2 as well as microglial mediated neurotoxicity. More importantly, 4-AP reduced neuronal damage and microglial activation induced by $A\beta_{1-42}$ *in vivo* which strongly supports the *in vitro* actions of 4-AP as a modulator of $A\beta_{1-42}$ mediated pro-inflammatory responses. These results suggest that 4-AP modulates $A\beta_{1-42}$-induced intracellular signaling pathways and functional responses in human microglia including microglial-mediated neurotoxicity.

The final set of studies examined the effects of the chemokine CXCL8 (IL-8) on $A\beta_{1-42}$-induced expression and production of the pro-inflammatory cytokines IL-6, IL-1β, TNF-α, the inducible enzyme COX-2 and chemokine CXCL8 (IL-8). Microglial treatment with CXCL8 (IL-8) added with $A\beta_{1-42}$ led to enhancement in both expression

and production of all of these pro-inflammatory factors compared with peptide alone. The expression of the anti-inflammatory cytokines IL-10 and $TGF\beta_1$ remained unchanged from basal levels with stimulation using either $A\beta_{1-42}$, CXCL8 (IL-8) or the peptide together with CXCL8 (IL-8). Since CXCL8 (IL-8) is elevated in AD brain and highly expressed by Aβ stimulated microglia, these results would suggest that other factors such as CXCL8 (IL-8) could be acting in concert with $A\beta_{1-42}$ to additively enhance inflammatory responses by microglia in AD.

The results summarized above illustrate that inflammatory stimuli such as $A\beta_{1-42}$ have profound signaling effects on microglia and that modulating these intracellular signaling pathways can either inhibit (as shown with 4-AP) or potentiate (as shown with CXCL8 (IL-8)) the pro-inflammatory effects of microglia. These results offer further insight into the interactions that microglia have with surrounding stimuli, the importance of particular intracellular signaling pathways in mediating microglial responses and most importantly, how modulation of these signaling pathways inhibits microglial inflammatory responses. The significance of these results is that modulating microglial mediated inflammatory functional responses to stimuli such as $A\beta_{1-42}$ with agents that inhibit intracellular signaling pathways could serve as a therapeutic strategy for use in neurodegenerative diseases such as AD.

Results from this thesis work indicate that 4-AP would be a suitable candidate for the treatment of AD. 4-AP has favourable properties over other K^+ channel inhibitors since it is able to cross the BBB. It should be noted that 4-AP has been used clinically for the treatment of AD with some positive results. However, careful consideration must be taken in its use clinically since 4-AP has unwanted side effects most notably at higher

doses. Thus, 4-AP could be used at low doses or as an adjunctive therapy in the treatment of AD. Further studies on the potential use of specific K^+ channel blockers, such as a specific blocker of Kv3.1, in inflammatory diseases of the CNS should also be considered.

Future research could also be carried out to determine the nature of the Ca^{2+} influx pathway induced by $A\beta_{1-42}$ in human microglia. It would also be of interest to determine the role of the FcγRII receptor in mediating microglial inflammatory responses and whether inhibition of the FcγRII receptor could serve as potential modulator of microglial functional responses. Since several intracellular factors are implicated in linking $A\beta_{1-42}$ to functional responses of microglia including $[Ca^{2+}]_i$, p38 MAPK and NFκB, each of these factors could serve as potential modulatory sites of microglial functional responses. Furthermore, an inhibitor of CXCL8 (IL-8) could be used as a modulator of microglial mediated inflammatory responses in AD. Moreover, the potential modulatory actions of combination therapies i.e. a K^+ channel inhibitor in the presence of another modulator of $A\beta_{1-42}$-induced intracellular signaling, could be investigated using *in vitro* assays of $A\beta_{1-42}$-induced microglial mediated inflammatory responses as well as in transgenic AD animals.

The positive results reported in this thesis of modulating microglial mediated inflammatory responses both *in vitro* and *in vivo* by using an inhibitor of stimulus-induced signaling will hopefully lead to further studies on potential modulators of intracellular signaling pathways in microglia. Ultimately, it is hoped that potential modulators of microglial mediated inflammatory responses with significant beneficial effects *in vitro* and *in vivo* will be developed for application in a clinical setting.

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