CELLULAR SIGNALING OF HUMAN MICROGLIA IN RESPONSE TO β-AMYLOID 1-40

by

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B.Sc., Washington State University, (U.S.A.) 1998

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Pharmacology and Therapeutics, Graduate Program in Neuroscience)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 2001

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Department of Program in Neuroscience

The University of British Columbia
Vancouver, Canada

Date Oct 2, 2001
Abstract

Microglia are resident immune cells of the brain that are activated in response to trauma and inflammation. Activated microglia exhibit characteristics similar to peripheral macrophages, such as the expression of immunomolecules, secretion of pro-inflammatory substances and phagocytic activity. Like macrophages, microglia exhibit these characteristics in order to defend the brain from infection and aid in the repair of damaged tissue. However, in Alzheimer’s Disease (AD) microglia can become over-activated resulting in the release of substances that escalate inflammation and ultimately cause neuronal death.

A protein implicated in the progression of AD is β-amyloid (Aβ). Aβ production is increased in AD and deposits of Aβ form throughout the brain, which are correlated to the activation of microglia and neuronal death. Studies have shown that Aβ can activate microglia and cause changes in the cellular functions of these cells. For example, in microglia Aβ has been shown to cause increases in the production of pro-inflammatory cytokines and reactive oxygen species.

The objective of this work was to characterize the actions of Aβ40, a commonly expressed form of Aβ, on the mobilization of intracellular calcium ([Ca$^{2+}$]) in human microglia. The rational was that subsequent pharmacological modulation of the calcium signals induced by Aβ40 could then be used to alter the cellular functions of microglia, such as the secretion of neurotoxic factors. The first study used calcium sensitive micro-fluorescence to examine Aβ40 actions on [Ca$^{2+}$]i mediated signaling pathways. Aβ40 application (4 and 10 μM) to microglia caused a rise in [Ca$^{2+}$]i to a plateau level which was sustained following the removal of the peptide. Calcium-free external solution (Ca-
free PSS) was used to show that the primary contribution to the \([Ca^{2+}]_i\) rise came from the influx of extracellular calcium. A small amount of intracellular calcium release is also possible since Ca-free PSS did not totally inhibit the Aβ40-induced \([Ca^{2+}]_i\) response. The Aβ40 mediated calcium influx was sensitive to depolarization since low chloride solution applied extracellularly inhibited the influx of calcium. Additional experiments suggested that a store-operated channel (SOC) did not mediate the Aβ40-induced calcium influx since an inhibitor of this pathway, SKF96365, had no effect on the \([Ca^{2+}]_i\) rise. At present, a specific modifier of the Aβ40-induced influx pathway has not been identified.

The next study examined the actions of Aβ40 on COX-2 expression. COX-2 is an enzyme responsible for prostaglandin synthesis and free radical formation that is over-expressed in AD. Microglia cultures were treated with Aβ40 for 24 hrs and the expression of COX-2 was determined through RT-PCR analysis. The results show that Aβ40 significantly increased COX-2 expression. However, it is not know if the enhancement of COX-2 is linked to the Aβ40-induced increase in \([Ca^{2+}]_i\).

The third study examined the potential of Aβ40 to induce the production of neurotoxic substances by human microglia. Neuroblastoma cells were treated with supernatant from microglia exposed to Aβ40 and the neurotoxic effects were evaluated by assessing cell viability. The results indicate that supernatant from Aβ40 treated microglia decreased neuroblastoma viability, however the decrease was not significantly different from Aβ40 applied directly to neuroblastoma cells. This result suggests that a larger number of human microglia are required to record the effects of neurotoxic substances in the cell viability assays used.
The final study was based on preliminary data indicating that \([Ca^{2+}]_i\) signaling mediated by adenosine triphosphate (ATP) and platelet activating factor (PAF) in microglia from AD brain is abnormal. This study examined the actions of chronic Aβ40 treatment (one or two days) on the \([Ca^{2+}]_i\) responses of microglia to ATP and PAF. The results show that chronic Aβ40 treatment of human microglia diminished PAF (but no ATP) responses and also elevated basal \([Ca^{2+}]_i\) levels, suggesting that the peptide perturbs calcium signaling. These results provide a viable \textit{in vitro} model since Aβ40 treatment of human fetal microglia mimics the properties of adult microglia in AD.

In summary, Aβ40 in human fetal microglia induces a rise in \([Ca^{2+}]_i\) due to a SOC independent pathway and also increases the expression of neurotoxic factors such as COX-2. Furthermore, it appears that Aβ40 treatment perturbs calcium signaling in human fetal microglia in a manner similar to what is observed in AD microglia.
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
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<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<td>Aβ</td>
<td>β-amyloid</td>
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<td>Aβ40</td>
<td>β-amyloid 1-40</td>
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<td>Aβ42</td>
<td>β-amyloid 1-42</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>ionic calcium</td>
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<tr>
<td>cDNA</td>
<td>complementary-deoxyribonucleic acid</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<td>Ca-free PSS</td>
<td>calcium free physiological saline</td>
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<td>Ca-PSS</td>
<td>normal physiological saline</td>
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<tr>
<td>[Ca$^{2+}$]_i</td>
<td>intracellular calcium</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>Fura 2 AM</td>
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<td>GAPDH</td>
<td>glyceraldehydes-3-phosphate dehydrogenase</td>
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<td>hrs</td>
<td>hours</td>
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<td>low Cl PSS</td>
<td>low chloride physiological saline</td>
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<tr>
<td>Symbol</td>
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<tr>
<td>mg</td>
<td>milligrams</td>
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<td>mRNA</td>
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<tr>
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<tr>
<td>PAF</td>
<td>platelet activating factor</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase</td>
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<tr>
<td></td>
<td>chain reaction</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SOC</td>
<td>store operated channel</td>
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<td>SKF963651</td>
<td>SKF</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet light</td>
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Acknowledgements

I would first like to thank Dr. McLarnon for his guidance and instruction. I would also like to thank all the other members of the McLarnon laboratory past and present; Sonia Franciosi, Choi Hyun Beom, Clarence Khoo, and Vikram Goghari.

I would also like to thank Dr. Seung U. Kim and all the members of his laboratory for their assistance.

Special thanks to Dr. Nagai and Choi for their help with the PCR work, and Andis Klegeris for his help on the neurotoxicity work, I couldn’t have done it without you guys!

Cosmic thanks goes out to my father, the greatest scientist and man that I know.
1. Introduction

1.1. Microglia

Rio-Hortega's early work in 1932 described the morphology and three functional states of microglia that are known today; the resting or ramified state, an activated phagocytic state and an intermediate state [Haerter-Gebicke 1996, Kreutzberg 1996, Stoll 1999]. These three functional states of microglia are their most striking characteristic and allude to their functional role within the brain. The function of the ramified form of microglia has been overshadowed by the activated morphologies, largely because it is much easier to study the activated states since resting microglia are very sensitive to changes in the extracellular environment, as a consequence they become activated very easily. In other words, due to the cell preparation process needed for the study of microglia in vitro the cells are, for all practical purposes, in a state of low activation and are not in a fully resting state [Haerter-Gebicke 1996, Kato 2000]. However, this low level of activation does not prevent the study of microglial activation or the examination of activated microglia.

The activated phagocytic state of microglia closely resembles circulating monocytes; indeed, microglia express many of the same receptors as macrophages in the blood stream [Gehrmann 1995, Kato 2000, Moore 1996]. This macrophagic behavior of microglia, along with observations of activated microglia at sites of brain injury, first implicated microglia as immunoreactive cells of the central nervous system (CNS) [Kreutzberg 1996, McGeer 1995]. Upon activation microglia exhibit a number of common cellular responses: increased proliferation, localization to the site of injury/stimulation, upregulation of immunomolecules and morphological changes to a more phagocytic phenotype [Gehrmann 1995, Stoll 1999]. These cellular responses of activated microglia bring into question the lineage of these cells. On one hand they appear to be very similar to macrophages of the blood stream; however, microglia also have a ramified
morphology unlike macrophages and they appear to be able to proliferate independent of bone marrow cells [Gehrmann 1995, Haerter-Gebicke 1996]. Regardless of the developmental origin of microglia it is important to stress that microglia are a unique cell type and are not simply peripheral macrophages that cross the blood-brain barrier (BBB) and enter the CNS.

Microglia have a unique role in the brain and are very sensitive to changes in the CNS. As a consequence, a major focus of microglia research centers on signaling pathways of microglia activation. There is a large body of evidence that suggests that increases in the activation state of microglia are associated with neurodegenerative diseases of the brain. A common factor in cellular signaling, and consequently cellular activity, is modulations of intracellular calcium ([Ca$^{2+}$]$_i$).

Calcium does much more than modulate the electrophysiological properties of the cell membrane, proper regulation of calcium is also important for cell signaling and survival [Felder 1994, Verkhratsky 1996]. Across all cell types calcium has been shown to signal modulations in channel and receptor properties, peptide function, and gene transcription [Carafoli, 1999. Ginty 1997]. Thus it makes sense that cells do not rely entirely on membrane channels and pumps for [Ca$^{2+}$]$_i$ regulation. A number of organelles have been shown to contain significant stores of calcium for uptake and release, the two primary ones being the endoplasmic reticulum and mitochondria [Bode 1996]. The calcium stores provided by these organelles are referred to collectively as intracellular stores and they have been shown to play a large role in the maintenance of [Ca$^{2+}$]$_i$ levels.

Microglia are no different in regards to other cell types in their use of calcium in cellular signaling. Indeed, changes in microglia calcium are of particular interest in the study of microglia function since it is thought that the transformation from a resting to an activated state in microglia is governed by changes in gene expression [Bader 1994]. Since the majority of transcription modulators are sensitive to calcium, it would stand to reason that a signal for microglia activation...
could be carried by modulations in intracellular calcium levels [Ghosh 1995, Sola 1999]. Thus research has been directed towards studying the calcium signaling responses induced by substances that are thought to activate microglia. The obvious candidates are substances that initiate inflammatory responses in the brain or are released as a result of cell damage since activated microglia have been observed in these conditions.

Inflammatory initiators such as bacterial lipopolysaccharide (LPS) have been shown to cause changes in basal $[\text{Ca}^{2+}]_i$. Work by Bader et al. (1994) illustrated that LPS causes a transient increase in $[\text{Ca}^{2+}]_i$ in cultured rat microglia. Furthermore, work from the McLarnon laboratory has shown that inflammatory cytokines such as TNF-α and IL-1β, also cause sustained rises of basal $[\text{Ca}^{2+}]_i$ in human microglia [Goghari 2000, McLarnon 2001]. Thus it appears that pro-inflammatory substances that activate microglia also cause changes in the $[\text{Ca}^{2+}]_i$ levels of microglia. Proteins found in neurodegenerative pathologies such as complement and β-amyloid also cause changes in $[\text{Ca}^{2+}]_i$, as do molecules such as adenosine tri-phosphate (ATP) and platelet activating factor (PAF), which are often released in conditions of cell damage and ischemia [Wang 2000, Wang 1999]. These activators of microglia will be discussed in more detail in later sections, their brief mentioning at this point is only to illustrate that changes in microglia $[\text{Ca}^{2+}]_i$ can be caused by a diverse group of activating agents.

Current research suggests that $[\text{Ca}^{2+}]_i$ and the level of microglia activation are closely linked since substances that modulate $[\text{Ca}^{2+}]_i$ also appear to activate microglia. The next section will discuss how the level of microglia activation may be cause for concern in inflammatory diseases of the brain.
1.2. Microglia and Disease

One might assume that since microglia aid in the repair of damaged tissue and the prevention of infection in the CNS, that the activation of microglia is a beneficial event in all cases. However, microglia have been implicated in actively contributing to the development of pathology in a number of neuronal diseases such as multiple sclerosis, HIV dementia, and Alzheimer's disease (AD) [Haerter-Gebicke 1996, Moore 1996]. In these diseases microglia become over-activated and start producing substances that either inhibit proper healing or are directly neurotoxic. It appears that a complex network of activation and inhibition controls the actions of microglia. Thus, chronic activation can trigger a lethal potentiality in microglia, that is usually only reserved for foreign antigens or diseased cells, to spill over onto healthy bystander neurons and glia that surround the area of insult [McGeer 2000].

A primarily example of a breakdown in the normal control mechanisms of microglia is illustrated in the pathology of AD. The dominant theory of AD pathogenesis holds that microglia are not the initiators of the disease but that they contribute to the pathology. Research has shown that one of the initial events in the development of AD is the abnormal processing of the amyloid precursor protein (APP), which leads to the production of abnormally high levels of the β-amyloid protein (Aβ) [Selkoe 1999, Wilson 1999]. APP and Aβ are both constitutively expressed in glia and neurons throughout the brain and are present in normal healthy individuals [Fukumoto 1999, Neve 1998]. Although the function of APP is not clear, knockout mice studies suggest that APP is necessary for development [Seabrook 1999]. The increase in APP processing leads to high concentrations of Aβ that then aggregate into insoluble plaques that form extracellularly throughout the brain [Busciglio 1993, Koo 1999]. Naturally microglia attempt to clear the excess Aβ debris as
would be expected from any resident macrophage; however, the plaques are dense and difficult to metabolize thus it is not possible to clear them quickly enough.

Microglia react to the Aβ plaques on two different levels, one that regards the plaque as extracellular debris, and another level of activation that is specific to the Aβ peptide itself. The premise that microglia react to Aβ as debris is supported by observations that show that microglia react to changes in the extracellular matrix (ECM) [Monning 1995] and also internalize Aβ fibrils [Paresce 1996]. Indeed, the main theory explaining the presence of microglia at amyloid plaques is that they are attempting to clear the lesion [Wisniewski 1998]. In addition, Aβ has been shown to activate microglia directly through cells surface receptors such as the scavenger receptor (SR) and a Clq complement protein receptor [Jiang 1994, Khoury 1996]. The complement-signaling pathway that leads to microglia activation has also been shown to be upregulated in the AD brain [Eikelenboom 1996, Yasojima 1999]. Aβ can activate this pathway because the 1-16 region of Aβ has a high binding affinity to the Clq complement protein; consequently, the Aβ1-16 region of Aβ can bind to the Clq receptors present on microglia and activate the complement-signaling pathway [Jiang 1994]. This may be a major pathway of microglia activation in AD since it has been shown that Clq is one of the most over expressed complement proteins found in AD brain [Yasojima 1999]. Further studies have also suggested that the 10-16 region of Aβ preferentially binds to microglia and induces the induction of a neurotoxin and an increase in phagocytosis [Giulian 1996]. These two pathways, along with the generalized response of microglia to debris would be enough to implicate microglia in AD, but there are still other potentially harmful microglia interactions that occur.

Upon activation microglia secrete a number of substances that amplify the inflammatory response; cytokines increase the general level of activity and recruit other microglia and astrocytes
into the inflammatory response [Kato 2000, Mattson 1997, McGeer 1995] and free oxygen species and other neurotoxic agents kill nearby neurons [Haerter-Gebicke 1996, Gehrmann 1995, Giulian 1995, McGeer 1995]. Neuron death contributes to the inflammatory cascade because dying neurons can release substances such as ATP that have been shown to activate microglia [Moller 2000, Wang 1999]. There is also evidence that healthy neurons secrete substances that inhibit the inflammatory activity of astocytes and microglia [Galoyan 2000]; thus the more neuronal death, the more inflammation, which subsequently leads to increased rates of neuronal death. In addition, the elevation of inflammation and activation of microglia with pro-inflammatory cytokines has been shown to increase the production of cyclooxygenase-2 (COX-2) and subsequent prostanoids, which have the potential to cause neuronal damage through vasoconstriction and the production of oxidative free radical species [Minghetti 1998]. COX-2 is the rate-limiting enzyme in prostaglandin synthesis and it is upregulated in brain inflammation, and in cases of ischemia and AD in humans [Pasinetti 1998, Sairanen 1998]. Microglia also initiate a positive-feedback loop in regards to Aβ since activated microglia have been shown to secrete Aβ as well as iron which facilitates Aβ aggregation and plaque formation [Busciglio 1993, Chung 1999, Halliday 2000]. Thus a cycle of Aβ stimulation, microglia activation, and inflammation develops which is visualized in Figure 1.

In addition to the web of microglia activators, preliminary studies from this laboratory using post-mortem adult microglia from cases of AD suggest that normal microglia responses are impaired in AD. ATP and PAF were used to induce [Ca\textsuperscript{2+}], responses in microglia from confirmed cases of AD and control; in the latter case adult microglia were obtained from individuals with no diagnosis of AD. Preliminary data gained thus far from this study suggest that responses to ATP and PAF in AD microglia are attenuated in comparison to control [unpublished].
Figure 1: The network of interactions in the formation of an autotoxic loop in Alzheimer’s Disease. All inputs in this illustration are excitatory in nature.
ATP and PAF were used as stimuli since previous work has established that rapid transient increases in intracellular calcium occur in response to both agonists and these responses have been well characterized and are highly reproducible [McLarnon 2000, Wang 2000]. These data suggest some component of AD causes microglia to alter their normal response patterns to stimuli. The cause of this change in microglia responsiveness as well as its relationship to the development of AD is currently unknown.

By considering all the inflammatory interactions involved in the pathology of AD it is evident that a relatively small change in Aβ processing can lead to an exponential increase in pathological symptoms and microglia autotoxicity. Therefore it is reasonable to conclude that any effort to control the activity and subsequent inflammation that microglia express in the brain would be of great therapeutic use in treating AD.

1.3. β-Amyloid

As the previous discussion of microglia in disease would indicate, research on the Aβ peptide is currently one of the major lines of study in AD. A pathological feature of the AD brain is the presence of amyloid plaques throughout the cortical and hippocampal region that are often found in the presence of activated microglia and neuronal cell death [Koo 1999, Wilson 1999, Wisniewski 1998]. The primary component of the amyloid plaques is β-amyloid, which is primarily expressed as Aβ40 and Aβ42, which are two different splice variants derived from the amyloid precursor protein (APP) [Koo 1999, Sinha 1999, Wilson 1999]. As previously mentioned, although the function of APP remains unknown, it is clear that APP is necessary in normal development since genetic knockouts of the APP and APP-like gene are lethal [Seabrook 1999, Slunt 1994]. Aβ is produced by differential processing of the APP protein by three different enzymes α, β, and γ secretase [Sinha 1999, Wilson 1999]. Normally α and β secretase cleave the
APP protein and then γ-secretase cleaves the fragments once again to produce Aβ40 and Aβ42 respectively [Citron 1996]. Both Aβ variants are produced in healthy individuals and are thought to play a role in normal development and cellular functioning [Busciglio 1993] and both neurons and glia have been shown to produce APP and Aβ [Fukumoto 1999].

Current work implicates that a dysfunction in APP processing is the causative factor in the development of AD. Mutations of the APP gene have been linked to cases of AD and Down syndrome, which displays plaques and cognitive deficits similar to AD [Koo 1999, Lansbury 1999, Larner 1999]. Mutations in the other two major genetic markers for AD, PS-1 and PS-2 have also enhanced Aβ production in both transfected cells and transgenic animals [Duff 1999, Seabrook 1999, Sinha 1999]. Thus there are many lines of evidence that link Aβ to the development of AD-like pathological symptoms. It is also clear from the earlier discussion of microglia that Aβ is capable of initiating a signaling cascade that ultimately leads to a cycle of microglia activation, neuronal death, and further microglia activation. In order to understand the involvement of Aβ in the escalating cycle of microglia activity and inflammation a brief review of some of the research in the area of Aβ signaling in microglia follows.

1.4. Aβ and Microglia

From the previous discussion of microglia in disease, it is clear that the Aβ peptide is capable of causing changes in microglia function. Aβ has been shown to modulate the secretion of cytokines, receptor expression, properties of ion channels, calcium levels, morphology, gene expression, production of neurotoxic factors, and even the secretion of Aβ by the microglia itself [Araujo 1992, Bader 1994, Bitting 1996, Giulian 1996, Silei 1999, Tan 1999]. In the scope of relating microglia to neurodegeneration most of the work can be put into two general categories;
studies dealing with upstream signaling elements that could lead to microglia activation, and
studies that deal with downstream products of activated microglia.

Studies of the upstream signaling events of Aβ in microglia are exclusively centered on
[Ca^{2+}]_i levels. This can be linked to two separate causal factors. First, the availability of calcium
sensitive dyes such as Fura-2 and Indo has made the study of [Ca^{2+}]_i a relatively simple and
straightforward procedure. Secondly, calcium modulation is the most likely candidate for carrying
the activation signal since many activators of microglia cause changes in [Ca^{2+}]_i levels as well (see
section 1.1). Thus, it is likely that the initial signaling actions of Aβ will be observable in
modulations of [Ca^{2+}]_i in microglia.

One of the first publications to show that Aβ modulates [Ca^{2+}]_i in microglia was from the
Korotzer laboratory. Incubation of an active fragment of the Aβ peptide, Aβ25-35 at 25 μM, in rat
microglia cultures caused a 50% increase in basal [Ca^{2+}]_i levels over a period of one hour when
compared to control conditions without Aβ present [Korotzer 1995]. The same study also observed
that a longer incubation time of 3 and 6 hours with the full-length Aβ peptide (Aβ42) resulted in a
significant change in the basal [Ca^{2+}]_i. However this work did not attempt to determine the
characteristics of the [Ca^{2+}]_i response to Aβ beyond measuring an overall increase when compared
to untreated control cultures. Work by Silei and colleagues (1999) went a step further and
attempted to examine the source of the [Ca^{2+}]_i response. Silei et al. used human fetal microglia
cultures incubated with Aβ25-35 at 40μM for 90 minutes. The results show that Aβ caused an
increase in [Ca^{2+}]_i that was blocked in calcium-free solution suggesting that the Aβ response in
microglia was governed by the influx of calcium from the extracellular solution and not from
intracellular stores of calcium. Furthermore, work from the McLarnon laboratory has shown that a
low concentration of the full-length Aβ peptide (Aβ42) in human microglia at 1 μM is sufficient to
cause large increases in the [Ca\textsuperscript{2+}]i [unpublished]. These studies provide evidence that both full length and active fragments of A\beta have the potential to modulate [Ca\textsuperscript{2+}] levels in microglia with the expectation that this could signal changes in the activation and function of microglia downstream of the initial [Ca\textsuperscript{2+}] response.

Evidence that A\beta exerts actions downstream of the initial [Ca\textsuperscript{2+}] signal is provided by Araujo & Cotman (1992), who showed that pro-inflammatory cytokines are released as a result of A\beta treatment. In this study 10 \mu g/ml of full-length A\beta42 was sufficient to induce a significant increase in IL-1 release in rodent microglia. Furthermore, a number of papers out of the Landreth laboratory provide evidence that A\beta activation of pro-inflammatory cytokines and chemokines causes the initiation of tyrosine kinase and MAP kinase dependent pathways. Work by MacDonald (1998 & 1997) has found that A\beta40 and A\beta25-35 at 50-60 \mu M stimulates tyrosine phosphorylation, ERK, and p38 MAPK expression. Studies from the same laboratory (Combs 1999 & 2000) have also shown that A\beta acting through the same kinase dependent pathways stimulates the production of pro-inflammatory and reactive oxygen species in monocytes and rat microglia that are potentially neurotoxic.

Work from the Giulian laboratory suggests that A\beta signals the production of a unique neurotoxic agent that is secreted by microglia. The results from \textit{in vitro} experiments have shown that 1\mu M of A\beta42 or A\beta40 was sufficient to induce the production of a neurotoxin by microglia [Giulian 1996]. This, as of yet unidentified, neurotoxin induced roughly 75-80% neuronal death. The same concentrations of A\beta, applied without the presence of microglia, resulted in no significant neuronal cell loss. Furthermore, the neurotoxin in question was determined, through a number of molecular assays, to be neither a reactive oxygen species nor a known cytokine [Giulian
1996], which are the two most common secretory products of microglia thought to contribute to neuronal damage.

The studies cited previously provide evidence that Aβ can signal changes in microglia that can account for a number of pathological symptoms that are observed in AD. Aβ has the capacity to activate and induce possible pathological events in microglia through signals carried by changes in \([\text{Ca}^{2+}]\). Once activated by Aβ microglia can then enter a seemingly never ending and ever escalating cycle of inflammation and neuronal death with the production of pro-inflammatory molecules, neurotoxic substances, and increasing amounts of Aβ. If more can be done to understand the signaling pathways that Aβ initiates in microglia then therapeutic approaches can be developed that can treat AD and other inflammatory diseases of the brain. To that end the focus of this thesis is to examine the signaling characteristics of Aβ in human microglia cells.

1.5. Which Aβ to use?

The purpose of this work is to characterize the effects of Aβ on signaling pathways in microglia, however, there are many different Aβ peptides that have been used, as noted from the previous discussion of earlier work in this area. Active fragments of Aβ such as Aβ25-35 are popular since they are relatively easy to use compared with the longer full-length Aβ peptides Aβ40 and Aβ42. However, active fragments of Aβ are not found in the brain of AD inflicted individuals [Neve 1998]. In addition, small active fragments of Aβ do not have the same activating potential as do the full-length peptide because they usually have only one active site. For example, the binding area of the Aβ peptide for both the Clq and the induction of the neurotoxin described by Giulian (1996) is in the 1-16 region so the popular Aβ25-35 fragment would be unable to activate either of these two pathways. Thus it seems premature to limit the activating potential of
Aβ through the use of Aβ fragments until all the pathways of Aβ signaling have been characterized. Therefore, the more common full length Aβ40 and Aβ42 were the only Aβ peptides considered for this thesis since they are the most relevant Aβ peptides concerning the pathology of AD and they have the greatest activating potential.

The majority of AD research, with microglia or otherwise, has focused on Aβ42 since it is thought to be the main Aβ variant found in AD plaques and the more neurotoxic and insoluble of the two Aβ isoforms [Sinha 1999, Wilson 1999]. However, there is some dispute as to whether Aβ is directly neurotoxic and the role of solubility in the activity of the Aβ peptide is also debated. There are a large number of studies that have shown that the Aβ peptide is not directly toxic to neurons since neurons can be grown in the presence of Aβ peptides [Wujek 1996], plaque and Aβ peptides directly added to neurons do not cause cell damage [Giulian 1996], and the infusion of Aβ peptides into the brain does not cause neuronal damage [Games 1992]. Thus it appears that at the level of direct neurotoxic activity there is no preference for either Aβ variant.

Many researchers also question the relevance of the solubility factor of the Aβ peptide. Giulian's work on the microglia neurotoxin has shown Aβ40 and Aβ42 to be equally effective in causing the production of a microglia-derived neurotoxin [Giulian 1996]. Further work by Giulian illustrated that the beta-pleated sheet structure, which is formed by aggregated Aβ, is unnecessary in inducing the microglia neurotoxin. These studies support the view that microglia respond to specific binding domains of the Aβ peptide that are, at least in the case of the neurotoxin and complement pathway, present in both the Aβ40 and Aβ42 peptide. Thus it appears that Aβ40 is as likely a candidate in signaling pathological changes in microglia as Aβ42. Overall the choice
between using either of the two peptides comes down to the issues of solubility, specificity, and practicalities of use.

The goal of this thesis is to examine the effects of Aβ signaling in microglia. To truly look at Aβ signaling an effort must be made to ensure that responses elicited are specific to the Aβ peptide. Since Aβ42 is more insoluble and more likely to form aggregates in solution there is a chance that any result obtained could be clouded by more generalized responses of microglia in their attempt at clearing the amyloid plaques. Furthermore, the relative insolubility of Aβ42 also introduces solvents that are not present in the brain that could confound any results obtained. This could result in a lack of reproducibility in data obtained using the Aβ42 peptide variant. In addition, communications with researchers at Astra Zeneca, in preparation for a possible collaborative project, advised the use of Aβ40 based on a greater degree of reproducibility with its use in their research protocols. Thus, due to the insolubility of Aβ42 and the possible complications that working with the peptide could present, it was decided that Aβ40 would be the peptide studied in this thesis work.

In summary, Aβ40 was used because it is physiologically relevant in the study of AD and has been shown to be a potent activator of microglia. Furthermore, the relative solubility of Aβ40 has the potential to produce more reproducible results than Aβ42 since there is less chance of non-specific effects due to peptide aggregation. The goal of this thesis was to characterize the actions of Aβ40 over a wide spectrum of human microglia properties that address both signaling pathways and cellular functions. The specific goals of this thesis are outlined in four separate aims.
1.6. Study Aims

1.6.1. Aim 1 / Study 1: To determine if acute application of Aβ40 induces changes in [Ca\(^{2+}\)]\(_i\) in human microglia and to determine the characteristics of the Aβ40 induced [Ca\(^{2+}\)]\(_i\) response.

The focus of this Aim is the characterization of changes in [Ca\(^{2+}\)]\(_i\) that occur as a result of Aβ40 mediated signaling in microglia. As mentioned previously, changes in [Ca\(^{2+}\)]\(_i\) have the potential to induce microglia activation. Although previous studies have shown that active Aβ fragments can cause changes in [Ca\(^{2+}\)]\(_i\) in rodent and human microglia [Araujo 1992, Combs 2000, Giulian 1996, Korotzer 1995], no previous published work has examined the effects of Aβ40 on [Ca\(^{2+}\)] in human microglia. Moreover, it is important to note that previous work examining changes in [Ca\(^{2+}\)]\(_i\) due to Aβ have used very high concentrations of Aβ. For example, levels of Aβ40 in the 40-60 μM range were routinely used [Combs 2000, MacDonald 1998] when other studies have illustrated that full-length Aβ at 1μM is sufficient in altering microglia function [Gulian 1996]. Furthermore, preliminary work with Aβ42 in the McLarnon laboratory has also shown that Aβ42 applied acutely at 1μM is sufficient in inducing a rise in [Ca\(^{2+}\)]\(_i\) in human microglia. Due to this, Aβ40 at 4 and 10 μM concentrations were used and specific protocols were applied in order to characterize the mechanisms underlying the response induced by the peptide.

The examination of the Aβ40 induced calcium response is important because the characteristics and properties of the [Ca\(^{2+}\)]\(_i\) response can contribute to understanding the mechanisms by which Aβ signals functional changes in microglia. This work is also important because no published research has yet examined the effect of Aβ40 on [Ca\(^{2+}\)]\(_i\) levels in human microglia. Furthermore, determining how microglia respond to Aβ will contribute to the
understanding of microglia activation and the role of Aβ and microglia in inflammatory diseases such as AD.

1.6.2. **Aim 2 / Study 2:** To determine if Aβ40 treatment of human microglia results in the upregulation of the COX-2 enzyme.

In the first Aim the focus was on initial signaling events caused by Aβ40 application to human microglia. This Aim’s focus is on functional actions of Aβ40 downstream of the [Ca$^{2+}$]$_i$ response. As outlined in the discussion of microglia and disease in section 1.2., COX-2 has been implicated in inflammation and has also been shown to be upregulated in AD. These studies suggest that COX-2 expression may be one pathway in which an autotoxic inflammatory response, as seen in neurodegenerative diseases such as AD, is perpetuated. Furthermore, although it has been shown that COX-2 is upregulated in neurons in AD and Aβ40 treatment of neuroblastoma [Pasinetti 1998], no study has examined Aβ40 induced COX-2 expression in microglia cells. Thus the goal is to determine if human microglia treated with Aβ40 upregulate COX-2 expression. If a specific modulator of the Aβ40-induced calcium response is identified in the first study, then the effects of that modulator on Aβ40-induced COX-2 expression will also be studied. In this way, the relationship between the Aβ40 calcium signal and COX-2 expression would be established.

1.6.3. **Aim 3 / Study 3:** To determine if incubation of Aβ40 in human microglia can induce the production of neurotoxic agents.

Once again the focus of this Aim is on the functional effects of Aβ40. As mentioned previously, Aβ has been shown to induce the production of neurotoxic substances in rodent and human microglia. However, neurotoxin production induced by Aβ40 in human microglia has not yet been studied. The purpose of this study is to determine whether concentrations of Aβ40 at 4
and 10 μM can induce the production of a neurotoxin in human microglia cultures. If modulators of the Aβ40-induced calcium signal are available, then experiments examining the relationship between the Aβ40 calcium signal and neurotoxin production will be examined. Once again, the goal is to try to understand the potential mechanisms through which Aβ40 activates microglia into a pathological state that contributes to the inflammatory diseases such as AD.

1.6.4. **Aim 4 / Study 4: To determine if the treatment of human microglia with Aβ40 alters \([Ca^{2+}]_i\) signaling pathways.**

The rationale for this Aim follows preliminary work mentioned in section 1.2, which suggests that human microglia from confirmed cases of AD respond differently to ATP and PAF when compared to human microglia not diagnosed with AD. The purpose of this study is to examine whether Aβ40 can alter microglia responses to ATP and PAF in a manner similar to the altered responses in AD microglia. One of the reasons for the use of ATP and PAF is that the calcium responses for both agonists are different. Both stimuli cause the release of calcium from internal stores in the endoplasmic reticulum (ER), but the ATP response consists primarily of ER release. On the other hand PAF induces a small initial depletion of internal ER stores followed by a sustained influx of calcium through store-operated calcium channels (SOC) [McLarnon 2000, Wang 2000, Wang 1999]. Thus the actions of Aβ40 on calcium release from ER stores and influx through the SOC can be studied in human microglia.

This is the first study to use methodology of this nature to examine the effects of Aβ40 treatment on the normal cellular responses of human microglia. If long-term incubation of Aβ40 does produce changes in microglia responses to ATP and PAF it will provide evidence that Aβ40 can alter normal \([Ca^{2+}]_i\) signaling pathways in microglia. In addition, such a result would validate that this *in vitro* model mimics responses observed in AD microglia.
1.6.5. Summary of Aims

Although the four aims of this thesis center around different characteristics of microglia, they all address the same general question; what are the possible mechanisms and actions of Aβ40 that induces microglia into a state of activation that is potentially pathogenic? Although much work has been done in the area of Aβ and microglia, the majority of previous studies have used either rodent microglia or cell lines. Such cells do not represent a model of disease in the human brain as well as human microglia. For example, rodent microglia exhibit properties such as the expression of inducible nitric oxide synthase (NOS), which is a substance not produced in human microglia [Lee 1993]. Furthermore, most of the previous work also used active fragments of Aβ that are not normally present in the human brain, or they have used concentrations of Aβ that were much higher than what is needed to induce responses in human microglia.

All of the studies proposed for this thesis will provide new insight in the study of microglia and Aβ. The combination of human microglia and the use of the full length Aβ40 peptide makes the study of the Aβ40 induced calcium response in Aim 1, and the expression of a neurotoxin in Aim 3, novel. The examination of Aβ40 induced COX-2 expression in human microglia, as outlined in Aim 2, is also unique. Although COX-2 expression has been shown to be upregulated in the AD brain and in Aβ treated neurons, no study has yet looked at COX-2 expression in human microglia treated with Aβ. Furthermore, the study of Aβ40 treatment on microglia responses in Aim 4 is a completely new approach in the study of microglia. No previous laboratory has attempted to examine the integrity of calcium signaling pathways in microglia after Aβ treatment.

The intention of the first three studies is to examine the actions of Aβ40 at the initiation of the [Ca^{2+}]_{i} signal on through to the functional effects that are produced in microglia as a result of that signal. The first study's purpose is to characterize the Aβ40-induced [Ca^{2+}]_{i} response and the
expression of COX-2 and neurotoxic substances examines the effects of Aβ40 further downstream. The final study examines whether the diminished responsiveness observed in preliminary work with AD microglia can be modeled in human microglia treated with Aβ40. All of these experiments are novel and will provide insight into the effects of Aβ40 on human microglia. Although there is no single unifying hypothesis in this thesis, this work represents a diverse characterization of the actions of Aβ40 on human microglia. The intention of this characterization is to provide the initial framework needed to progress the study of Aβ40, microglia and disease.

2. Materials and Methods

2.1. Preparation of human microglia

Human microglia cultures were isolated from human fetal brain tissue following legalized therapeutic abortions. Approval for this work was granted from the Ethics Committee of the University of British Columbia. Brain tissue was incubated in phosphate buffered saline (PBS) solution containing 0.25 % trypsin and DNase (40 μg/ml) for 30 minutes at 37°C and then dissociated into single cells. The dissociated cells were then cultured in T75 flasks in medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 5% horse serum, 5mg/ml glucose and 20 μg/ml gentamicin. After 2-4 weeks of growth in flasks, free-floating microglia were harvested and plated on glass coverslips or in culture dishes depending on the protocol to be used. Immunostaining of cells following this procedure was used to verify that the overall purity of the cultures exceeded 98%.
2.2. Study 1; the examination of a Aβ40 induced calcium response in human microglia

2.2.1. Calcium spectrofluorometry

Glass coverslips plated with microglia were incubated with the calcium dye Fura-2 acetoxyethylester (Fura-2 AM) for 25 minutes. Methods for Fura-2 usage are reviewed in Hirst 1999. Fura-2 is a radiometric dye with an excitation spectrum that shifts when it binds to calcium. When bound to calcium maximum Fura-2 fluorescence is observed at a 340 nm wavelength and in calcium free conditions optimum fluorescence is emitted at 380 nm. The concentration of free [Ca\(^{2+}\)], can then be calculated by using the ratio of bound calcium at 340 nm over free calcium at 380 nm (340/380) which is proportional to [Ca\(^{2+}\)]\(_{i}\) levels within the cell. Acetoxyethylester (AM) is applied in combination with Fura-2 in order to facilitate the loading of Fura-2 into the cell as well as the accurate recording of [Ca\(^{2+}\)]\(_{i}\) levels. The addition of AM on Fura-2 renders the molecule calcium insensitive and permeable to the cellular membrane. Once inside the cell esterases cleave off the AM group allowing the Fura-2 to bind to calcium and also preventing the molecule from leaving the cell. Thus the addition of AM prevents Fura-2 from binding and fluorescing calcium in the extracellular solution as well as keeping the dye inside the cell. Fura-2 AM was applied with pluronic acid at equal concentrations (1 μM) in a normal physiological saline solution (Ca-PSS) at room temperature in order to facilitate solubilization of the Fura-2 AM. After loading the dye the coverslip was transferred to a dye-free solution of PSS to wash out excess Fura-2 for 5-7 min. The coverslip was then mounted on a Zeiss Axiovert inverted microscope with a x40 quartz objective lens. Alternating wavelengths of 340 and 380 nm of UV light was applied at 8 second intervals and fluorescence signals were obtained at an emission wavelength of 510 nm. Fluorescent emissions were captured by a digital camera (DVC-1310, DVC Co. Austin, TX) and were
processed using an Empix imaging software package by recording and calculating the 340/380 UV ratio which is used as a quantitative measure of \([\text{Ca}^{2+}]_i\) levels. Table 1 shows 340/380 UV ratios for calcium and the approximate corresponding \([\text{Ca}^{2+}]_i\) that is represented by the specific ratio value.

**Table 1:** Approximate conversions of \(F_{340} / F_{380}\) ratios to \([\text{Ca}^{2+}]_i\)

<table>
<thead>
<tr>
<th>(F_{340} / F_{380})</th>
<th>([\text{Ca}^{2+}]_i) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>550</td>
</tr>
<tr>
<td>0.6</td>
<td>365</td>
</tr>
<tr>
<td>0.4</td>
<td>200</td>
</tr>
<tr>
<td>0.2</td>
<td>45</td>
</tr>
</tbody>
</table>

2.2.2. *Solutions and chemicals*

Human Aβ 1-40 was purchased from California Peptides (Napa, CA) and prepared in a 1% ammonium hydroxide (NH₄OH) solution as recommended by Astra Zeneca or solublized in sterile distilled water or Ca-PSS in a stock solution of 1mM. Application of the peptide followed closely after its preparation, however for short-term storage the stock solution was kept at 4°C. The physiological calcium-containing saline solution (Ca-PSS) contained (in mM): NaCl (126), KCl (5), MgCl₂ (1.2), CaCl₂ (1), D-glucose (10) HEPES (10), and was set to pH 7.4. In calcium-free physiological saline solution (Ca-free PSS), 1 mM of EGTA was used instead of CaCl₂, otherwise the ingredients and concentrations matched the Ca-PSS solution. A low chloride solution was also applied in some experiments and it had two changes from the regular Ca-PSS mixture; the NaCl was replaced with Na-gluconate (126 mM), and KSO₄ and MgSO₄ replaced the Cl⁻ salt so that the
total Cl− concentration was at 2 mM. SKF963651 (SKF) was solubilized in double distilled H2O and stored at a stock concentration of 50 mM. Fura 2 AM was purchased from Molecular Probes (Eugene, OR) and ionomycin was obtained from Sigma (St. Louis, MO).

Experiments were performed by applying Aβ40 directly to the coverslips at the concentration of 4 or 10 µM. Two general types of experiments were performed, Aβ40 in Ca-free PSS or in normal Ca-PSS solution. Experiments in Ca-free PSS were done to determine the contribution of internal stores on the Aβ40 induced calcium response. Ca-free PSS, low chloride solution, and SKF (50 µM) were applied directly in other experiments in order to determine the characteristics of the calcium response induced by Aβ40.

2.3. Study 2: the examination Aβ40 treatment of human microglia on COX-2 expression

Aβ40 was solubilized in sterile distilled water for all the experiments in this study and the microglia cells were isolated and prepared the same as in Study 1. The basic RT-PCR procedures used in this study are described previously by Nagai (2001). RT-PCR was performed with two different sense and antisense oligonucleotide primers of COX-2

Primer1: sense; 5’-TTC-AAA-TGA-GAT-TGT-GGG-AAA-ATT-GCT-3’
antisense; 5’-AGA-TCA-TCT-CTG-CCT-GAG-TAT-CTT-3’; 304 base pair (bp) product.

Primer2: sense; 5’-CAC-AAT-GTG-GCT-GAG-GGA-ACA-CAA-CA-3’
antisense; 5’-GAC-TGG-TAT-TTC-ATC-TGC-CTG-CTC-TGG-3’; 489 bp product.

Aβ40 at 4 and 10 µM was incubated in microglia cultures for ~24 hrs. Total RNA was extracted using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD). Complimentary cDNA templates from each sample was prepared from 2 µg of total RNA primed with random hexamers
(Pharmacia, Gaithersburg, MD) using 200 units of MMLV reverse transcriptase (GIBCO-BRL) followed by 40 PCR amplification cycles (94°C for 30 sec, annealing at 55°C for 60 sec, and extension at 72°C for 90 sec). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reaction standard [Ercolani 1988]. Ten µl of each PCR product was analyzed by 1.5% agarose gel electrophoresis. Authentic bands were determined by selective enzyme digestion.

2.4. Study 3; the examination of Aβ40 induced neurotoxic potential in human microglia

2.4.1. Reagents

The following reagents were used in this study and were purchased from Sigma (St. Louis, MO): bacterial lipopolysaccharide (LPS, from Escherichia coli 055:B5); diaphorase (EC 1.8.1.4, from Clostridium kluvyeri, 5.8 U/mg solid); p-iodonitrotetrazolium violet; nicotinamide adenine dinucleotide (NAD+); MTT [3-(4,5-dimethylthiazol2-gl)-2,5-diphenyl tetrazolium bromide]. Human recombinant interferon-g (IFN-γ) was purchased from Bachem (Torrance, CA), and both calcein/acetoxy methylester (AM), and ethidium homodimer (EthD-1) were purchased from Molecular Probes (Eugene, OR).

2.4.2. Cell Culture

The human microglia cells were obtained and prepared the same as in the previous two studies. Instead of being plated on glass coverslips the microglia were seeded in 12-well plates at the concentration of 6-8 x 10⁴ cells per well in 0.8 ml of Dulbecco's modified Eagle's medium nutrient mixture F12 ham (DMEM-F12) containing 5% fetal bovine serum (FBS).
Undifferentiated human neuroblastoma SH-SY5Y were also plated in 12-well plates at the concentration of 2 x 10⁵/ml in 0.5 ml of DMEM-F12, with 5% FBS.
2.4.3. Neurotoxicity of microglia supernatants

The procedure is similar to those outlined in Klegeris (2000). Aβ40 at 4 or 10 μM was solubilized in sterile distilled water was added to the plated microglia cultures and the wells were then incubated for 24 hrs in a humidified 5% CO₂, 95% air atmosphere at 37°C. For a negative control, plastic wells with Aβ40 but no microglia were used as well as microglia containing wells without any Aβ40 stimulus. A positive control stimulus of LPS (0.5 μg/ml) & IFN-γ (333U/ml) on microglia cultures was also used since this mixture has been used as a positive control before in neuroblastoma. All control wells were incubated for the same amount of time under the same conditions as the treated microglia wells. At the end of the 24 hrs incubation period the supernatant from the SH-SY5Y neuroblastoma wells was removed and then replaced with 0.5 ml of supernatant from the treated microglia and control wells. The neuroblastoma were then incubated for 72 hrs and the supernatant was analyzed for lactate dehydrogenase (LDH) as a measure of cell death and the MTT cell viability assay for formazan production was also performed.

2.4.4. Cell death and viability assays

The level of neuronal cell death was determined by measuring the release of LDH from dead neuroblastoma cells. The enzymatic assay for measuring LDH activity is described by Decker (1988) and Klegeris (2000). After the 72 hrs incubation of the neuroblastoma with supernatant from the treated microglia cultures 100 μl of supernatant was transferred to another set of wells with the addition of 15 μl of lactate solution (36mg/ml in phosphate-buffered saline (PBS)) and 15 μl of p-iodonitrotetrazolium violet solution (2 mg/ml in PBS). A reaction was initiated by 15 μl of NAD+/disaphorase solution (3mg/ml NAD+, 2.3 mg solid/ml diaphorase). After a 15 min incubation period the reaction was halted with the addition of 15 μl oxamate (16.6 mg/ml). Optical densities were measured by a Model 450 microplate reader (Bio-Rad Laboratories, Richmond, CA)
with a 490-nm filter. The amount of LDH released was expressed as a percentage of the value obtained in comparison to wells where 100% of the cells were lysed with 1% Triton X-100.

Cell viability was determined by the MTT assay described by Mosmann (1983) and Klegeris (2000). This assay is based on the ability of viable cells to convert tetrazolium salt (MTT) to colored formazan. The viability of the neuroblastoma cells was determined by adding 1 mg/ml of MTT to the cell cultures. After a 2 hrs incubation the formazan was extracted by adding an equal volume of extraction buffer (20% sodium dodecyl sulfate and 50% N,N-dimethyl formamide, pH 4.7). The well plates were then incubated for 16-20 hrs at 37°C. The amount of formazan formed was determined by measuring 100 µl aliquots at 570 nm with the plate reader. A percentage value was obtained by comparing treated cells to cells incubated in fresh medium only.

**2.5. Study 4; the examination of Aβ40 treatment on human microglia responses to ATP and PAF**

Aβ40 was solubilized in sterile double distilled water for all the experiments in this study. All other procedures and solutions used for the cell culture and calcium spectrofluorometry were identical to the procedures outlined in the first study. Aβ40 was incubated with plated coverslips of human microglia in DMEM at 37°C for 1 or 2-day treatment (~20 or 48 hours). After the incubation period spectrofluorescence of the [Ca^{2+}]i was examined in these cells in response to ATP and PAF. ATP was solubilized in distilled water at a stock concentration of 10 mM and applied at a concentration of 100 µM. PAF was also solubilized in distilled water at a stock of 100 µM and was applied at a concentration of 100 nM. Responses from the treatment group were compared to responses from untreated cells that were tested on the same day.
3. Results

3.1. Study 1; the examination of a A\(\beta\)40 induced calcium response in human microglia

Initial experiments investigated the effects of acute application of A\(\beta\)40 (4 and 10 \(\mu\)M) on \([\text{Ca}^{2+}]_i\) levels in cultured human microglia. Control experiments were performed where \([\text{Ca}^{2+}]_i\) levels were recorded without the application of A\(\beta\)40, a representative experiment is shown in Figure 2A. The trace shown is of a mean of all the \([\text{Ca}^{2+}]_i\) responses (mean of 22 cells), note the lack of any noticeable change in \([\text{Ca}^{2+}]_i\) for up to 10 minutes. In addition, the application of fresh Ca-PSS at approximately 300 sec did not cause any observable changes in \([\text{Ca}^{2+}]_i\). A\(\beta\) studies employed the application of A\(\beta\)40 in calcium containing physiological saline solution (Ca-PSS). A representative experiment is shown in Figure 2B which illustrates that A\(\beta\)40 application at 10 \(\mu\)M cases a slow progressive linear rise in \([\text{Ca}^{2+}]_i\) (mean of 26 cells). The rise in \([\text{Ca}^{2+}]_i\) was not immediate but occurred within two minutes after A\(\beta\)40 application. A plateau level of \([\text{Ca}^{2+}]_i\) was reached after approximately 8 min. of treatment and did not change following the removal of A\(\beta\) (data not shown). A similar rise in \([\text{Ca}^{2+}]_i\) due to A\(\beta\)40 application was replicated for both 4 and 10 \(\mu\)M concentrations in a total of 14 experiments (see Table 2). The change in \([\text{Ca}^{2+}]_i\) before A\(\beta\)40 application was compared to the change in \([\text{Ca}^{2+}]_i\) with A\(\beta\)40 application in all experiments with a standard \(t\)-Test and the results were highly significant (see Table 2).

In order to establish whether the \([\text{Ca}^{2+}]_i\) induced response to A\(\beta\)40 is mediated by external or internal sources similar experiments were carried out in calcium-free physiological saline solution (Ca-free PSS). If a component of the \([\text{Ca}^{2+}]_i\) response is due to internal stores then A\(\beta\)40 application in Ca-free PSS will still illicit a response, on the other hand, if the \([\text{Ca}^{2+}]_i\) rise is
primarily due to the influx of calcium from the extracellular solution then no rise in $[Ca^{2+}]_i$ would be seen. A representative control experiment where $[Ca^{2+}]_i$ levels were recorded in Ca-free PSS without Ab40 is shown in Figure 3A (mean of 17 cells). No evident changes in $[Ca^{2+}]_i$ occurred over the course of the experiment (10 min). A typical response of Ab40 application in Ca-free PSS is shown in Figure 3B (mean of 22 cells), and shows that no significant rise in $[Ca^{2+}]_i$, was observed with the application of Ab40 in Ca-free PSS. The introduction of Ca-free PSS caused a slight reduction in basal $[Ca^{2+}]_i$ and when calcium was reintroduced into the extracellular solution a rise in $[Ca^{2+}]_i$ was observed. A lowering of basal $[Ca^{2+}]_i$, in response to the removal of extracellular calcium is commonly observed in these microglia cultures thus it is unlikely that the rise in $[Ca^{2+}]_i$, following the re-introduction of Ca-PSS is due to Ab40 treatment. The lack of a Ab40 $[Ca^{2+}]_i$, response in Ca-free PSS for both Ab40 concentrations (4 & 10 mM) was replicated in a total of 9 separate experiments (Table 2). Once again the change in $[Ca^{2+}]_i$, before Ab40 application in Ca-free PSS was compared to the change in $[Ca^{2+}]_i$, with Ab40 application in all experiments using a standard $t$-Test (see Table 2). The results from these experiments indicate that Ab40 applied in calcium-free solution does not cause a significant increase in $[Ca^{2+}]_i$, which suggests that Ab40 induces an influx of calcium from the extracellular solution with no depletion of internal stores of calcium.

A series of experiments were next carried out using Ca-free PSS solution applied following the response induced by Ab40 in Ca-PSS. These studies were used to investigate if introduction of Ca-free PSS subsequent to the Ab induced $[Ca^{2+}]_i$, rise inhibited the increase in $[Ca^{2+}]_i$. If calcium influx was the cause of the $[Ca^{2+}]_i$, response then removal of calcium from the external solution at the height of the $[Ca^{2+}]_i$, rise should eliminate the influx of calcium and the $[Ca^{2+}]_i$, concentration should drop back down to basal levels. To test this hypothesis Ab40 was applied in Ca-PSS and a
rise in \([Ca^{2+}]_i\) was induced (Figure 4A). Upon the removal of extracellular calcium, near the peak of the response, \([Ca^{2+}]_i\) dropped quickly, however the calcium influx was not fully inhibited. A second application of Ca-free PSS did inhibit the Aβ40-induced influx even further, but not all the way to initial basal \([Ca^{2+}]_i\) levels, suggesting a small internal stores component to the Aβ40 calcium response (mean of 9 cells). A second example of this protocol is presented in Figure 4B (mean of 26 cells) where the application of Ca-free PSS at the height of the Aβ40 \([Ca^{2+}]_i\) response caused a sharp drop in \([Ca^{2+}]_i\), yet once again the \([Ca^{2+}]_i\) rise was not completely inhibited. The re-introduction of Ca-PSS after Ca-free PSS reversed the fall in \([Ca^{2+}]_i\) (Figure 4B). Inhibition of the Aβ40-calcium response was also observed when Aβ40 was maintained during the application of Ca-free PSS (Figure 4C, mean of 23 cells).

Both of the experiments shown in Figure 4A & B suggest that while the main component of the Aβ40-induced \([Ca^{2+}]_i\) response in microglia is due to calcium influx, there may still be a small contribution of internal calcium stores since the removal of calcium from the extracellular solution did not fully inhibit the \([Ca^{2+}]_i\) rise. Inhibition of the \([Ca^{2+}]_i\) rise induced by Aβ40 with the removal of extracellular calcium was replicated in a total of 11 experiments (Table 2). However, in only 3 of the 11 experiments was the Aβ40-induced rise in \([Ca^{2+}]_i\) inhibited fully. These experiments suggest that although calcium influx constitutes the major component of the calcium response of human microglia to Aβ40, there also appears to be evidence that Aβ40 causes limited mobilization of calcium from intracellular stores.

In order to better elucidate the nature of the influx pathway activated by Aβ40 a series of experiments were performed that attempted to modulate this pathway. A high conductance anion channel has previously been described in human microglia that is activated with both depolarizing and hyperpolarizing steps from holding potential, suggesting a role in the maintenance of cell
potential [McLarnon 1997]. More recent work has shown that a reduction in the expression of these anion channels with a low chloride physiological saline solution (low \( \text{Cl}^- \) PSS) inhibits calcium influx through store-operated calcium channels (SOC) in human microglia [McLarnon 2000]. It was further suggested that the inhibition of SOC was secondary to cell depolarization induced by a decrease in the expression of \( \text{Cl}^- \) channels [McLarnon 2000]. Furthermore, although detailed properties of the anion channels are unknown, the data suggest that these ion channels are active under basal conditions. As shown in Figure 5 (mean of 32 cells) low \( \text{Cl}^- \) PSS was applied at the peak of the A\( \beta \)40-mediated \( [\text{Ca}^{2+}]_i \) response. The application of low chloride solution caused an immediate and sharp drop in \( [\text{Ca}^{2+}]_i \), that approached baseline levels. The inhibition of the A\( \beta \)40-mediated \( [\text{Ca}^{2+}]_i \) response was replicated in a total of 5 separate experiments (Table 2). This result suggests that the influx pathway activated by A\( \beta \)40 is sensitive to changes in membrane potential modulated by altering the activity of anion channels in microglia.

The major calcium influx pathway in microglia is the SOC (store-operated or capacitative) pathway that is sensitive to anion channel modulation [Wang 2000]. Since A\( \beta \)40-mediated influx is also sensitive to anion channel modulation it is possible that the A\( \beta \)40 effect is mediated through the SOC pathway. However, there was no change in \( [\text{Ca}^{2+}]_i \), with A\( \beta \)40 application in Ca-free PSS (Figure 3B), indicating that there was no depletion of intracellular stores, thus no SOC activation would be possible. On the other hand, experiments which attempted to block the \( [\text{Ca}^{2+}]_i \) rise with the removal of extracellular calcium do suggest that a small amount of internal release of calcium is occurring which may come from ER stores. Thus the ability of A\( \beta \)40 to deplete ER stores in order to activate the SOC pathway remains uncertain. However, the SOC is the major calcium influx pathway in microglia because microglia, like other unexcitable tissue, do not express voltage-gated calcium channels [Eder 1998, McLarnon 1997]. Therefore A\( \beta \)40 could be activating the SOC
pathway directly without signaling depletion from ER stores or a slow ER depletion may be causing SOC activation.

A pharmacological maneuver was next used to investigate the dependence of the Aβ40-induced rise in [Ca^{2+}]_{i} on the SOC pathway. SKF963651 (SKF), a known inhibitor of the SOC pathways [Li 1999], was applied at the peak of the Aβ40-induced [Ca^{2+}]_{i} rise. If Aβ40 was activating SOC pathways directly then SKF application should inhibit calcium influx and the [Ca^{2+}]_{i} level should drop. On the other hand, if Aβ40 is causing an influx of calcium through some other pathway, SKF application should have little or no effect on [Ca^{2+}]_{i} levels. Figure 6A illustrates that SKF does inhibit the SOC pathway in microglia since it causes a sharp drop in [Ca^{2+}]_{i} levels when applied to the SOC-dependent plateau phase of a [Ca^{2+}]_{i} response to platelet activating factor (PAF) [Wang 2000] (mean of 10 cells). Figure 6B illustrates a representative experiment and shows that SKF application had no effect on the Aβ40-induced influx of calcium (mean of 12 cells). The inability of SKF to modulate the Aβ40-induced calcium influx was replicated in 3 separate experiments (Table 2). These experiments suggest that Aβ40 is causing an influx of calcium that is independent of the store-operated influx pathway.

The goal of this study was to characterize the [Ca^{2+}]_{i} response initiated by Aβ40 in human microglia. Initial experiments with Aβ40 in Ca-PSS and Ca-free PSS indicated that Aβ40 induced a rise in [Ca^{2+}] due to calcium influx from the extracellular solution. Further experiments then showed that the Aβ40 induced calcium influx was blocked by the removal of calcium from the extracellular solution and the depolarization of the cell membrane with low Cl- PSS. However, complete inhibition of the Aβ40-induced rise in [Ca^{2+}]_{i} did not occur with Ca-free PSS, which suggests that Aβ40 may also cause a small release of calcium from internal stores. At this point the Aβ40-induced calcium response appeared to be mainly due to calcium influx and have the
properties of the SOC influx pathway. Thus it was necessary to determine the possible role of the SOC pathway in the Aβ40 induced \([\text{Ca}^{2+}]_i\) response. The SOC inhibitor SKF was applied to the Aβ40 induced influx and had no observable effect. Therefore the results of this study indicate that Aβ40 induces a rise in \([\text{Ca}^{2+}]_i\) that is mainly due to an influx pathway in human microglia that is not mediated by the SOC pathway.

The ability to block the mechanism by which Aβ40 alters \([\text{Ca}^{2+}]_i\) would allow for the modulation of cell functions, such as the secretion of inflammatory factors, that contribute to the role of microglia in pathologies such as AD. At present, however, no pharmacological agent has been identified which would specifically inhibit the Aβ40-induced calcium pathway. The use of low Cl\(^-\) PSS would not be feasible as a modifier since this procedure would result in sustained cell depolarization that would presumably alter many cellular functions. Nevertheless, this work has identified a putative target for pharmacological manipulation in order to modulate the actions of Aβ40 that are mediated through the mobilization of \([\text{Ca}^{2+}]_i\). Further studies are needed to test for agents with the capacity to block the Aβ40-induced \([\text{Ca}^{2+}]_i\) response and thus the downstream actions of Aβ40 as well. The next study addressed a cellular function that is modulated as a result of Aβ40 treatment of human microglia. The specific function investigated was the expression of COX-2 in microglia, an enzyme that is over expressed in AD.
Figure 2: The effects of Aβ40 on [Ca$^{2+}$]$_i$ in human microglia with Ca-PSS. Graphs are representative experiments showing the mean of the responses measured. The error bars presented illustrate the SEM at points along the trace. (A) [Ca$^{2+}$]$_i$ activity recorded in human microglia cells in Ca-PSS solution with no Aβ40 present (22 cells). Note that no rise or drop in [Ca$^{2+}$]$_i$ is observed. A Ca-PSS solution change was also applied at approximately 300 sec with no apparent effect on [Ca$^{2+}$]$_i$. (B) A rise in [Ca$^{2+}$]$_i$ induced by Aβ40 in Ca-PSS (26 cells). Aβ40 application (10 μM) induces a rise in [Ca$^{2+}$]$_i$ within 2 minutes that increases in a linear manner. Analysis of [Ca$^{2+}$]$_i$ levels with a standard $t$-Test indicates that the rise is significant (***, $P < 0.0001$) when compared to initial baseline levels. Washout of the Aβ40 from the extracellular solution does not slow or diminish the rise induced by Aβ40.
## Table 2: Study 1; Aβ40 induced calcium responses in human microglia

<table>
<thead>
<tr>
<th>Study</th>
<th>Concentration (μM)</th>
<th>n value</th>
<th>Total # of Cells</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ40 induced calcium rise in Ca-PSS</td>
<td>4</td>
<td>9</td>
<td>157</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>72</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>No Aβ40 induced calcium rise in Ca-free PSS</td>
<td>4</td>
<td>6</td>
<td>129</td>
<td>0.0672</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Ca-free PSS effects on Aβ40 induced influx</td>
<td>4</td>
<td>5</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Low chloride effects on Aβ40 induced influx</td>
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<td>5</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

SKF effects on Aβ40 induced influx
Figure 3: The effects of Aβ40 on \([\text{Ca}^{2+}]_i\) in human microglia with Ca-free PSS. Graphs are representative experiments showing the mean of the responses measured. The error bars presented illustrate the SEM at points along the trace. A standard t-Test was used in all statistical analysis.

(A) \([\text{Ca}^{2+}]_i\) activity recorded in human microglia cells in Ca-free PSS solution with no Aβ40 present (17 cells). Note that no rise or drop in \([\text{Ca}^{2+}]_i\) is observed. A Ca-free PSS solution change was also applied at approximately 480 sec with no apparent effect on \([\text{Ca}^{2+}]_i\). (B) Application of Aβ40 (4 μM) in Ca-free PSS resulted in no significant increase in \([\text{Ca}^{2+}]_i\) levels (22 cells). Note that the drop in basal \([\text{Ca}^{2+}]_i\), seen with the application of Ca-free PSS is commonly observed in these cells. Thus the rise in \([\text{Ca}^{2+}]_i\), upon re-introduction of extracellular calcium should not be construed as the actions of Aβ40.
Figure 4: Removal of extracellular calcium inhibits Aβ40 induced [Ca^{2+}]_{i} responses. Graphs are representative experiments showing the mean of the responses measured. The error bars presented illustrate the SEM at points along the trace. A standard t-Test was used in all statistical analysis.

(A) Aβ40 caused a significant rise in [Ca^{2+}]_{i} (**, P = 0.0027). Removal of extracellular calcium after the Aβ40 (4μM) induced [Ca^{2+}]_{i} response resulted in a quick drop in [Ca^{2+}]_{i} (9 cells). A second application of Ca-free PSS caused a further drop in [Ca^{2+}]_{i}, however full inhibition of the [Ca^{2+}]_{i} response was not obtained. (B) Aβ40 caused a significant rise in [Ca^{2+}]_{i} (***, P = 0.0001). Removal of extracellular calcium after the Aβ40 (10μM) induced [Ca^{2+}]_{i} response resulted in a sharp drop in [Ca^{2+}]_{i} (26 cells). Once again full inhibition of the Aβ40-induced [Ca^{2+}]_{i} response did not occur. Replacement of extracellular calcium resulted in a reversal of the [Ca^{2+}]_{i} drop suggesting that the calcium influx pathway remains activated. PAF application (100nM) produced a robust [Ca^{2+}]_{i} response illustrating that the microglia remain viable. (C) In this experiment Aβ40 (4μM) was maintained with the application of Ca-free PSS (23 cells). Aβ40 caused a significant rise in [Ca^{2+}]_{i} (***, P = 0.0001). Even in the presence of Aβ40, Ca-free PSS inhibited the influx of calcium into the cell.
Figure 5: Aβ40 (4μM) induced calcium influx in Ca-PSS followed by low chloride PSS (32 cells).

The graph shown is a representative experiment showing the mean of the responses measured. The error bars presented illustrate the SEM at points along the trace. A standard t-Test was used in all statistical analysis. Aβ40 caused a significant rise in $[\text{Ca}^{2+}]_i$ (***, $P = 0.0001$). Low chloride PSS inhibited Aβ40 induced influx in a manner similar to Ca-free PSS.
Figure 6: The effects of SKF on Aβ40-induced influx. The graphs shown are representative experiments showing the mean of the responses measured. The error bars presented illustrate the SEM at points along the trace. A standard t-Test was used in all statistical analysis. (A) This experiment illustrates that SKF (50 μM) does inhibit the SOC pathway in microglia since the SOC-influx phase of the PAF response is inhibited by SKF application (10 cells). (B) Aβ40 caused a significant rise in $[\text{Ca}^{2+}]_i$ (**, $P = 0.0037$). Aβ40-induced (10μM) calcium influx in Ca-PSS was not inhibited by the SOC inhibitor SKF (50μM) (16 cells).
Figure 6A

Figure 6B
3.2. Study 2; the examination Aβ40 treatment of human microglia on COX-2 expression

The goal of this study was to determine if Aβ40 treatment of human microglia can modulate cellular functions downstream of the initial Aβ40-induced increase in [Ca^{2+}]; the functional process examined was COX-2 expression. The initial hope of this study was that a method for modulating the Aβ40-induced [Ca^{2+}] response would be found that could be used as a maneuver for altering the functional actions of Aβ40, such as COX-2 expression or the production of neurotoxic substances studied later on. However, as the earlier section indicated, no modulatory agent for the [Ca^{2+}] response mediated by Aβ40 was found. Therefore, the action of Aβ40 on COX-2 expression was examined without any modulation of the Aβ40 signal.

In order to address the concern that Aβ40 treatment over 24 hrs diminished the viability of cultured human microglia, experiments were first performed that were similar to those outlined in the fourth study where the responsiveness of the microglia was assessed with ATP and PAF after treatment with Aβ40. It was determined that after 24 hrs treatment with Aβ40 (4 & 10 μM) microglia still retained their responsiveness and showed no indication that their viability was compromised (data not shown). The expression of COX-2 RNA in human microglia following Aβ40 treatment was then characterized by RT-PCR.

In the first of the three experiments (Figure 7A) a large increase in COX-2 expression was seen for both doses of Aβ40 (4 & 10 μM) and a dose response is evident. Primer2 was used for this PCR amplification.

The results of the second experiment are shown in Figure 7B. In the top gel, low expression of COX-2 is observed even in control where no Aβ40 was added. Aβ40 treatment of the microglia at 4 and 10 μM caused a dose dependent increase in COX-2 expression. Replication of the PCR
amplification produced an observable increase in COX-2 expression only at the high dose of Aβ40 (10μM). The difference in expression and molecular weight of both PCR products can be attributed to different primers that were used. In the top band Primer1 was used and Primer2 was used for the bottom gel (see section 2.3. in Material and Methods).

The results from the third experiment (Figure 7C) also show an increase in COX-2 expression with Aβ40 treatment and the use of Primer1. No band was observable in both control or with 4μM of Aβ40 treatment; however, Aβ40 treatment at 10 μM did produce faint bands in both PCR amplifications. GAPDH bands demonstrate that the amount of mRNA for each group was equal since GAPDH is a constitutively expressed enzyme and acts as a positive control.

Data from three separate experiments show that Aβ40 treatment of human microglia over a 24 hrs period enhances COX-2 expression. Although these results are non-quantitative, a clear increase in COX-2 expression is observable with Aβ40 treatment. When taken together with the findings of the first study which focused on [Ca^{2+}]_i signaling of Aβ40 it is clear that the levels of Aβ40 found to cause a [Ca^{2+}]_i response in human microglia are also capable enhancing COX-2 expression. Thus the results from these first two studies suggest that the [Ca^{2+}]_i response initiated by Aβ40 could signal the enhanced expression of COX-2. Further work should examine this possible connection and investigate whether modulators of the Aβ40-induced [Ca^{2+}]_i response can also modulate COX-2 expression. The third study investigated Aβ40 actions on another pathogenic action of microglia, the production of neurotoxic substances.
**Figure 7:** The effects of Aβ40 on COX-2 expression in human microglia. The gels shown in each section are PCR amplifications of cDNA from a single experiment where human microglia was exposed to Aβ40 at 0, 4, and 10 µM. The expression of GAPDH, a constitutively expressed enzyme, was used as a positive control to ensure equal levels of mRNA in all of the wells. Molecular markers were also routinely run on the gels to determine the approximate molecular weight of the PCR products. The brightest marker for all gels corresponds to the molecular weight of 600 bp. (A) Primer2 was used for this experiment (see section 2.3.). Increased expression of COX-2 due to Aβ40 treatment is observable with both 4 and 10 µM concentrations and no basal COX-2 expression was observed in control. (B) In the first PCR run primer1 was used and a small amount of basal COX-2 expression is observable and Aβ40 treatment resulted in a dose-dependant increase in COX-2 expression. However, the second PCR amplification with primer2 produced no observable bands in control or 4 µM, faint COX-2 expression was only visible with Aβ40 treatment at 10 µM. The difference in molecular weights seen between both PCR runs is because of the different COX-2 primers used. Primer2 produces slightly larger COX-2 PCR products of 489 bp. (C) Primer1 was used for both PCR runs. Both control and Aβ40 treatment at 4 µM caused no observable COX-2 expression while Aβ40 at 10 µM caused visible expression of COX-2.
Figure 7A

Figure 7B

Figure 7C
3.3. Study 3; the examination of Aβ40 induced neurotoxic potential in human microglia

The objective of this study was to determine whether Aβ40 treatment of human microglia could alter cellular functions and induce the production of neurotoxic substances. Aβ40 (4 and 10 μM) was incubated for 24 hrs with or without microglia cells, after which the media was transferred to neuroblastoma cells that were incubated for an additional 72 hrs. Aβ40 incubated without the presence of microglia served as a control for any possible neurotoxicity of the Aβ40 peptide alone.

The viability of the neuroblastoma cultures after incubation was determined using the MTT assay and cell death was determined with the LDH assay as outlined in the materials and methods section. According to the LDH assay, supernatant from Aβ40-treated microglia caused very low levels of cell death, less than 10% (Figure 8A). This result was not significantly different from control conditions where the supernatant from untreated microglia was used. A further comparison of supernatant from Aβ40-treated microglia with Aβ40 application directly to neuroblastoma cultures did not yield any significant differences.

Analysis of cell viability with the MTT assay yielded some unexpected results. Supernatant from Aβ40 treated microglia did decrease cell viability of neuroblastoma cultures when compared to untreated microglia; however, direct application of Aβ40 to neuroblastoma cultures also decreased cell viability (Figure 8B). A comparison of the level of cell viability between Aβ40 treated and microglia & Aβ40 supernatant treated neuroblastoma did not show any significant differences.

The degree in the reduction of cell viability with Aβ40 treatment alone is somewhat surprising because previous work with solubilized Aβ40 in this neuroblastoma cell line did not
cause a decrease in cell viability at concentrations twice as high as what was used in the present work [Lambert 1994]. However, aggregated forms of Aβ have been shown to cause degeneration of neuroblastoma cells [Cedazo-Minguez 2001] and it is possible that some of the Aβ40 aggregated into more potentially neurotoxic forms during the course of treatment in these cells. Regardless, the initial hypothesis was that Aβ40 treatment of microglia would cause a greater degree of neurotoxicity than Aβ40 alone since other studies have shown this to be the case with Aβ treatment of rodent microglia and monocyte cell lines [Combs 1999, Giulian 1996].

If microglia do in fact produce neurotoxic agents, as numerous studies would suggest, then the most obvious recourse is to activate microglia to the point where their neurotoxin production surpasses the direct neurotoxic action of Aβ40 in neuroblastoma cells. The two most obvious changes in the current protocol that could accomplish this would be to increase the density of microglia per treatment well and/or increase the length of Aβ40 incubation in the microglia cultures. Because both Aβ40 treated microglia and Aβ40 directly both cause decreases in neuroblastoma viability at levels that are not significantly different from each other, no conclusion can be made at this time as to whether or not Aβ40 treated microglia produce neurotoxic substances.
Figure 8: The neurotoxic effects of supernatant from Aβ40 treated microglia or Aβ40 alone on neuroblastoma. A standard t-Test was used in all statistical analysis. (A) The level of cell death in neuroblastoma cells as determined by the LDH assay is shown. No significant differences were calculated between any of the treatment groups. (B) The level of cell viability in neuroblastoma as determined by the MTT assay is shown. Significance was only found between Aβ40 free and Aβ40 treated groups. Within the Aβ40 treated microglia group both 10 μM ( *, P = 0.0399) and 4 μM ( **, P = 0.0043) treatment significantly decreased neuroblastoma viability. With Aβ40 treatment alone on neuroblastoma significant reduction was seen at 10 μM (#, P = 0.0343). Aβ40 treated microglia did not exhibit any significant neurotoxic effect greater than Aβ40 treatment alone on neuroblastoma cultures.
3.4. Study 4; the examination of Aβ40 treatment on human microglia responses to ATP and PAF

The first three studies were designed to characterize [Ca\textsuperscript{2+}]\textsubscript{i} signaling pathways induced by Aβ40 in human microglia and to determine Aβ40 actions on the cellular functions of microglia. The specific functions examined were the expression of COX-2 and neurotoxic substances. The objective of this final study is not directly related to the previous three and addresses whether treatment of human microglia with Aβ40 alters calcium-mediated signaling pathways. As outlined in section 1.2., preliminary data from this laboratory suggests that microglia responses to the agonists ATP and PAF are attenuated in adult human microglia from confirmed cases of AD relative to adult microglia from non-AD subjects.

In this study, Aβ40 was incubated for either a one or two-days in cultures of human microglia. Following treatment, responses to ATP and PAF were recorded using calcium-imaging procedures similar to those described in study 1. A number of different measurements were recorded and responses were compared between treated (Aβ40 at 10 μM) and untreated microglia. The variables that were studied included basal levels of calcium, amplitude of agonist response, and the sustained influx component of agonist response. Figure 9A and B illustrates normal ATP and PAF responses and shows how the characteristics of the responses were analyzed. As noted, the ATP response is primarily due to the mobilization of calcium from ER stores. The PAF response also has an initial ER store component followed by an additional sustained influx of calcium mediated by the SOC pathway. There were a total of 4 control experiments to 8 treated for each incubation time point. A standard student \(t\)-test was then used between treated and control groups to determine the level of significance in all analysis.
Significant differences in basal $[\text{Ca}^{2+}]_i$ were seen in both the one and two-day treated microglia as compared to control (Figure 10). Differences in basal $[\text{Ca}^{2+}]_i$ were determined by pooling the 340/380 calcium ratios at time zero for all the control and treated cells for each treatment group. This result is consistent with other studies that show elevated $[\text{Ca}^{2+}]_i$ levels in rodent and human microglia after incubation with active fragments of Aβ [Korotzer 1995, Silei 1999].

Amplitudes of PAF responses following Aβ40 treatment were significantly diminished for both one and two-day treatments (Figure 11). The influx component of the PAF response due to the SOC pathway was also examined in Aβ40 treated microglia by comparing the $[\text{Ca}^{2+}]_i$ level 1 min after the initial PAF peak. A significant decrease in the SOC mediated $[\text{Ca}^{2+}]_i$ plateau of PAF was seen for both treatment groups (Figure 12). These results show that Aβ40 treatment may be altering normal calcium-mediated signaling in response to PAF in human microglia. Furthermore, this data also suggests that the actions of Aβ40 in human fetal microglia can model the functional responses found in microglia from AD brain.

An analysis of the ATP responses resulted in no significant differences in the ATP amplitude or level of influx between the treated and control groups. Both PAF and ATP utilize the same internal calcium stores in their initial response peaks, however the signaling pathways for both agonists are not identical since they utilize different receptors and second messenger systems [McLarnon 2000]. Therefore, Aβ40 could be only acting on the PAF signaling pathways in the alteration of $[\text{Ca}^{2+}]_i$ signaling.

The purpose of this study was to examine if Aβ40 treatment in cultured human microglia mimics conditions found *in vivo* from AD patients where calcium-mediated cellular responses to the common agonists ATP and PAF were altered. Significant elevation of basal $[\text{Ca}^{2+}]_i$ was
observed in all Aβ40 treated cells over both one and two-day treatments. Furthermore, significant attenuation of the PAF response was also seen in both the one and two-day Aβ40 treated microglia in regards to both internal stores release and the level of calcium influx through SOC. These results mimic the preliminary data from AD microglia and thus suggest that the use of fetal human microglia cultures is a viable model in studying conditions that occur in the adult human brain. Furthermore, this data also shows that Aβ40 can effectively alter normal [Ca^{2+}]_{i} responses to common signaling agents of microglia.
Figure 9: Normal PAF and ATP $[Ca^{2+}]_i$ responses in human microglia. Graphs are representative experiments showing the mean of the responses measured from control groups that received no Aβ40 treatment. The green line indicates what was determined as the amplitude of the response and the blue line indicates where the time point for determining the level of influx was taken. (A) A characteristic response to PAF in human microglia from a control experiment that was treated for one day with Aβ40 free medium (24 cells). Note the large initial peak in $[Ca^{2+}]_i$ followed by a sustained elevated $[Ca^{2+}]_i$ level. The initial peak in $[Ca^{2+}]_i$ is primarily reliant on internal stores of calcium and the subsequent sustained elevation in $[Ca^{2+}]_i$ is reliant on the influx of extracellular calcium through the SOC pathway. (B) A characteristic response to ATP in human microglia from a control experiment that was treated for one day with Aβ40 free medium (55 cells). Note that the major observable difference when compared to the PAF response is that there is no elevated shoulder after the initial $[Ca^{2+}]_i$ peak.
Figure 9A

Ca-PSS

\[ \frac{F_{sat}}{F_{act}} \]

PAF amplitude

influx level
time point

ionomycin

Ca-free PSS

0.8

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0

50

100

150

200

250

300

350

400

450

500

Time (sec)

Figure 9B

Ca-PSS

\[ \frac{F_{sat}}{F_{act}} \]

ATP amplitude

influx level
time point

Ca-free PSS

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0

50

100

150

200

250

300

350

400

450

500

Time (sec)
Figure 10: Aβ40 incubation (10 μM) in human microglia cultures for one and two-day treatment significantly increased basal levels of [Ca$^{2+}$]. The level of basal [Ca$^{2+}$]$_i$ was taken as the first [Ca$^{2+}$]$_i$ measurement at time zero for all experiments. A standard t-Test was used in all statistical analysis. (A) This graph represents the basal [Ca$^{2+}$]$_i$ levels after one-day treatment of media without Aβ40 (control) or with Aβ40 (10 μM). Control represents 4 experiments with a total of 233 cells. Aβ40 treatment represents 8 experiments with a total of 166 cells (*** = p < 0.0001). (B) This graph represents the basal [Ca$^{2+}$]$_i$ levels after two days of Aβ40 treatment (10 μM) or control. Control represents 4 experiments with a total of 127 cells. Aβ40 treatment represents 8 experiments with a total of 208 cells (* = p < 0.05)
**Figure 11:** Aβ40 incubation (10μM) in human microglia cultures for one and two-day treatment significantly attenuated the amplitude of the PAF induced calcium response. A standard t-Test was used in all statistical analysis. (A) This graph represents the amplitude of the PAF [Ca$^{2+}$]$_i$ response after one-day treatment of media without Aβ40 (control) or with Aβ40 (10 μM). Control represents 2 experiments with a total of 79 cells. Aβ40 treatment represents 4 experiments with a total of 85 cells (** = p < 0.0001). (B) This graph represents the PAF amplitudes after two days of Aβ40 treatment (10 μM) or control. Control represents 2 experiments with a total of 52 cells. Aβ40 treatment represents 4 experiments with a total of 112 cells (** = p < 0.0001).
Figure 11A

The figure shows the PAF calcium response amplitude for Control and Aβ 40 Treatment groups. The Control group has a higher amplitude compared to the Aβ 40 Treatment group.

Figure 11B

The figure displays the PAF calcium response amplitude for Control and Aβ 40 Treatment groups. The Control group shows a higher amplitude than the Aβ 40 Treatment group, indicated by the asterisks.
Figure 12: Aβ40 incubation (10μM) in human microglia cultures for one and two-day treatment significantly decreased the level of Ca$^{2+}$ influx through the SOC as observed in the calcium plateau that is characteristic of a normal PAF response (see Figure 9). A time point one minute after the peak of the PAF response was used as a measure of the level of influx. A standard t-Test was used in all statistical analysis. (A) This graph represents the level of Ca$^{2+}$ influx in response to PAF after one-day treatment of media without Aβ40 (control) or with Aβ40 (10 μM). Control represents 2 experiments with a total of 79 cells. Aβ40 treatment represents 4 experiments with a total of 85 cells (*** = p < 0.0001). (B) This graph represents the PAF amplitudes after two days of Aβ40 treatment (10 μM) or control. Control represents 2 experiments with a total of 52 cells. Aβ40 treatment represents 4 experiments with a total of 112 cells (** = p < 0.001).
4. Discussion

This thesis has characterized some of the effects of Aβ40 on signaling pathways in human fetal microglia cells. The relevance of such work lies in a growing body of research implicating that the actions of Aβ on microglia contribute to the progression of Alzheimer’s Disease (AD). The current hypothesis regarding the role of microglia in AD is that the microglia become chronically over-activated which leads them to damage diseased and healthy tissue alike [McGeer 2000]. Aβ is a peptide that activates microglia directly through specific binding domains as well as through amyloid plaques, which are difficult for the microglia to metabolize and subsequently cause chronic activation of microglia.

The initial causative event in AD development is thought to be abnormal processing of APP that leads to disproportionately large amounts of Aβ forming and being deposited in the brain [Selkoe 1999, Koo 1999]. Thus, it is theorized that excess Aβ is made which accumulates into large Aβ plaque deposits. High concentrations of Aβ, in and around these plaques, activate microglia and cause them to produce substances that ultimately kill neurons, increase Aβ production, and activate microglia even further [Eikelenboom 1996, Jiang 1994, McGeer 1995] (see Figure 1). Although Aβ40 is one of the major forms of Aβ that is found in the brain, little work has been done to characterize the actions of this amyloid peptide on human microglia. This lack of research, along with the fact that Aβ40 is more soluble and potentially provides more reproducible results than Aβ42, determined that Aβ40 would be the Aβ peptide studied in this thesis. The individual studies of this thesis project were designed to examine different potential effects of Aβ40 on microglia with the first study examining signaling characteristics induced by Aβ40 and the other three studies looking at downstream effects of Aβ40 treatment on human microglia.
4.1. The use of primary cell cultures of human microglia

One major challenge in the execution of these studies was the use of primary cultures of human microglia from fetal brain tissue. This was the one critical factor in these experiments that was beyond the direct control of our laboratory and the availability and quality of tissue was not consistent. At times new cells were not available for long periods since fresh brain tissue was not available. In other instances the tissue samples themselves were not of good quality, the likely result of extra long storage and small sample sizes. As a consequence, cells isolated from these tissues showed abnormalities including high and unstable \([\text{Ca}^{2+}]_i\) baselines, low cell density, inadequate Fura-2 uptake, and little or no responsiveness to the common agonists ATP and PAF. Even when good quality cultures were available the viability of the cultures would become compromised over a period of time and, after approximately 2 weeks on average, the cells would fail to respond normally and take on some of the abnormal characteristics previously mentioned. Thus, the availability and quality of the primary human microglia cultures was a limiting factor in all of these studies. Nevertheless, cultured human microglia comprise a novel \textit{in vitro} preparation since very few studies have reported results from such cells.

Given the difficulties in obtaining consistent primary cultures of human microglia, it may be prudent to examine if microglia cell lines could be used instead. Such cell lines would have to be human in origin since rodent microglia have different cellular properties in comparison to human cells. At present no human microglia cell line is available. THP cells derived from blood monocytes are commonly used instead of human microglia, however monocytes, while being similar to microglia, do not always respond the same as human microglia. However, if a monocyte cell line were shown to respond to Aβ40 in a manner similar to the human microglia used in this study then perhaps initial screening and pilot studies in a monocyte cell line would be feasible.
Such use of an appropriate cell line could narrow down areas of study that would allow for the more efficient use of primary human microglia cultures. In addition, there are a number of laboratories that are developing a functional human microglia cell line and there is the possibility that one will become available in the near future. Consequently, if a viable microglia cell line was established, future experiments would be free of the limitations imposed by the use of primary microglia cultures and studies that require large numbers of cells, such as the neurotoxicity study, could be undertaken more easily. The use of a human microglia cell line would also allow for the more efficient screening of possible agents that could target the Aβ40-induced \([\text{Ca}^{2+}]\) signaling pathway characterized in this work. Therefore future research expanding the findings of this thesis would benefit greatly from the development and use of a human microglia cell line.

4.2. The use of Aβ in vitro as a stimulus

Another central issue in the methods surrounding this thesis is the use of Aβ40. Aβ is a difficult molecule to work with, and as mentioned in the introduction, issues of solubility and aggregation constantly surface. The problem with much of the research around Aβ is that very little effort is made to characterize the aggregation and solubility level of the peptide in solution. The degree of aggregation is often described in vague terms with solutions of Aβ either assumed to be mostly aggregated or mostly solubilized. Although it is understandable that most laboratories do not have the resources to characterize the rates of Aβ aggregation in a variety of solvents, it is surprising that none of the well-financed laboratories in this field (of which there are many) have thought to undertake this project. The activity of Aβ cannot be accurately determined if the functional state of the Aβ is not known and controlled for. Furthermore, it would be prudent to be able to characterize the level of solubility and the conformational state of Aβ in vivo in order to perform in vitro studies where the physical characteristics of the Aβ peptide are controlled for in a
manner that mirrors the in vivo conditions. Therefore, further studies that progress the findings of this thesis should consider characterizing the solubility and aggregation rate of Aβ over a number of solvents, time courses, and temperatures. Then accurate comparisons between levels of Aβ aggregation and Aβ activity can be made.

4.3. Aβ40-induced [Ca^{2+}]_i signaling pathway

The first study of this thesis work examined the [Ca^{2+}]_i signaling pathway induced by Aβ40 in human microglia. The results have shown that the application of Aβ40 (4 and 10 μM) to microglia in Ca-PSS causes a rise in [Ca^{2+}]_i that reaches a plateau level that is maintained even when the peptide is washed out of the extracellular solution. When this experiment was repeated in Ca-free PSS, Aβ40 caused no evident alteration of [Ca^{2+}]_i levels. The removal of extracellular calcium at the peak of the Aβ40-induced [Ca^{2+}]_i rise caused a decrease in [Ca^{2+}]_i levels, however there was not complete inhibition of the [Ca^{2+}]_i rise suggesting a small intracellular stores component to the response. This data indicates that Aβ40 induces a change in [Ca^{2+}]_i that is mediated primarily through the influx of extracellular calcium into the cell.

The Aβ40-induced influx response was examined further with the application of low Cl^- PSS, which has been shown previously to inhibit influx through the SOC pathway in microglia [McLarnon 2000]. The application of low Cl^- PSS at the peak of the Aβ40-induced rise in [Ca^{2+}]_i caused a decrease in [Ca^{2+}]_i, thereby suggesting that the actions of Aβ40 in human microglia are sensitive to changes in anion channel expression. It is possible that depolarization caused by low Cl^- PSS is responsible for inhibiting the calcium influx response. These results show that Aβ40-induced influx of calcium in microglia responds to the removal of extracellular calcium and the modulation of anion channels in a manner similar to the SOC pathway. However, it is possible that Aβ40 is causing the influx of calcium through a pathway independent of the SOC since the
contribution of intracellular stores to the Aβ40 response appears to be very small. This possibility was addressed by using SKF96365 (SKF), a known inhibitor of this pathway [Li 1999]. SKF caused no observable effect on the Aβ40-induced calcium influx in human microglia indicating that the SOC pathway did not mediate the calcium influx response. Furthermore, it is unlikely that a voltage-gated calcium channel contributes to the influx since microglia, like other unexcitable cells, do not express such channels [McLarnon 1997]. Therefore it appears that Aβ40 initiates a calcium influx pathway, which is not mediated by SOC.

Aβ40 seems to be activating an influx pathway independent of SOC, thus a review of some possible routes for calcium entry into microglia that would explain the results of this study is needed. One possibility is that the Aβ molecule itself forms channels in the cell membrane. Work by Arispe (1993) has shown that Aβ40 at a very high concentration of 0.46 mM is capable of forming calcium channels in synthetic bilayer membranes and the resulting current and channel properties were similar to other known calcium channels. More recently, work by Lin (1999) has also shown that Aβ40 forms calcium channels in lipid vesicles at a concentration of 0.12 mM. However, the concentrations of Aβ40 used in both of these studies were much higher than the concentrations used in this thesis work; the Arispe study, for example, used Aβ40 concentrations more than 40 times larger. Other work by Fukuyama (1994) that examined [Ca^{2+}]_i increases of neuronal cells with Aβ40 application at 46 μM ruled out the possibility of a large effect of Aβ-channels because the magnitude of the Ca^{2+} current recorded was too small to account for the level of calcium-uptake observed. If 46 μM of Aβ40 is too small an amount to cause the formation of Aβ-calcium channels then it would stand to reason that concentrations of Aβ40 at 4 and 10 μM would be even less likely to form channels. Furthermore, it is unlikely that the formation of Aβ-calcium channels would result in the slow increase in [Ca^{2+}]_i that is observed in these results. A
calcium pore in the cell membrane should cause a much faster and greater rise in \([\text{Ca}^{2+}]_j\) than what is observed in these cells. Thus it seems unlikely that the formation of \(\text{A}\beta40\) calcium channels is the explanation for the \(\text{A}\beta40\)-induced \([\text{Ca}^{2+}]_j\) responses observed in this study.

Another possible mechanism for the effects of \(\text{A}\beta40\) would be that it activates specific receptors that modulate calcium entry into the cell such as the Clq receptor that has been shown to be present on microglia and be activated by \(\alpha\beta\) peptides [Jiang 1994]. However, Clq activation is not linked to an influx pathway, activation of the Clq receptor has been shown to modulate \([\text{Ca}^{2+}]_i\) levels by mobilizing ER stores of calcium [Kishore 2000, Lovik 2001]. This activation of the Clq receptor could explain the small intracellular store component of the \(\text{A}\beta40\)-induced response that is suggested in those experiments where the \([\text{Ca}^{2+}]_i\) rise is not completely inhibited by the removal of extracellular calcium. However, Clq activation cannot explain the major component of the \(\text{A}\beta40\) response that is due to calcium influx, although it does illustrate the possibility that \(\text{A}\beta40\) could be inducing a \([\text{Ca}^{2+}]_i\) response through more than one signaling pathway.

Yet another explanation for the actions of \(\text{A}\beta40\) on \([\text{Ca}^{2+}]_i\) in microglia is possible. If the peptide acted as an inhibitor of the ER calcium pump (SERCA inhibitor) then a leak of calcium from the ER could result in a slowly developing influx of calcium. Thus rather than an all or nothing signal from the ER to activate the SOC pathway, an enhancement of a calcium leak channel would occur. Such channels have been proposed previously and may even be active in regulating normal basal \([\text{Ca}^{2+}]_i\) levels [Bode 1996]. However, this mechanism does not explain the lack of a \([\text{Ca}^{2+}]_i\) increase with \(\text{A}\beta40\) application in Ca-free PSS experiments since a small leak of calcium from the ER should be observable. It is quite possible that such a calcium leak from ER stores may be too small to be detected with the imaging apparatus used in these experiments.
The results from this study are consistent with previous studies that have shown that Aβ peptides cause an elevation of [Ca$^{2+}$]i in rodent and human microglia as well as monocyte cell lines [Combs 1999, Korotzer 1995, Silei 1999]. Previous work by Silei (1999) has also shown that the actions of Aβ25-35 on [Ca$^{2+}$]i in human microglia are mainly due to the influx of extracellular calcium which they attribute to voltage sensitive calcium channels. However, studies have shown that voltage gated calcium channels are not found in human microglia [Elda 1998] and the channel inhibitors used in the Silei study could be having non-specific effects. Nevertheless, the finding that Aβ induced a [Ca$^{2+}$]i response which is primarily mediated by a calcium influx pathway is consistent with the results of this thesis.

Recent work from Combs et al. (1999) is also relevant to the discussion of the present results. They showed that aggregated Aβ40 (40 μM) causes a [Ca$^{2+}$]i increase in THP cells which was attributed to the mobilization of calcium from internal stores. However, examination of their data indicates that calcium influx is the primary contributor of the [Ca$^{2+}$]i increase induced by Aβ40. Specifically, the [Ca$^{2+}$]i rise in Ca-PSS is roughly three times greater than the rise induced in Ca-free PSS. Thus it appears that the primary component of the Aβ40 response in the Combs study is actually due to influx, which is consistent with the results with human microglia reported in this thesis.

Although the experiments from this first study have not pinpointed the precise mechanism of calcium entry into microglia as a result of Aβ40 treatment, they have narrowed the focus of the search and have characterized the features of the [Ca$^{2+}$]i response. The major action of Aβ40 is an influx of extracellular calcium into microglia independent of the SOC pathway that is maintained even when Aβ40 is removed from the extracellular solution. Intracellular stores of calcium may also contribute a small part to the [Ca$^{2+}$]i rise. A future set of experiments that could determine the
pathway of calcium-influx would be to screen inhibitors of the Clq receptor, as well as Aβ-channels, and modulators of the SERCA pump in order to look for modulatory actions on the Aβ-induced influx. If the actions of the Aβ40 induced rise in 
\[ \text{Ca}^{2+} \] can be narrowed down to a precise receptor pathway or influx mechanism then it is possible that the over-activation of microglia in cases of neurodegeneration can be controlled and the progression of diseases such as AD can be slowed or even halted. The next two studies move on from the initial signaling actions of Aβ40 to functional consequences of Aβ40 signaling.

4.4. Aβ40 induced COX-2 expression in microglia

This study moves its focus downstream from the Aβ40 \([\text{Ca}^{2+}]\), signal to the examination of Aβ40 actions on COX-2 expression in human microglia. It was undertaken because of a large body of evidence showing that COX-2 is upregulated in conditions of inflammation, ischemia, and neurodegeneration such as in cases of AD. It is also thought that inhibition of COX-2 is how NSAID type drugs exert their anti-inflammatory effects. Furthermore, pro-inflammatory cytokines and activators of microglia also have been shown to upregulate COX-2 expression [Minghetti 1998]. Therefore it appears that COX-2 expression is enhanced in response to inflammation thus Aβ40, a further activator of microglia, has the potential to also upregulate COX-2 expression.

The results indicate that application of Aβ40 (4 and 10 μM) is able to enhance COX-2 expression in human microglia. As mentioned in the discussion, COX-2 can exert neurotoxic actions through vasoconstriction and the production of reactive oxygen species [Minghetti 1998]. Thus Aβ40 has the potential to cause neurotoxic effects in microglia by enhancing the expression of COX-2. Furthermore, since COX-2 is over-expressed in the pathology of AD, Aβ can account for yet another physiological symptom of AD since this data shows that Aβ40 also enhances COX-2 expression. Although increasing the expression of COX-2 in microglia has the potential to cause
neuronal damage COX-2 is an enzyme in prostaglandin synthesis that has the capability of
catalyzing the production of a number of different prostaglandin products that have a diverse range
of functions [Aloisi 1999, Minghetti 1998]. Therefore the end result of increased COX-2
expression in microglia, be it neurotoxic or not, remains unclear.

The rationale for this study was to link the imaging studies on Aβ40 mediated [Ca^{2+}]_i
signaling with changes in the cellular function of microglia. Ideally a direct link between the Aβ40
[Ca^{2+}]_i signal and COX-2 expression would be established with a procedure that could be applied to
both experimental situations which would modulate both the calcium signal and COX-2 expression.
However, since the precise mechanism of the Aβ40-induced [Ca^{2+}]_i response was not determined, a
pharmacological agent that could be applied to block the actions of Aβ40 on [Ca^{2+}]_i was not
identified. Although Ca-free PSS and low Cl⁻ PSS did inhibit the Aβ40-induced calcium response
the use of such solutions in an attempt to modulate Aβ40-induced COX-2 expression would not be
feasible since long-term exposure to such solutions would cause many changes in microglia
independent of Aβ40 effects. Thus, it can only be hypothesized at this time that the [Ca^{2+}]_i
response to Aβ40 could contribute to the increase in COX-2 expression observed in this study.
Future experiments that examined the effects of agents which blocked the Aβ40-induced [Ca^{2+}]_i
response on COX-2 expression would be useful in determining the characteristics of Aβ40
signaling in microglia.

Since the effects of COX-2 expression in microglia remains unclear another set of future
studies that assay for prostaglandin production and characterize the inflammatory effects of the
prostanoid products produced would be useful in determining the implications of COX-2
expression as a result of Aβ40 treatment. In this way the specific downstream products that are
produced due to COX-2 upregulation can be determined and therapeutic approaches that target
these end products can be developed. Drugs that are designed to control these end products of COX-2 expression would potentially have a greater therapeutic impact then targeting the COX-2 enzyme with NSAID drugs since the inhibition of COX-2 causes the inhibition of prostaglandins that are both inflammatory and anti-inflammatory in nature [Minghetti 1998]. The third study addresses another possible action of Aβ40 on microglia function, the production of neurotoxic substances, of which enhanced COX-2 production may be a contributing factor.

4.5. Aβ40 induction of neurotoxic substances from microglia

The production of an Aβ-induced neurotoxin in microglia is an important concept in relating microglia to the pathogenesis of AD and it has been documented in rodent microglia, macrophage cell lines, and postmortem human microglia [Giulian 1996]. However, no previously published work has examined the potential of Aβ40 in inducing the production of neurotoxic substance in fetal human microglia. If neurotoxin production was found in Aβ40 treated microglia then it would provide direct evidence that Aβ40 treatment can be linked to a dominant pathological symptom of AD. The other three studies, while being related to the pathology of AD, are not end point functions of neurodegeneration that can be linked to the cognitive deficits seen in AD. [Ca^{2+}]_i signaling is an initial event that can lead to many different outcomes, COX-2 is elevated in the AD brain but its role in pathogenesis is still not fully understood, and the role that diminished microglia responsiveness has in neurodegeneration is at this time purely conjecture. All of the other studies have the potential to illuminate the pathogenesis of AD but neurotoxicity is an endpoint symptom that can easily explain the behavioral outcome of AD. Thus this was an important study not only from the view that Aβ40 induced neurotoxicity in human microglia is novel, but it was also examining the possibility that Aβ40 can cause a direct pathological symptom that is seen in AD.
Two standard molecular assays; MTT, which determined cell viability, and LDH, which determined cell death, measured the level of neurotoxicity. The LDH assay proved ineffective since all the treatment groups had similar low levels of cell death. The MTT assay on the other hand showed that both Aβ40 treated microglia and Aβ40 alone caused a decrease in neuroblastoma viability without microglia involvement. Furthermore, the decreased level of neuroblastoma viability was not significantly different from neuroblastoma treated with supernatant from microglia and Aβ40. Thus the results from the MTT assay indicate that no conclusions as to the ability of microglia treated with Aβ40 to produce neurotoxic substances can be made. The possibility remains that Aβ40 treatment of microglia does cause the production of neurotoxic substances but the methods used were unable to show this effect.

Assuming that human microglia do react to Aβ40 and produce neurotoxins then it becomes a matter of stimulating the microglia enough so that the neurotoxin production of microglia in response to Aβ40 becomes greater than the neurotoxic effects of Aβ40 alone. This issue was briefly touched upon in the results section, but to paraphrase it once again, there are two obvious courses of action that could be taken. The first method would be to increase the incubation time of Aβ40 with microglia cells, with a greater time frame of Aβ40 treatment it is possible that the microglia will produce more neurotoxic substances. Another possible approach would be to increase the density of microglia per treatment well. The reasoning being that a greater number of microglia cells could increase the concentration of neurotoxin in the supernatant. Thus it is possible that a slight alteration in the procedure would be able to document the induction of neurotoxic substances in microglia as a result of Aβ40 application.

However, one of the greatest difficulties in performing these experiments is the amount of microglia cells that are needed. In order to have a single well of microglia approximately $6 \times 10^4$
cells are needed. Thus one run of three concentrations of Aβ40 (0, 4, and 10 μM) would use $1.8 \times 10^5$ microglia cells. This large amount of microglia is often difficult to obtain due to factors discussed earlier on of the use of microglia cultures in section 4.1. Thus, in addition to the protocol changes suggested previously, it would be useful to determine the neurotoxin inducing potential of Aβ40 in an appropriate microglia cell line. A cell line could be used in this work to optimize the experimental conditions needed for Aβ40 induction of neurotoxic substances. Once the neurotoxic potential of Aβ40 was characterized in a cell line then experiments using the same optimized methods with primary human microglia cultures could be preformed. In this way valuable primary cultures of human microglia would be used more efficiently and would not be needed for the assessment of experimental protocols.

4.6. The actions of Aβ40 on ATP and PAF $[Ca^{2+}]_i$ responses

This study examined the effects of Aβ40 exposure on the reactions of human microglia to ATP and PAF. Preliminary data from this laboratory suggests that responses to both ATP and PAF are attenuated and basal $[Ca^{2+}]_i$ levels are elevated in human microglia from AD cases. These preliminary results suggest that normal $[Ca^{2+}]_i$ signaling is altered in AD microglia.

The effect of Aβ40 on human microglia was studied in an effort to determine if Aβ has a role in causing this alteration of $[Ca^{2+}]_i$ signaling that is observed in AD microglia. The results indicate that Aβ40, over both a 20 and 48 hrs time frame (approximately one and two day treatment), was able to cause elevated $[Ca^{2+}]_i$ levels and attenuated PAF responses in both ER store depletion (amplitude peak) and SOC amplitude ($[Ca^{2+}]_i$ plateau). However, ATP responses in Aβ40 treated microglia were not diminished. Although both ATP and PAF utilize similar stores of internal calcium the signaling pathways for these agents are different. Thus it appears that Aβ40 is able to disrupt $[Ca^{2+}]_i$ signaling in response to PAF but not ATP. These data provide evidence that
Aβ40 is able to alter normal \([\text{Ca}^{2+}]_i\) signaling responses in microglia. The results also support the theory that Aβ40 actions on microglia cause some of the alterations in cellular signaling observed in AD since diminished PAF responses were also observed in preliminary work with AD microglia.

One explanation for the elevated \([\text{Ca}^{2+}]_i\) levels observed in the Aβ40 treated cells can be drawn from the results obtained from the first study (section 3.1.). A clear result from the first study is that Aβ40 induces a SOC independent influx pathway in microglia. Therefore, it would be reasonable to hypothesize that long-term activation of this Aβ40-influx pathway could cause increased basal \([\text{Ca}^{2+}]_i\) levels.

Raised \([\text{Ca}^{2+}]_i\) levels may also explain a possible reason for the decreased levels of SOC influx seen in the PAF response of Aβ40 treated cells. Since the purpose of the SOC is to replenish stores after depletion, if another influx pathway was activated previously then there would be less need for a large influx through the SOC since there would be more available calcium already present in the cell. However this possibility still does not explain the attenuated PAF amplitudes and there is no current evidence to support this theory. Furthermore, the use of calcium for signaling within the cell is often very localized to specific domains of activation [Verkhratsky 1996]. Therefore an influx of calcium through one pathway may not necessarily supply another calcium signaling pathway.

The clear result of this study is that Aβ40 is capable of altering normal \([\text{Ca}^{2+}]_i\) responses of microglia. Furthermore, since attenuated \([\text{Ca}^{2+}]_i\) responses are also seen in AD microglia, this data provides evidence that Aβ40 can account for yet another physiological symptom of AD. The similarity in the responsiveness of adult human AD microglia and fetal human microglia also supports the use of primary fetal microglia cultures as a viable model in the study of disease in the adult human brain.
4.7. Final Summary

Research undertaken for this thesis provides evidence that Aβ40 activates microglia primarily through a SOC-independent calcium influx pathway in human microglia. A small internal stores component may also be present in response to Aβ40 since removal of extracellular calcium did not completely inhibit the [Ca\(^{2+}\)]\(_i\) rise. These experiments characterized the initial [Ca\(^{2+}\)]\(_i\) signal in response to Aβ40 and the further two studies focused on downstream functional effects of this [Ca\(^{2+}\)]\(_i\) signal. However, a direct connection between the Aβ40 [Ca\(^{2+}\)]\(_i\) signal and downstream functional effects cannot be established at this time since no pharmacological intervention is available that is capable of blocking the actions of Aβ40-mediated [Ca\(^{2+}\)]\(_i\) signaling without compromising cell viability.

In the second study it was determined that Aβ40 enhances the expression of COX-2 in human microglia. While COX-2 has been shown to produce neurotoxic free oxygen species, COX-2 also produces many different prostaglandins with diverse functions, thus the full implications of this increase in COX-2 expression remain unclear. Future experiments in this area should focus on the expression of the prostaglandins that are produced in response to Aβ40 treatment and subsequent COX-2 expression.

The third study examined the potential of Aβ40 to induce the production of neurotoxic substances in human microglia. The production of neurotoxic substances from Aβ40 treated microglia was not found. However, this might be due to the limitations of the methods used, which relied on the availability of a large number of cultured human microglia.

The final study focused on Aβ40's potential to cause alterations in normal [Ca\(^{2+}\)]\(_i\) responses in microglia. This work was pursued because of preliminary data showing that AD microglia have elevated basal [Ca\(^{2+}\)]\(_i\) levels and attenuated ATP and PAF responses. Aβ40 treatments of fetal
human microglia caused elevated [Ca$^{2+}$]$_i$ levels and attenuated PAF responses. These results show that an *in vitro* model of Aβ application to cultured microglia can mimic the characteristics of AD microglia.

This thesis focused on a continuum of Aβ40 actions in microglia; from the initial [Ca$^{2+}$]$_i$ signal to the functional expression of enzymes and the putative production of neurotoxic substances. In addition, it provided evidence the Aβ40 alters the normal [Ca$^{2+}$]$_i$ responses in fetal human microglia; a result which preliminary work suggests is also a characteristic of AD microglia. These studies provide fresh insight into the involvement and role of microglia in diseases of the brain and new directions in future research have emerged as a result.
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