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TRIPTOLIDE IS A POTENTIAL THERAPEUTIC AGENT FOR ALZHEIMER'S DISEASE

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TRIPTOLIDE IS A POTENTIAL THERAPEUTIC AGENT FOR ALZHEIMER'S DISEASE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

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Major Director: Dr. Guo-Huang Fan
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Mounting evidence indicates an involvement of inflammation in the pathogenesis of Alzheimer's disease. While there are other mechanisms involved, it is this role of inflammatory processes that we wish to investigate. Triptolide is the major constituent in the Chinese herb, *Tripterygium wilfordii* Hook F, and has been used for centuries as part of Chinese herbal medicine. The four ringed structure has close homology to drugs of the steroid class and it has been shown to be beneficial as an anti-inflammatory for rheumatoid arthritis and for treatment of certain cancers. The aim of this study was to evaluate the potential therapeutic effect of Triptolide on the neuropathology and deficits of spatial

learning and memory in amyloid precursor protein (APP) and presenilin 1 (PS1) double-transgenic mice, a well established Alzheimer's disease (AD) mouse model. After treatment of APP/PS1 mice with Triptolide (40μg/kg, three times weekly,), initiated when the mice were 5 months old, for as little as 8 weeks, significant decrease in β-amyloid (Aβ) deposition and microglia activation was observed. Moreover, Triptolide treatment robustly rescued spatial memory deficits observed in APP/PS1 mice. However, APP processing, tau hyperphosphorylation, and the activities of the two major kinases involved in tau hyperphosphorylation, cyclin dependent kinase 5 (cdk5) and glycogen synthase kinase 3β (GSK3β) were not affected by the Triptolide treatment. Based on the recent finding for the inhibitory effect of Triptolide on Aβ-induced production of pro-inflammatory cytokines from microglia, we propose that Triptolide treatment may have beneficial properties in halting glial activation and help restore an immune system that fights plaque deposition. Although the exact mechanism of action has yet to be deduced, the increase in APP CTFs while having a significant decrease in amyloid plaque deposition suggests that alterations in gamma secretase activity may be a possible answer. Currently, these results support the use of Triptolide as an effective therapeutic to prevent the progression of Alzheimer's disease.

Key words: Alzheimer's disease, triptolide, APP processing, microglia, astrocytes

Introduction

Today, Alzheimer's disease (AD) is the number one cause of dementia in the elderly. Dementia refers to a progressive decline in cognitive function due to neuronal damage that is unlike the normal aging process. As of now, there is no cure and the degeneration of neurons coupled with secondary complications from sedentary behavior will eventually lead to the death of the patient. It was first described by Alois Alzheimer in 1901, a German psychiatrist who described his patient as having symptoms of strange behavior and short-term memory loss. Subsequent examination of the patient's brain showed amyloid plaque deposition and neurofibrillary tangles, two major hallmark pathologies associated with AD. Current treatments consist of acetylcholinesterase inhibitors (donepezil, galantamine, rivistigmine) and the drug memantine, which is an NMDA receptor antagonist. The NMDA receptor is an ionotropic glutamate receptor which when activated will open and allow the flow of cations in and out of the cell. Synaptic plasticity plays an important role for learning and memory and calcium flux through the NMDA receptor upon activation is thought to be critical in this process. It is well known that AD brains show a reduction in cholinergic neuron activity due to degeneration of these neurons, thus acetylcholinesterase inhibitors are used to increase the concentration of acetylcholine in the brain, thereby maintaining the activity of cholinergic neurons. Although hotly debated, some believe that excitotoxicity from excess glutamate is also a feature of AD and memantine's activity at NMDA receptors blocks this

overstimulation from glutamate. These drugs represent symptomatic treatments, not mechanistic or cause-based treatment and thus have no indication for delaying or halting the progression of neurodegeneration. As a side note, part of memantine's controversial mechanisms of action are a result of some clinical trials showing that patients receiving memantine retained cognitive functions longer than those not taking it, suggesting a delay in the progression of AD.

The need for a definitive treatment that has the ability to halt or delay further neurodegeneration cannot be emphasized enough. Currently, there are approximately 5 million cases of AD in the United States, costing over $350 billion per year. These numbers are expected to escalate to over 15 million cases at a cost of over $1 trillion per year by 2050. This is predicted because the average lifespan has more than doubled since 1840 and furthermore, we face increasing numbers of people over the age of 65 that require more and more medical care (Figure 1). If nothing is done to reverse and/or treat this disease, we face an epidemic of neurological diseases that encompass not only AD, but Parkinson's, Amyotrophic Lateral Sclerosis (ALS), and many more.

Percentage of Older Adults who have Alzheimer's Disease, by Age

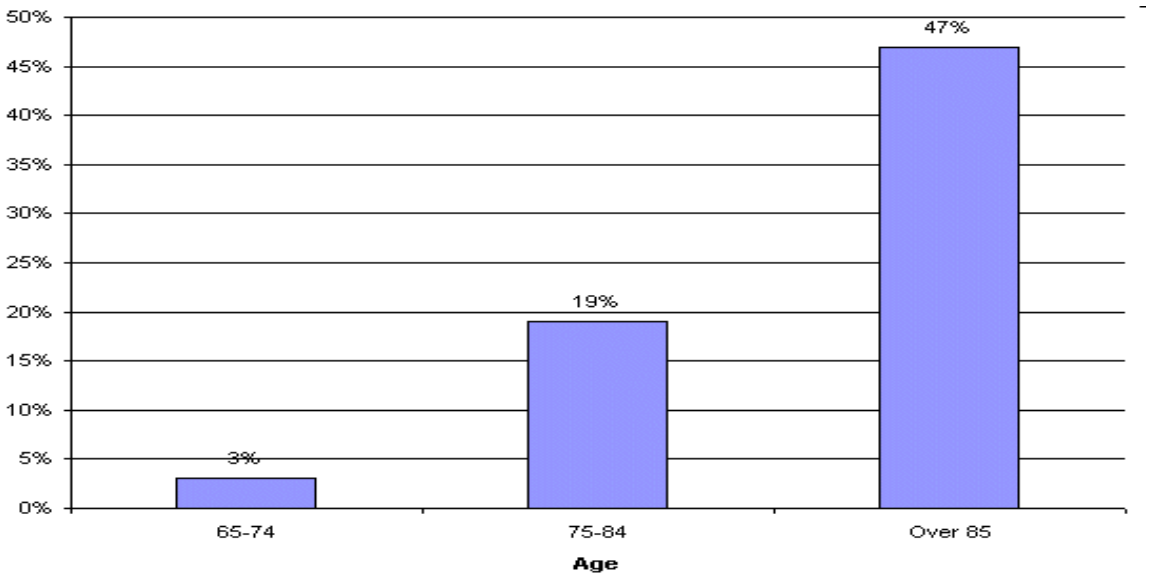


Figure 1 Chart reflecting the percentage of adults in the United States who have AD. As the age of the population increases, the chances of developing AD increases exponentially.

(Figure from **http://aspe.hhs.gov/health/Reports/medicalinnovation/, US Dept. of HHS)**

APP Processing and the Amyloid Hypothesis

Aβ plaque deposition begins when the amyloid precursor protein (APP), a type I transmembrane glycoprotein, undergoes endoproteolytic cleavage by β-site APP cleaving enzyme 1 (BACE1, β-secretase), producing a secreted ectodomain of APP and the C99 membrane-bound fragment of the remaining APP protein. The C99 fragment becomes the substrate for subsequent cleavage by another protease, γ-secretase, which produces the carboxyl terminus of Aβ and subsequently, the mature peptide is secreted from the cell to aggregate with other Aβ peptides to form amyloid plaques. The cleavage by γ-secretase is

not always specific and will produce fragments that vary in their amino acid length. However, the majority of fragments produced by γ-secretase contain either 40 or 42 amino acids, referred to as $A\beta_{40}$ and $A\beta_{42}$. It has been shown that insoluble, oligomeric $A\beta_{40/42}$ (Jiao, Xue et al. 2008) is more pathologically relevant to the degenerative effects of AD than other monomers and oligomers of different lengths. Another enzyme involved in APP processing, α- secretase, cleaves APP in the middle of the Aβ domain which precludes the formation of Aβ plaques. This can be considered 'normal' APP processing and is referred to as the non-amyloidogenic pathway in APP processing (Vassar 2002). The importance of BACE1 in the progression of AD is that it has been identified as the first enzyme that cleaves the APP protein and results in an increase in Aβ deposition. This may be the initial cause which "gets the ball rolling" in a pathological state. Indeed, the introduction of BACE1 siRNA through lentiviral vectors has been shown to ameliorate AD pathologies and neural deficits in vivo (Singer, Marr et al. 2005).

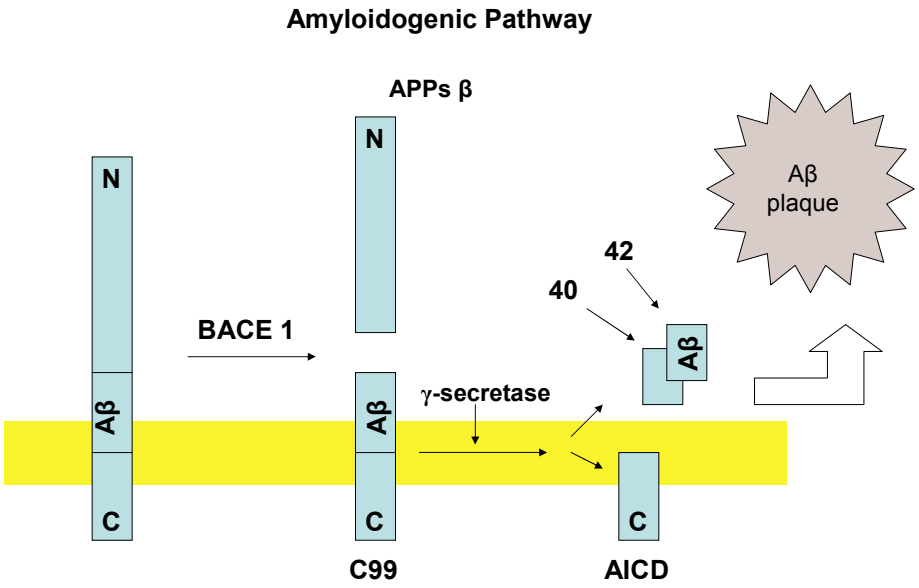


Figure 2 Diagram of APP processing. In the amyloidogenic pathway, sequential cleavage of APP by BACE1 and γ-secretase yields fragments of Aβ 40 and 42, referring to the amino acid length. These dimerize and oligomerize into amyloid plaques.

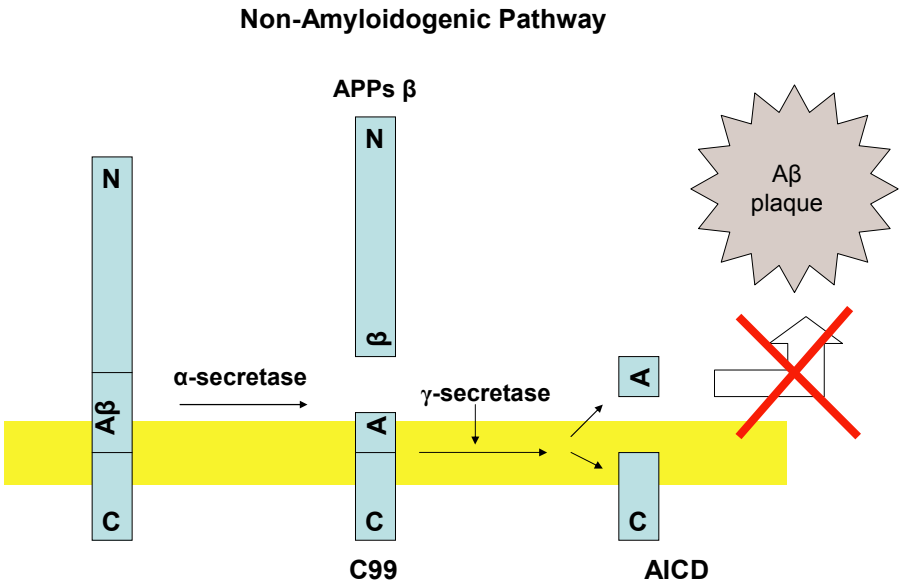


Figure 3 In the non-amyloidogenic pathway, APP is sequentially cleaved by α-secretase followed by γ-secretase which yields CTFs and AICD. The cleavage of APP is within the Aβ domain which precludes the formation of Aβ plaques. This can be thought of as the "normal" processing of APP.

Until recently, the amyloid hypothesis was by consensus the leading theory on which the research community based their work around. It allowed scientists to focus on a specific set of pathologies that could possibly yield therapeutic breakthroughs. Part of the reason that there has been some malcontent among the scientific community regarding the amyloid hypothesis revolves around the thought that all amyloid oligomers and plaques are toxic. While Aβ oligomers are clearly implicated in synaptic interruption, it is difficult to definitively say that Aβ is toxic to neurons since no convincing data has yet to be presented. Also, reports of successfully clearing of Aβ plaques from mice brains using immunization techniques does not clearly indicate the effects of Aβ on behavioral changes

(Schenk, Barbour et al. 1999). As far as clinical success with immunization techniques, it was concluded that although successful clearance of Aβ can be achieved, the end result of a rescue in cognitive function was not guaranteed since late stages of the disease are irreversible. However, administration of a vaccine or antibodies early in the disease progression could offer hope in delaying or halting the neurodegeneration (Boche, Zotova et al. 2008; Hardy 2009). Finally, there is a possibility that a double hit hypothesis is implicated more in onset of late-onset Alzheimer's disease (LOAD). It provides a cause for how neurodegeneration induced by tau hyperphosphorylation appears in LOAD. But, it still leaves fundamental questions about the relationship between APP/Aβ and tau protein (Hardy 2009).

Tau hyperphosphorylation

Tau is a microtubule-associated protein abundant in neurons of the CNS and helps maintain normal neuronal functions, such as axonal transport and neuron stabilization. The components of microtubules are globular proteins called tubulins that weigh roughly 55kDa. The tubulins come in many forms but the most common forms are the α-tubulins and β-tubulins. The interaction of tau with these tubulins helps stabilize the microtubule structure and promote its assembly within the cell. This is beneficial because it permits neurite expansion. The phosphorylation of tau results in the destabilization of the microtubule assembly. This results in a breakdown of microtubules that are necessary stages in many cell cycle functions, including cell division and mitosis. In Alzheimer's disease, one of the major hallmark pathologies are aberrant structures of intracellular

neural tangles of hyperphosphorylated tau called neurofibrillary tangles (NFTs). NFTs are composed of tau proteins which aggregate to form structures called paired helical filaments (PHFs) (Maccioni, Munoz et al. 2001). These are the components to NFTs. Phosphorylation of tau occurs at more than thirty different serine/threonine residues in the AD brain by several kinases. Proline-directed kinases include GSK3β, CDK5, p38/MAPK, and JNK. Non-proline directed kinases include PKA, PKC, CaMKII, and MARK (Avila 2006). Major focus has been towards GSK3β since this kinase has been extensively studied and characterized under pathological conditions. In familial Alzheimer's disease (FAD), there are several mutations in genes that code for proteins clearly implicated in the facilitation of AD pathologies. These include APP and the presenilin proteins, PS-1 and PS-2. Missense, deletion, and silent mutations in these genes have each been shown to result in the corresponding protein's inability to bind protein phosphatase 2A, an enzyme responsible for the dephosphorylation of tau (Goedert, Satumtira et al. 2000). Consequently, the reduced ability to dephosphorylate results in a constitutively phosphorylated state of the tau protein, which leads to accumulation of NFTs.

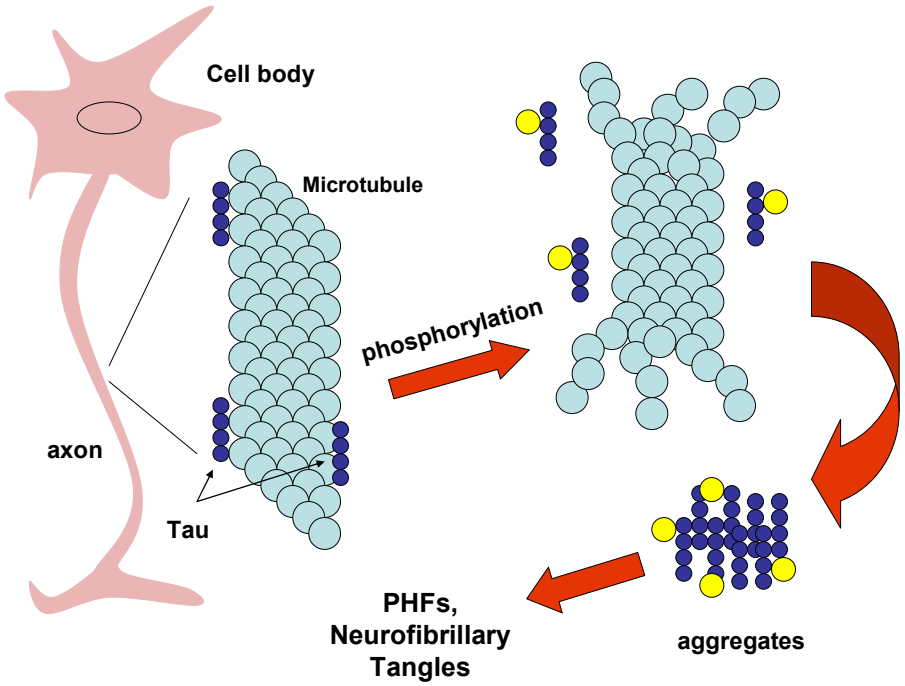


Figure 4 Tau hyperphosphorylation begins when the microtubule associated protein tau is phosphorylated by several kinases which results in microtubule disassembly and concomitant aggregation of hyperphosphorylated tau. These form the intracellular NFTs which are neurotoxic.

Amyloid deposition or Tau hyperphosphorylation?

It is still unclear which major hallmark pathology is implicated to exert the more significant cognitive and memory deficits on the diseased brain in Alzheimer's disease. The leading hypotheses have been narrowed down to amyloid plaque deposition and neurofibrillary tangles (NFTs). In the disease itself, the formation of NFTs strongly correlates with cognitive dysfunction, whereas amyloid plaque deposition does not produce cognitive dysfunction alone. Briefly, it has been studied and reported in a transgenic mouse model that tau phosphorylation plays an important and beneficial role in learning and memory and that only when tau becomes hyperphosphorylated does the cognitive decline

associated with AD and dementia begin (Boekhoorn, Terwel et al. 2006). A study published in the Journal of Neurochemistry in 2008 studied the temporal correlation between memory deficits and the activation of glycogen synthase kinase-3β (GSK-3β), which is a major kinase involved in tau phosphorylation and APP phosphorylation. The phosphorylation of these proteins facilitates the onset of the two major AD pathologies described above. The authors induced memory deficit and GSK-3β activation using WT/GFX, which has already been shown to induce spatial memory loss, and observed a time dependent correlation with GSK-3β activation and memory deficit (Wang, Zhang et al. 2008). Also, there was an increase in tau hyperphosphorylation that correlated with the memory deficit, but amyloid plaque deposition was not changed. Further, the amyloid plaque deposition increased after the memory deficits were regained by the injected mice. In this situation, these data suggest that tau hyperphosphorylation may be more important in AD-like memory deficits.

As stated above, the phosphorylation and dephosphorylation of tau is a normal process in cell function and is important in microtubule assembly/disassembly and axonal transport. If this process is natural to the living cell, then something must alter kinase and phosphatase functions involved with tau regulation. In another study, the authors sought to determine how tau might become hyperphosphorylated. They showed that aberrant glycosylation of tau proteins results in their becoming better substrates for major kinases involved in AD, such as CDK-5 and GSK-3β. The more efficient enzyme-substrate kinetics involved in tau hyperphosphorylation seems to be a possible answer because the

increase in tau phosphorylation does not correlate with an increase in CDK-5 and GSK-3 expression in AD brains.

Amyloid plaque formation is a somewhat ambiguous pathological hallmark but it is believed to be heavily involved in AD progression. There have been many studies showing that amyloid plaque formation occurs ahead of dementia and cognitive decline but this may be due to the eventual collapse of neurotrophic factors in the brain, including amyloid degrading enzymes and glial activation to remove the accumulated proteins. Also, the formation of amyloid does not always result in AD and may not even exert any form of mild cognitive impairment throughout the lifetime of affected individuals. But, the fact still remains that the presence of extracellular plaques disrupt neuronal synaptic connections and can induce a chronic inflammatory response, a situation in which neurotoxic chemokines and cytokines destroy neurons and their connections in the affected areas.

Presenilin 1 is a subunit of the gamma secretase complex which facilitates amyloid processing into insoluble fragments that aggregate into the amyloid plaques. Genetic inactivation of the PS1 subunit in transgenic mice resulted in a substantial decrease in Aβ peptides and plaque formation (Saura, Chen et al. 2005). This in turn, resulted in a brief rescue in spatial memory and learning. However, the rescue in cognitive effects only occurred temporarily in short term PS1 inactivation as opposed to long term inactivation (Saura, Chen et al. 2005). Interestingly, there is increasing evidence that other biological systems are heavily involved in AD progression other than the usual players (i.e. PS1, APP, secretases, etc.) The tumor necrosis factor death receptor 1 (TNFR1) has been implicated in the regulation of BACE1 and thus regulates amyloid plaque deposition. He et

al.used mice lacking the TNFR1 receptor and showed that the amount of plaque generated was significantly reduced. In correlation with the decrease in Aβ, the learning and memory deficits usually attributed to mice in an AD model were prevented and rescued (He, Zhong et al. 2007). This evidence further supports the suggestion that amyloid protein generation is the main cause of memory and learning deficits associated with AD. So, if the amyloid protein seems to be relevant to the issue of memory and cognitive deficits, although this is not proven yet, it is vitally important to know how and why this is occurring as opposed to just what is causing it. It has been proposed that the reason amyloid plaque deposition does not correlate well with cognitive decline is because the deposited plaque is less of a threat to synaptic connections than soluble Aβ-derived oligomers and also, the memory impairments can occur before any amyloid plaque deposition is observed. Briefly, the best correlation for AD related dementia is to observe the synaptic density and to compare it with non diseased brains and it is this loss in synaptic density explains the memory loss seen in AD patients. Lacor et al provided evidence to support the idea that the soluble oligomers of APP can initiate neurotoxic mechanisms in cultured neurons, and thereby result in abnormal and dysfunctional dendritic spines in the synapse. The authors used highly differentiated hippocampal pyramidal neurons and exposed the soluble Aβ oligomers to them. The results were that less expression of memory-related receptors, such as NMDA, occurred after exposure and that the dendritic spine morphology began to resemble spines seen in other cognitive related diseases (Lacor, Buniel et al. 2007). In addition, a study of all-trans retinoic acid (ATRA) as a potential therapeutic for treating AD has shown that ATRA can attenuate amyloid plaque deposition by inhibiting cdk5 and

BACE1, both heavily involved in APP processing (Ding, Qiao et al. 2008). The inhibition of cdk5 and GSK-3β also results in the decerease in tau hyperphosphorylation at multiple epitopes. In the Morris water maze test of the treated mice, the spatial memory deficits were comparable to those of wildtype, while the untreated APP/PS1 mice displayed a profound loss of memory and no ability of spatial memory retention. These results suggest the idea that although tau hyperphosphorylation and amyloid plaque deposition have different etiologies, they are both significantly connected in AD. Treatment in the future is almost certain to combine some form of control over kinases involved, thus allowing a measure of control over both tau aggregation and APP processing.

Genetics of Alzheimer's disease

There are two forms of AD; early onset, also known as familial AD (FAD), and late onset AD (LOAD). FAD occurs in less than five percent of the cases of AD, whereas late-onset comprises the majority of AD cases. Both forms share very similar characteristics such as plaque formation, NFT formation, and similar distribution throughout the brain even though the genetics involved are as different as night and day. Since the groundbreaking achievement of mapping the human genome, it has become ever clearer that genetics play an important role in the onset of Alzheimer's disease, especially familial AD. The human genome consists of roughly 30,000 genes. Within these genes, there are sequences of nucleic acids that contain the instructions to create proteins. Any alteration or mutation in this sequence can cause abnormal proteins to form and over time the build up of these abnormal proteins can cause disease. The genes involved with AD pathogenesis

are prevalent in early onset AD (familial AD) and manifest as an autosomal dominant form of inheritance. In familial AD cases, identification of the genes involved was narrowed to three: amyloid precursor protein (Goate, Chartier-Harlin et al. 1991), presenilin 1 (Sherrington, Rogaev et al. 1995), and presenilin 2 (Levy-Lahad, Wasco et al. 1995). Mutations in these genes have been confirmed to intiate APP processing at an earlier age and have come to be known as Swedish mutations, referring to the people whom these genes were identified from. Although the discovery of these three genes were indispensable in furthering the etiology of AD, more and more evidence has pointed to a myriad of effects that contribute to late-onset AD which comprises almost 95% of all AD cases. In late-onset AD, the overexpression of APOE ε4 has been shown to be linked to a four-fold increase in developing AD (Strittmatter, Weisgraber et al. 1993). This gene is heavily involved with cholesterol levels and consequently, incidences of heart disease are also increased with the increase in risk of AD.

Figure 5 describes the APP gene on chromosome 21. Mutations in the APP gene can result in cleavage and accumulation of the abnormal Aβ peptide which will lead to aggregation and formation of toxic neuritic plaques. It is also interesting to point out that aberrant expression of chromosome 21 (i.e. Trisomy 21, Down syndrome) in patients reveal a development of pathological hallmarks associated with AD. These include amyloid plaques, NFTs, and neuronal loss. The deposition of amyloid plaques occurs at an early age in Down syndrome patients and in late-onset AD patients, which further supports the notion that Aβ deposition precedes and may enhance other pathological changes.

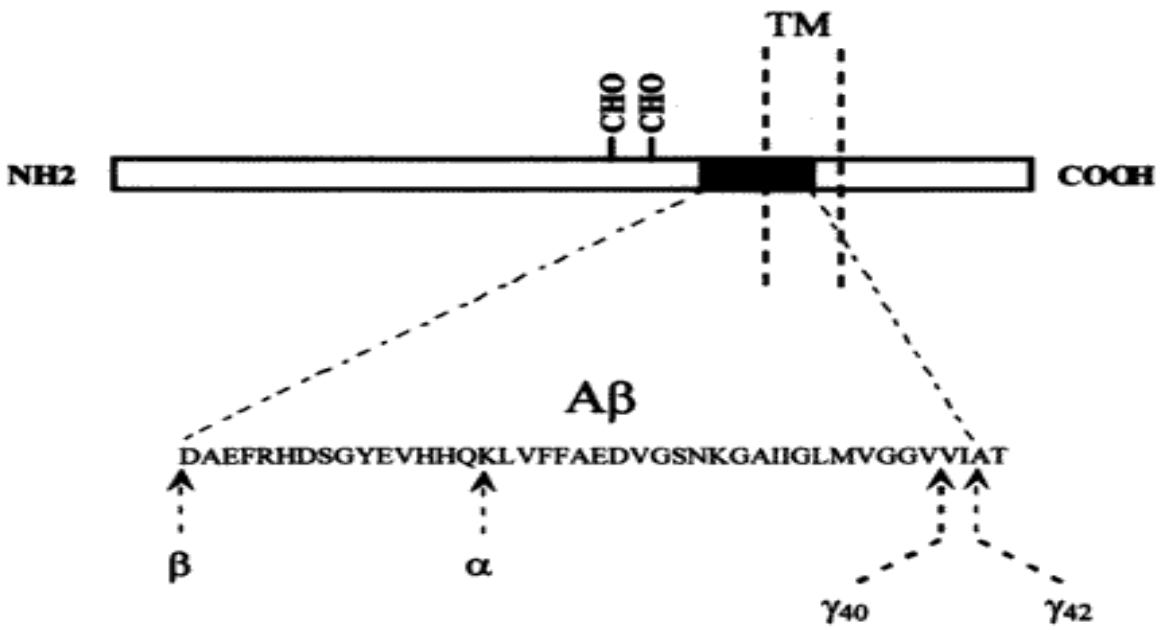


Figure 5 Schematic drawing of APP and Aβ. The vertical dashed lines represent the single membrane spanning domain and the Aβ domain is represented by the solid black box. The lower drawing shows the amino acids of Aβ domain with the cleavage sites.

(figure from http://www.scielo.cl/fbpe/img/rmc/v129n5/img14-01.gif)

Inflammatory Hypothesis

In most cases, the early symptoms of AD manifest through subtle losses in short term memory and gradually progress to total inability to recall any memory and dementia. What is interesting is that the formation of NFTs and amyloid plaque deposition do not account for every symptom associated with AD, especially in the early stages where neurodegeneration is not evident (Heneka and O'Banion 2007). One important facet of AD is the undisputed changes in inflammatory mechanism in the CNS that have been shown to facilitate the progression of AD symptoms. While it is true that all aging brains show signs of neuroinflammation, the diseased brain exhibits a much more profound case of activated

glial cells and inflammation (Heneka and O'Banion 2007). Amyloid plaques are diffusely covered with activated microglia and astrocytes. These glial cells, when stimulated, release several pro-inflammatory cytokines, chemokines, and complement that include TNF-α, IL-1β, and various free radicals (Griffin, Sheng et al. 1998). While the initial inflammatory response to foreign antigens and wound healing changes are an important component of CNS health, the chronic overstimulation and activation of the glial cells contributes to neuronal dysfunction. Once these stimulators of glial cells begin, it creates a perpetual cycle of neuronal death that is difficult to impede (Abbas, Bednar et al. 2002)(Figure 6). In brief, it has been reported that fibrillar Aβ interacts with the lipopolysaccaride receptor, CD14, which causes the cell to secrete proinflammatory cytokines that in turn activate resident microglial cells in the brain (Fassbender, Walter et al. 2004) (Yan, Zhang et al. 2003). Even more compelling is the report that microglia can kill neurons damaged by Aβ in a CD14 dependent process (Bate, Veerhuis et al. 2004). There is still much more work to be done in elucidating the exact mechanisms involved with immune system activation and response in relation to amyloid plaques because the role of fibrillar versus non-fibrillar Aβ and other forms have not been completely characterized. But, the significance of a specific receptor involved with Aβ signaling represents strong evidence that the innate immune response is an important player in the progression of AD.

Microglia are the resident macrophages within the CNS and play an important role in quick responses to foreign antigens, tumor invasion, and other forms of tissue injury. Representing roughly 10% of all cells in the CNS, they become activated and migrate in and around damaged cells to remove cellular debris and other protein fragments, such as

Aβ, through phagocytosis (Fetler and Amigorena 2005) (McGeer, Itagaki et al. 1988). In the case of AD, it has been shown that products of APP processing can activate glial cells and if this processing is inhibited, there is a corresponding decrease in glial activation (Schubert, Morino et al. 2000). As previously stated, the initial activation of microglia represents the body's reaction to a neurotoxic stress, and the microglia's ability to clear Aβ through phagocytosis is beneficial in that plaque deposition may be reduced (Yan, Zhang et al. 2003), although Landreth et al has shown that the same receptors responsible for phagocytosis cause super-oxide damage (Koenigsknecht and Landreth 2004) which contributes to neurotoxicity. Further, if chronically over activated, the constant release of cytokines initiates a perpetual cycle of neuronal death that will eventually overwhelm and destroy neuronal cells. This is the aspect of inflammation that is the target of therapeutics. If the chronic inflammatory state can be inhibited, then perhaps the beneficial aspects of microglial clearance and degradation can be enhanced and tip the balance of amyloid accumulation into a less damaging case.

Similar to microglia, the astrocytes have been shown to be able to clear amyloid plaques as well as provide neurotrophic support to neurons (Koistinaho, Lin et al. 2004) but this area is still debatable. They become over activated as well in a chronic inflammatory state. As is the case in microglia, it is not definitive that amyloid plaque deposition alone is enough to initiate this inflammatory response because it has been shown that activated astrocytes can occur in AD brains in the absence of amyloid deposition (Nunomura, Perry et al. 2001) since they respond to any neuron damage (Damiani and O'Callaghan 2007). Much work is being done now to discern the true cause

of glial activation in AD brains and whether or not this initiates APP processing or vice versa.

The human immune system is a complex network of mediators. The system as a whole must be taken into account when trying to deduce a mechanism of neuronal toxicity. Chemokines, cytokines, and other mediators such as complement can exert beneficial as well as detrimental effects on the CNS. For example, cytokines such as TNF-α and IL-1β have been shown to be able to suppress long term potentiation (LTP), a key process in memory formation, without damaging the neurons structurally (Tancredi, D'Antuono et al. 2000). Neurons communicate via chemical neurotransmitters in the synapse, and this is believed to be the underlying basis for memory formation. Although the mechanisms are still being worked out, LTP functions by strengthening synaptic connections which can strengthen and retain learning and memory skills. This clearly shows how mediators of inflammation may exert the detrimental effect well before any sign of neuronal loss is observed (Heneka and O'Banion 2007). It is a balance of these molecules that is desired to create a "homeostatic" state where the body eliminates toxic antigens and promotes neuronal integrity. Inflammation is a key process in AD pathogenesis; there is more inflammation in the AD brain than in a patient after knee replacement surgery. Interference in the process of glial activation is a novel target of drug therapeutics. Although it does not attack the amyloid deposition directly, it could be beneficial in halting the neurodegenerative aspects of AD by promoting effective clearance and degradation of Aβ.

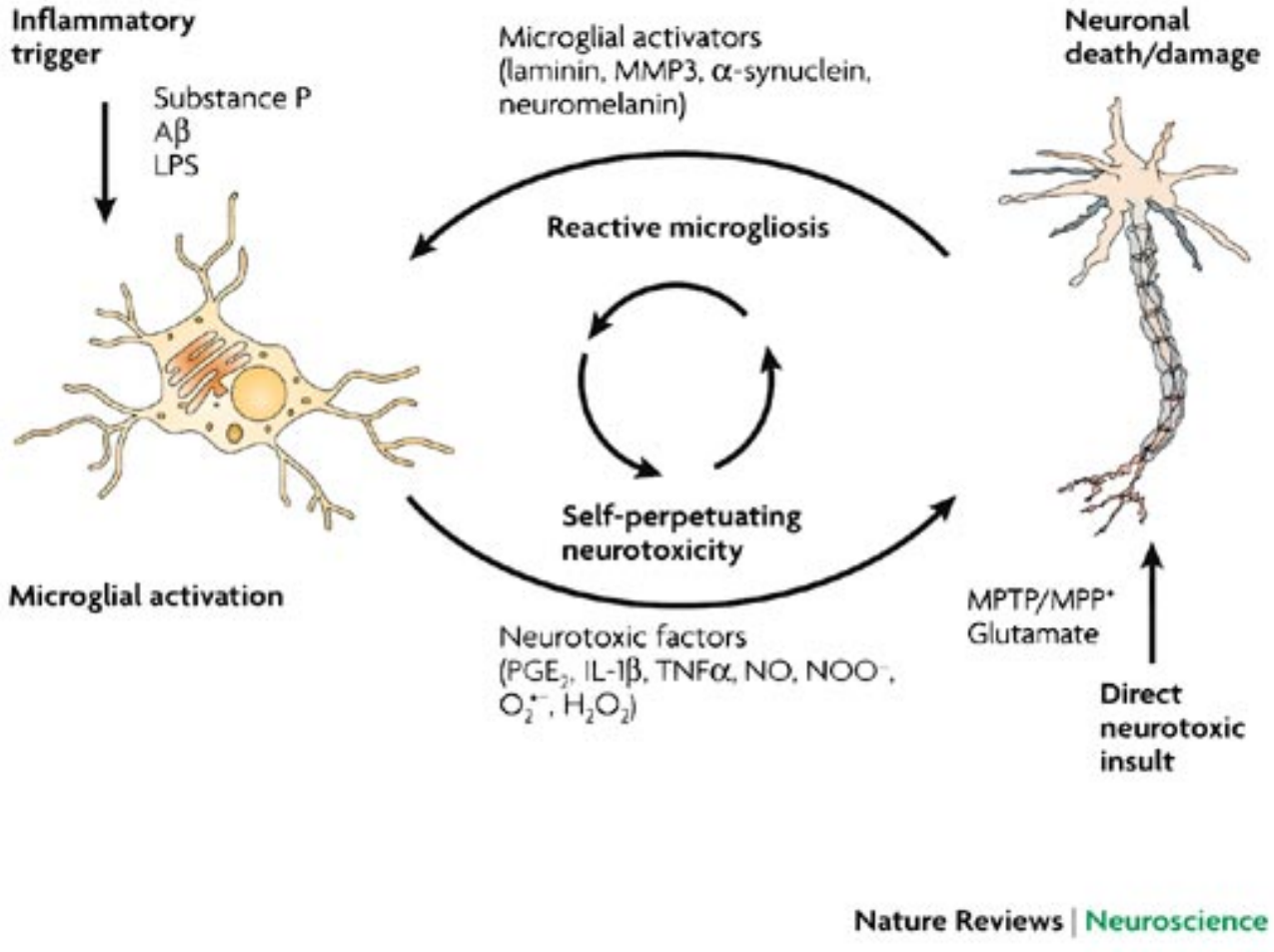


Figure 6 Diagram showing the perpetual "death" cycle initiated by activated microglia or neuronal damage. Intervention in this cycle is a therapeutic strategy that has been shown to alleviate neuronal damage and cognitive deficits associated with it.

(from Block et al. Nature Reviews Neuroscience 8, 57 69 January 2007)

Triptolide

The drug being tested in this study is a major constituent of the Chinese herb, *Tripterygium wilfordii* Hook F, called Triptolide. It is a diterpenoid triepoxide and has structural similarities with steroids (see figure 7). The rationale for choosing such a drug

can be traced to previous studies involving Triptolide being used to treat rheumatoid arthritis, an inflammatory disorder, and some cancers through its antiangiogenic properties (He, Liu et al. 2009). With the recent discovery that Alzheimer's disease brains display aberrant expression of cell cycle related proteins such as the cyclin-dependent kinases (CDKs), it was hypothesized that the use of anti-cancer drugs may have some effect in alleviating AD symptoms or even attenuates some pathology, including APP processing. For instance, treatment of APP/PS1 mice with indirubin, another Chinese herbal medicine for cancer, had alleviated AD pathologies including Aβ plaque load. It was later discovered through X-Ray crystallography that indirubin can bind to and inhibit CDK-2, eliminating its effects from over expression in diseased neurons. In the literature, Triptolide has been reported to suppress inflammation and cartilage destruction, inhibit prostaglandins via inhibition of cyclooxygenase (COX)-2, and reduce nuclear factor kappa B(NFκB) expression *in vivo* (Lin, Liu et al. 2007). Triptolide has also been shown to inhibit the growth and metastasis of solid tumors through apoptosis induction and the reduction in expression of several cell cycle related molecules (Yang, Chen et al. 2003). In this same study, it proved to be more effective than conventional anti-tumor drugs such as adriamycin and cisplatin at inhibiting tumor cell proliferation. One of the most important effects of Triptolide related to Alzheimer's disease was reported in 2008 in the Journal of Neuroimmunology. Triptolide inhibited the production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), both inflammatory cytokines known to be produced upon amyloid-β_{1-42} stimulation of neuronal cells (Jiao, Xue et al. 2008). This role of Triptolide is important because it is believed that the progression of Alzheimer's disease can be halted if

chronic activation of the neuronal glial cells such as microglia and astrocytes can be inhibited or reduced. Previous work in this laboratory has tested many other Chinese herbal anti-cancer agents and some experimental agents developed for specific inhibition of certain neural processes. These include retinoic acid, a metabolite of vitamin A, indirubin, lupeol, both anti-cancer agents, and the CXCR-2 antagonist SB225002. CXCR-2 is a chemokine responsible for many inflammatory mechanisms in the CNS. All of these drugs so far, have been proven to be beneficial in attenuating AD pathologies, such as APP processing and tau hyperphosphorylation, and able to rescue memory deficits in our AD mouse model as evidenced in the Morris Water maze acquisition test. The dose of 40µg/kg was decided as the appropriate dose to administer to the mice after carefully searching the literature and finding a study that administered varying concentrations of Triptolide to mice in order to discover its effects in arthritis (Lin, Liu et al. 2007). The effects of Triptolide were most prominent at a concentration of 32µg/kg. This corresponded to 0.625-2.5% of LD_{50} for Triptolide (1.278mg/kg). There was no evidence of drug toxicity and no viscera damage. Even more, several studies characterizing the pharmacological toxicology of triptolide in mice indicated a safe dose as 0.03 mg/kg (Xu, Pan et al. 2008). Triptolide can cross the blood brain barrier fairly easily due to its lipophilic character and size (Wang, Liang et al. 2008).

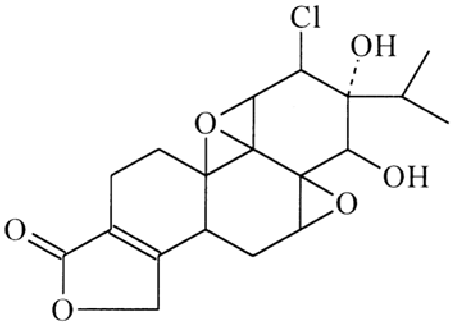


Figure 7 Structure drawing of the triptolide molecule. Its four rings are homologous to corticosteroids, thus, it has similar effects in immunosuppression.

(figure from **http://www.asiaandro.com/1008-682X/1/121f1.jpg**, *Wang et al, 1999*)

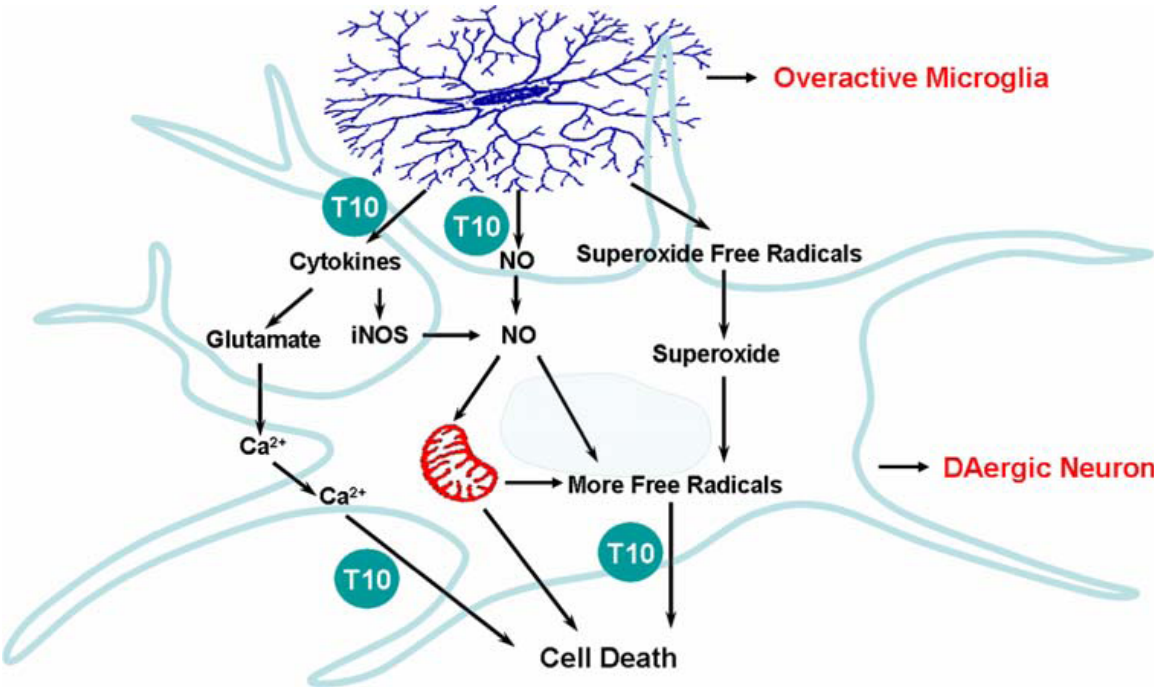


Figure 8 Mechanisms of the neuroprotective effects of triptolide. Overactive microglia in the CNS can lead to chronic release of cytokines and other inflammatory mediators which can kill dopaminergic neurons. Triptolide, aka T10, can inhibit the overactivation of microglia resulting in inhibition of the release of inflammatory cytokines, free radicals, NO, and Ca2+ overload.

(figure from *Wang, Liang et al 2008*)

Materials and Methods

Transgenic Mice and Triptolide treatment. There are many different mouse models available for the study of Alzheimer's disease. For this study, we utilized APP/PS1 double-transgenic mice from The Jackson Laboratory [strain name, B6C3-Tg (APPswe, PSEN1dE9)85Dbo/J; stock number 004462]. This particular strain of mice expresses a mutant chimeric mouse/human APP gene with Swedish mutations at K595N/M596L sites. There is also a presenilin 1 (PS1) human mutant with the exon 9-deleted variant. This variant is under the control of mouse prion promoter elements that directs the expression of these transgenes mainly to neurons of the CNS. The APP/PS1 mice were maintained as double hemizygotes by cross breeding with wild-type mice with a background strain of B6C3F1/J (stock number 100010), also from The Jackson Laboratory. To ensure proper genotype before each study, tail biopsies and PCR analysis of genomic DNA were performed. All animals were housed in the animal care facility at Virginia Commonwealth University medical campus and are in accordance with standard animal care protocols. Five month old wild-type mice and APP/PS1 mice were randomly assigned into four groups: untreated wild-type mice, treated wild-type mice, untreated APP/PS1 mice, and treated APP/PS1 mice. The treated groups received 40µg/kg of the drug Triptolide (Sigma, T3652), which was dissolved in normal saline with 5% DMSO in saline (vehicle). They were injected intraperitoneally three times weekly at 40µg/kg. The control groups received

an equal volume of 5% DMSO. Treatment of drug and vehicle control lasted approximately 8 weeks. The dose and regimen of treatment were based on previous reports describing effective therapeutic doses using Triptolide in mice and characterization of the pharmacokinetics involved (Blanchard, Moussaoui et al. 2003; Lin, Liu et al. 2007). The rationale for starting drug treatment at 5 months of age is based on previous reports that APP/PS1 mice of this particular strain begin to accumulate Aβ plaques as early as 5 months of age. Although the expression of the mutant forms of these genes do not necessarily accurately describe the cases in sporadic late-onset AD, the lesions seen in all cases point to a final common pathway of pathologies that all sufferers of AD develop, human or mouse.

Morris water maze test. After the treatment with Triptolide for 8 weeks, the memory and spatial learning was evaluated using the Morris water maze test (Morris 1984). The maze itself consists of a 120 cm diameter X 50 cm height circular and galvanized water tank. The area of the tank was divided into four equal quadrants and made opaque by the addition of milk powder. The water temperature was adjusted to 24 ± 1°C. An escape platform of 10 cm diameter was placed in one of the quadrants and submerged 2 cm below the water surface and at least 30 cm away from the side wall. Throughout the experiment, the platform was kept in the same quadrant. The mice were each gently placed in the maze always facing the tank wall, and were required to find the platform using only distal spatial cues in the testing room. For each trial, a different starting position was used. The mice had 120s to find the platform and once they found it they were allowed to remain on the

platform for 10s. Then, the mice were taken out, dried, and placed in a separate cage for ~60 minutes before the next trial. If a mouse failed to find the platform within the allotted 120s, the experimenter assisted the mouse and subsequently allowed to stay on the platform for 10s. Between the trials, the water was stirred to erase olfactory traces of the previous mice. Each animal was trained for four trials per day for five consecutive days to locate and escape onto the platform. Their spatial learning scores was measured as escape latency in seconds and recorded after each trial. In order to assess the memory consolidation, a probe trial was conducted two days after the five day acquisition tests. During this trial, the platform was removed and the mice were allowed to swim freely in the tank. Time spent in the quadrant that had the platform during the acquisition tests was taken to indicate the degree of memory consolidation. All time measurements were done using a stopwatch by an experimenter blinded to which experimental group to which each animal belonged.

Immunohistochemistry. After testing the spatial and learning memory of the mice, the mice were anesthetized with an intraperitoneal injection of ketamine (0.05 mg/kg) and perfused with PBS and subsequently 4% paraformaldehyde in PBS. The brains were removed and placed in 70, 96, and 99% ethanol solutions, respectively, for 2 hour intervals in order to dehydrate the brains completely. The brains were then left in xylene overnight and subsequently embedded in paraffin. The paraffin blocks were then sectioned horizontally by a microtome at 10µm. The sections were placed in a warm water bath and mounted onto SuperFrost Plus (Menzel-Glazer) glass slides. Sections were placed in 40° C for one hour

before being rehydrated and stained. Procedures for immunohistochemical staining were performed using coronal brain sections. The sections were deparafinned, rehydrated in various intervals of xylene and ethanol, and endogenous peroxide quenched with hydrogen peroxide [1% (v/v) in methanol]. They were then placed in 10mM sodium citrate buffer (pH 6) and microwaved for 15 minutes. After cooling to room temperature, they were incubated in blocking buffer [10% (v/v) goat normal serum (Millipore Bioscience Research Reagents) in PBSA containing 0.1% (v/v) Triton X-100 (Sigma)] for 60 minutes. Adding the appropriately diluted antibodies overnight at 4° C followed the blocking.

Histochemistry. The paraffin-embedded brain sections were first deparafinned and rehydrated in various xylene and ethanol solutions following this protocol: 15 minutes xylene I; 15 minutes xylene II; 5 minutes 100% ethanol; 5 minutes 100% ethanol; 5 minutes 95% ethanol; 5 minutes 85% ethanol; 5 minutes 70% ethanol; 5 minutes 50% ethanol; 5 minutes 30% ethanol; store in deionized water until ready for development. Subsequently, the sections were stained for the presence of Aβ plaque deposits using the Campbell-Switzer silver staining method (NeuroScience Associates). A detailed protocol for using this stain was generously provided by Dr. Bob Switzer of NeuroScience Associates in Knoxville, TN. The procedure begins after the rehydration of the brain sections by placing the sections in 2% ammonium hydroxide solution and shaking for 5 minutes then placing in deionized water for 1 minute twice. Then, the sections are covered and placed in the SPC solution, a pyridine silver solution for the induction of nucleation sites, for 40 minutes while gently stirring. After the 40 minutes, the sections are placed in

1% citric acid solution for 3 minutes while shaking. Subsequently, the slides are placed in 4.99 pH acetate buffer while the developing solution is prepared. The developing solution consists of making three separate solutions and then mixes them together to form the ABC solution. Solution A consists of 5g of sodium bicarbonate dissolved in 100mL of deionized water. Solution B consists of 0.2g of ammonium nitrate, 0.2g of silver nitrate, and 1.0g of silicotungstic acid all dissolved in 100mL of deionized water. Solution C consists of taking 20mL of solution B and adding 140µL of 37% formaldehyde. All three solutions are mixed and stirred well. This ABC solution is then added to the brain sections, making sure to completely cover the brain tissue, and then incubated at room temperature until the plaques are developed. Once the desired results are obtained, the brain slides are washed in 4.99pH acetate buffer for 3 minutes. They are subsequently washed in deionized water for 30 seconds, then a 0.5% thiosulfate buffer for 45 seconds, and then deionized water again for 2 minutes. The slides are then available for additional staining or to be mounted.

Image Analysis: Images for the DAB staining and Campbell-Switzer staining were taken with a Nikon TE2000-E inverted microscope, whereas immunofluorescent images were aquired with a Nikon TE2000-U confocal microscope under 40x oil immersion objective with numerical aperture (NA) 1.4, zoom 1.6. Fluorochromes were excited using a 488nm argon laser for FITC and a 543 nm helium-neon laser for Cy3, and the detector slits were configured to minimize any crosstalk between the channels.

Stereology: The stereological setup consisted of an Olympus BH-2 microscope (Olympus Life and Material Science Europe) with a high numerical aperture (NA 1.40) and oil immersion 100x objectives. This allows focusing in a thin focal plane inside a thick section. A camera transmits the image to a monitor on which a counting frame is superimposed using the computer-assisted stereological CAST-GRID software (Visiopharm). A motorized automatic stage was used to control movement in the *x*-*y* plane via a connected joystick. Movement in the *z* axis was done manually with the focus button on the microscope, and the distance between the upper and lower surfaces of the section and the height of the dissectors were measured with a Heidenhain microcator (model VRZ 401) with a precision of 0.5 µm. The number of Aβ plaques in the hippocampal and cortical regions were counted using the optical fractionator method of unbiased stereological cell counting techniques. Aβ plaques or cells were sampled in counting frames of 644-988 μm^2 [*a*(frame)] moved in *x* and *y* steps of 100 x 100 µm [*a*(step)]. The area sampling fraction (asf) was calculated as *a*(frame)/*a*(step). The thickness sampling fraction (tsf) was calculated as the height of the optical dissector probe (*h*) (8 or 10 µm) divided by the average height of the sections (*t*) (tsf=*h*/*t*). Aβ plaques were counted using a 20x immersion lens. Total Aβ plaque or cell number (*N*) was estimated using the following equation: $N=Q^-$ x 1/tsf x 1/asf x 1/ssf, where Q^- is the number of cells counted, and ssf is the section sampling fraction. In the case of clusters of Aβ plaques, each cluster was counted as one plaque or cell and identified by the most clearly defined nucleus. Coefficients of error and variation were calculated as described previously (Wirenfeldt, Dalmau et al. 2003). The same sections that were sampled for number estimates were used

to estimate volume of Aβ plaques in cortical or hippocampal regions. The system software superposed a point grid at random over low-power (2.5x) magnified images of each section. Total reference volume (V_{ref}) was estimated using the Cavalieri-point counting method (Gundersen and Jensen 1987), based on the sum of points that hit on each reference space, $a(p)$ is the area per point on the grid, t is the mean section thickness (in millimeters), and k is the sampling interval. The same $a(p)$ was used for estimating volume for both the cortical and hippocampal regions. The densities of Aβ plaques (number per cubic millimeter) were calculated by dividing the number counted by the total volume sampled of each reference space. The volume of sampled reference space was the number of dissectors multiplied by the volume of one dissector.

Western blot analysis. Brain tissues were homogenized in TBS (20mM Tris-HCl buffer, pH 7.4, 150mM NaCl) containing several protease inhibitors such as, 0.5mM phenylmethylsulfonyl fluoride, 20µg/mL aprotinin, 20µg/mL leupeptin, 20µg/mL pepstatin, and 1mM EDTA. Approximately, there was 0.150g of brain tissue per 2 mL of TBS buffer. The brain homogenates were briefly sonicated and subsequently centrifuged at 15,000 x g for 30 minutes. In order to determine protein concentration, the BCA protein assay (Pierce) was performed. The assay results were then used to determine proper loading amount. For each gel, 10µg of brain SDS supernatants were loaded in each well and run on 12% SDS polyacrylamide gel. The proteins were transferred at 300mA for 2 hours to a polyvinylidene difluoride membrane (BioRad). After transfer, the membranes with the newly aquired proteins were incubated for at least 4 hours or overnight in 4°C

with specific antibodies to the proteins of interest. After incubation, the membranes were washed twice in 0.1% tween 20/TBS for 5 minutes. Subsequently, the membranes were then incubated at room temperature with the corresponding HRP-labeled secondary antibody for 1 hour (concentration always 1:2000). The ECL system was used for detection of labeling (GE Healthcare). This entailed placing radiographic film over the exposed membrane in a dark room and then developing it in a Kodak film developer. To ensure equal loading, β-actin antibody was used in the same procedure outlined above at concentration of 1:5,000. The observed bands were then analyzed using densitometric software (Scion Image). The following antibodies were used for western blot analysis: rabbit polyclonal anti-phosphorylated (p)-CDK5 (Ser 159) (1:500; Santa Cruz Biotechnolgy), mouse monoclonal anti-CDK5 (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-p-tau (Ser 404) (1:300; Santa Cruz Biotechnology), rabbit polyclonal anti-p-GSK3β (Ser 9) (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-p-GSK3α/β (Tyr 279/Tyr 216) (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-GSK3β (1:500; Santa Cruz Biotechnology), mouse monoclonal anti-p-APP (Thr 668) (1:500; Cell Signaling Technology), rabbit polyclonal anti-APP-C-Terminal Fragments (CTFs) (1:3000; Sigma), mouse monoclonal anti-β-actin (1:5,000; Sigma), mouse monoclonal anti-GFAP (1:500; DakoCytomation).

Iba-1, BACE1, PS1

Statistical analysis. Presented data are expressed as the mean ± SEM and the analyses were performed using a two-way ANOVA followed by Fisher's least significant difference *post*

hoc analysis to identify effects deemed to be significant. Differences were deemed significant at $p < 0.05$.

Results

Triptolide treatment prevents Aβ plaque accumulation in APP/PS1 mice

Triptolide has been shown in the literature as a potent therapy for many cancers and rheumatoid arthritis but the effects on amyloid accumulation in APP/PS1 mice has not been characterized. After administration of triptolide intraperitonneally for 8 weeks on 5 month old APP/PS1 mice and wild type littermates, the results in figure 9 show that triptolide treatment substantially decreased the amount of Aβ plaque accumulation in APP/PS1 mice compared to vehicle treated APP/PS1 mice. The areas observed include the frontal cortex and the hippocampus, a region that is involved in memory and learning. Stereological analysis also portrays a significant decrease in the amount of Aβ plaque levels. Plaque number, average volume, and area occupied by the amyloid plaques were all reduced in both the frontal and hippocampal brain regions of the APP/PS1 mice. Also, the vehicle treated APP/PS1 mice had no significant effects when compared to untreated mice of the same genotype and age (data not shown). These results support the idea that triptolide may be a possible therapeutic agent for inhibiting amyloid plaque deposition. A caveat to these results is that out of three samples subjected to amyloid staining and stereological analysis, one set of samples did not show a decrease in Aβ levels, but rather an increase in plaque accumulation. The reason for the antithetical result has yet to be determined.

Triptolide does not inhibit APP processing

The current knowledge of amyloid processing indicates that APP plays a central role in the pathogenesis of plaque accumulation. APP CTFs are an early biomarker for the diagnosis for AD (Sergeant, David et al. 2002) and are a key step in the processing of APP. Western blot analysis using an anti-CTF antibody was used to determine whether APP was affected by the treatment with triptolide. This particular antibody recognizes the full-length APP protein as well as the CTFs. As shown in figure 10, we observed that APP expression is not changed between vehicle and drug treated mice. Interestingly, the CTFs were enhanced in the triptolide treated mice when compared to wild type. Also, the vehicle treated APP/PS1 mice did not show a significant difference in APP or CTF expression when compared to untreated mice of the same age (data not shown). To further explore the affects of triptolide on APP processing, an anti-BACE1 antibody was used to see whether the beta secretase enzyme was differentially expressed. Based on the western blot data in figure 10, it appears that BACE1 levels were not altered between the drug and vehicle treated groups. In keeping with the exploration of APP processing, an anti-phospho-APP antibody was used to see if the phosphorylation of the APP protein was affected in the drug treated mice. The phosphorylation of APP at Thr668 is important in the processing of this protein to cleavage and Aβ oligomer formation (Lee, Kao et al. 2003) and neurodegeneration (Chang, Kim et al. 2006). Again, as shown in figure 10, no significant change was seen across the group. The role of hyperphosphorylated tau is important when considering the clinical manifestations of AD. In this study, it was particularly difficult to obtain data that was consistent in showing whether tau was hyperphosphorylated or not.

Consequently, no data is presented that shows tau hyperphosphorylation in the APP/PS1 mice even though it has been well documented that these mice exhibit excessive phosphorylation at several tau epitopes (Kurt, Davies et al. 2003). Nevertheless, according to the amyloid hypothesis, the role of tau hyperphosphorylation in relation to memory loss appears to occur downstream of plaque formation and deposition which leads many to believe that its role in neurodegeneration is secondary to amyloid processing. This is, however, a very much debatable area in AD research and it should be emphasized that there is no definite consensus on how the mechanistic cascade of AD plays out. Kinases known to be implicated in AD include CDK5 and GSK3β. These are involved in tau hyperphosphorylation (Singh, Grundke-Iqbal et al. 1994; Hartigan and Johnson 1999) and APP phosphorylation (Aplin, Gibb et al. 1996; Iijima, Ando et al. 2000). According to figure 10, observing the activity of CDK5 and GSK3β, we can conclude that the administration of triptolide has no effect on the enzyme activity or expression levels. The expression remained unchanged between drug treated APP/PS1 mice and vehicle treated APP/PS1 mice. Assessing the activity of GSK3β, the results were similar to that of CDK5. The levels of protein expressed in drug treated versus vehicle treated APP/PS1 mice were unchanged, indicating that triptolide has no effect on changing the activity of GSK3β. Together, the data concludes that the activities of the major kinases of tau and APP processing are unchanged by triptolide treatment.

Triptolide treatment reduces the number of glia associated with Aβ plaques in APP/PS1 mice

It has been well documented that APP/PS1 mice as well as AD patients exhibit enhanced reactivity of glial cells such as microglia and astrocytes. These glial cells are in and around Aβ plaques and appear to be the results of an inflammatory reaction caused by these diffuse plaque depositions. This is very characteristic of inflammatory mechanisms that occur when injury presents itself in the brain (McGeer and McGeer 1999). Triptolide has been shown in vitro to inhibit inflammatory cytokines such as IL-1β and TNF-α in response to amyloid oligomer stimulation (Jiao, Xue et al. 2008). The activated microglia was visualized by immunohistochemical techniques using an Iba-1 antibody. Double staining of Iba-1 and amyloid plaques shows that activated microglia are localized in and around the deposited plaques. In figure 11, there is a significant increase in Iba-1 immunoreactivity around the amyloid plaque in the vehicle treated APP/PS1 mouse brain as compared to the triptolide treated APP/PS1 mouse brain. However, it is difficult to determine if the amount of microglial cells in the drug treated APP/PS1 mouse brain is the result of a significant inhibition by triptolide or from the significant decrease in Aβ plaque deposits.

The activated astrocytes were visualized using immunohistochemical techniques using a GFAP antibody. Similar to the microglia, the double staining of astrocytes and Aβ plaques showed that the astrocytes indeed were in and around the plaque deposits. The amounts of activated astrocytes around the plaques were significantly reduced in the triptolide treated APP/PS1 mouse brain when compared to the vehicle treated APP/PS1 mouse brain. This is similar to the microglia results but similar conclusions cannot be drawn from the data since astrocyte migration activity differs from microglia (Pihlaja,

Koistinaho et al. 2008). Thus, it is still unclear whether the reduction in glial activation is a result of triptolide's action as an immunosuppressant or from the substantial decrease in amyloid plaques.

Triptolide treatment rescues learning and memory deficits in APP/PS1 mice

The mouse model used in this study was an APP/PS1 double transgenic mouse line that is well characterized. This model has been consistently reported to develop Aβ plaques associated with cognitive deficits as the mice age (Trinchese, Liu et al. 2004). Based on the Morris water maze test, the results of our investigation in figure 12 show that after treatment with triptolide, the APP/PS1 mice exhibited a substantial improvement in memory as proven by the decrease in escape latency across trials. Improvement of memory retention in the drug treated APP/PS1 mice was also evidenced by the probe trial. In contrast, the data illustrate that the vehicle treated APP/PS1 mice portrayed an inability to acquire the learning and memory exhibited by wild type and triptolide treated mice. It should be noted that the deficits in the acquisition and probe trial were not attributable to different swim speeds since APP/PS1 mice and wild type mice exhibit similar swimming abilities. Based on these results, it can be concluded that triptolide is beneficial in retaining cognitive abilities in APP/PS1 mice.

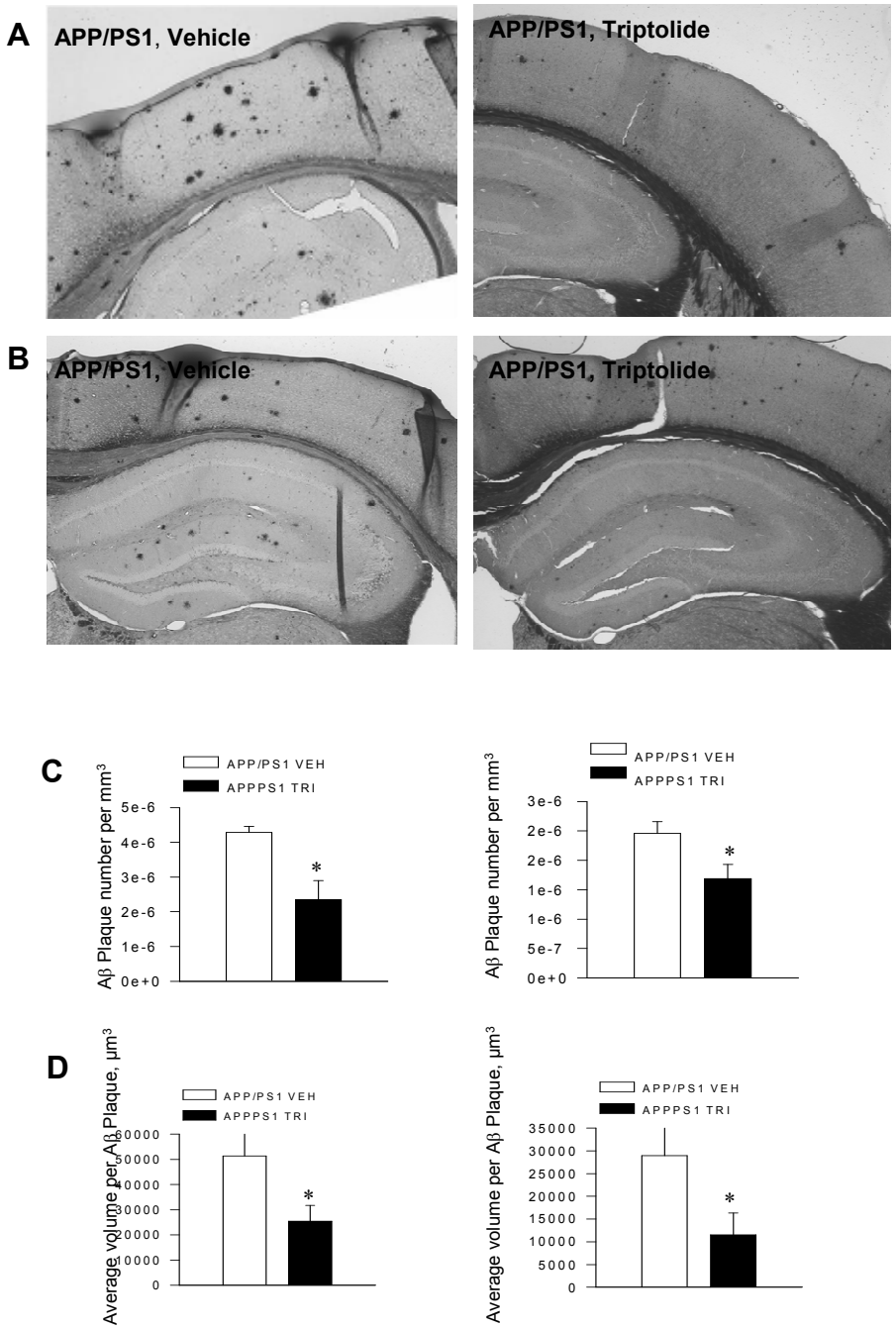


Figure 9 Triptolide treated APP/PS1 mice show reduced levels of Aβ deposits when compared with vehicle treated APP/PS1 mice. A represents Campbell-Switzer staining in the frontal cortex of

APP/PS1 mice treated with vehicle (left) and triptolide (right). B represents Campbell-Switzer staining in the frontal cortex of APP/PS1 mice treated with vehicle (left) and triptolide (right). C represents stereological quantification of Aβ plaque number in the hippocampus. D represents stereological quantification of Aβ volume in the hippocampus, all described in the Materials and Methods. Values from multiple images of each section were averaged per animal per experiment. Data are mean ± SEM from three mice per genotype. *p<0.05 versus vehicle treated control APP/PS1 mice.

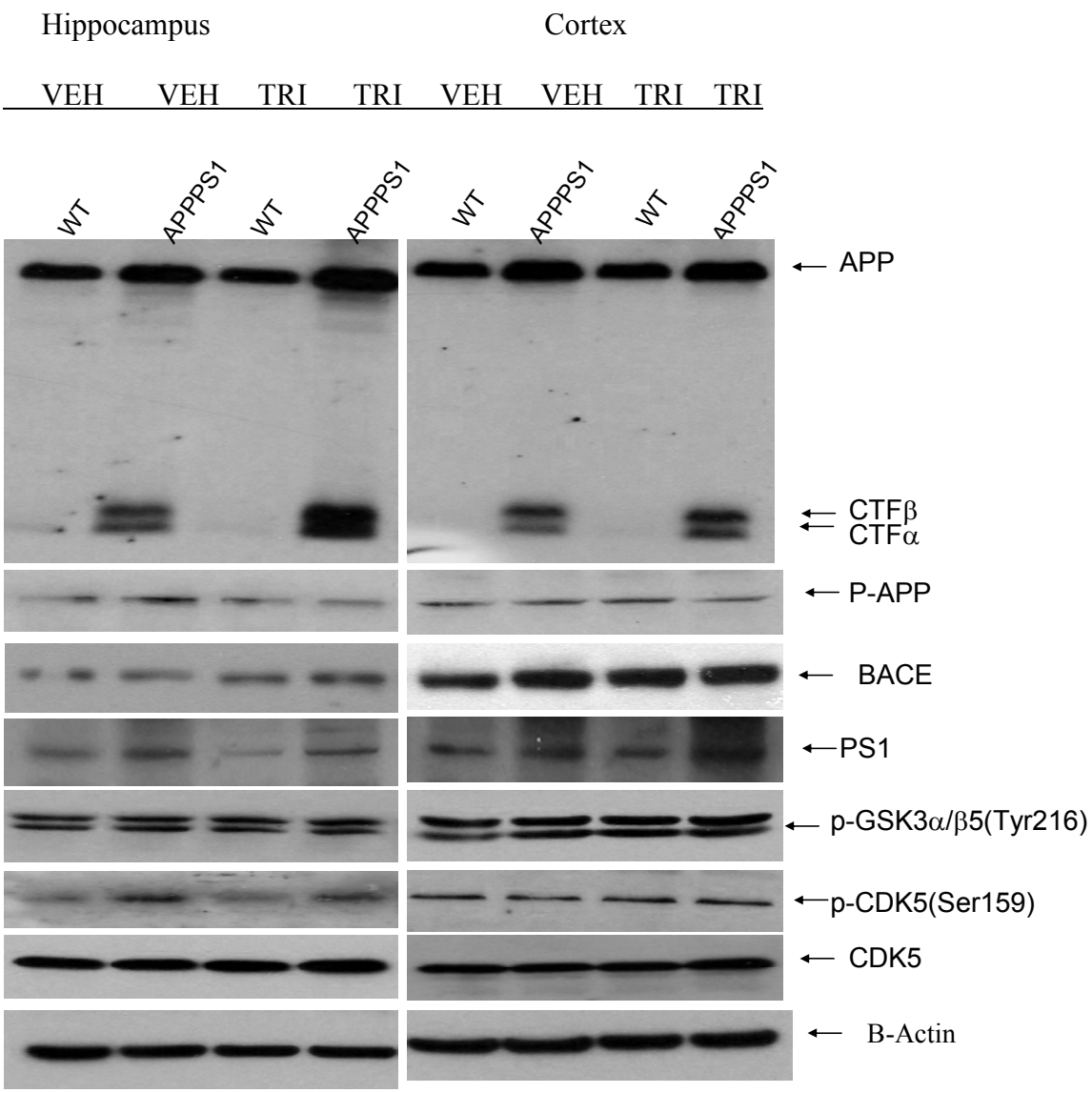
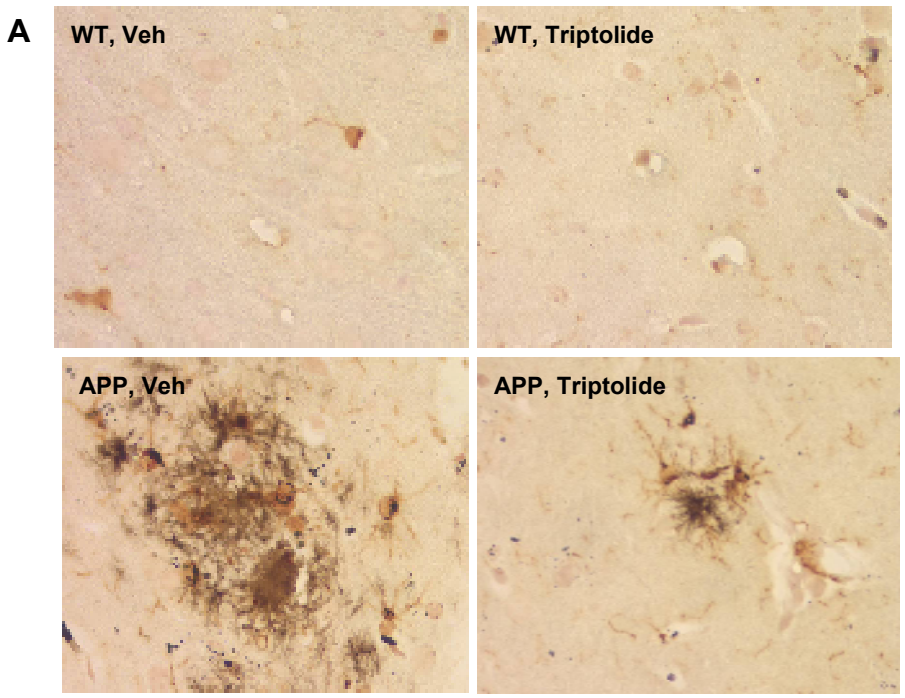


Figure 10 Triptolide treatment had no change on the expression of APP and did not reduce the amount of CTFs. Western blots for APP, APP-CTFs, phosphorylated-APP, BACE1, PS1, phosphorylated GSK3α,β (Tyr 216), phosphorylated CDK5 (Ser 159), CDK5, and β-actin.



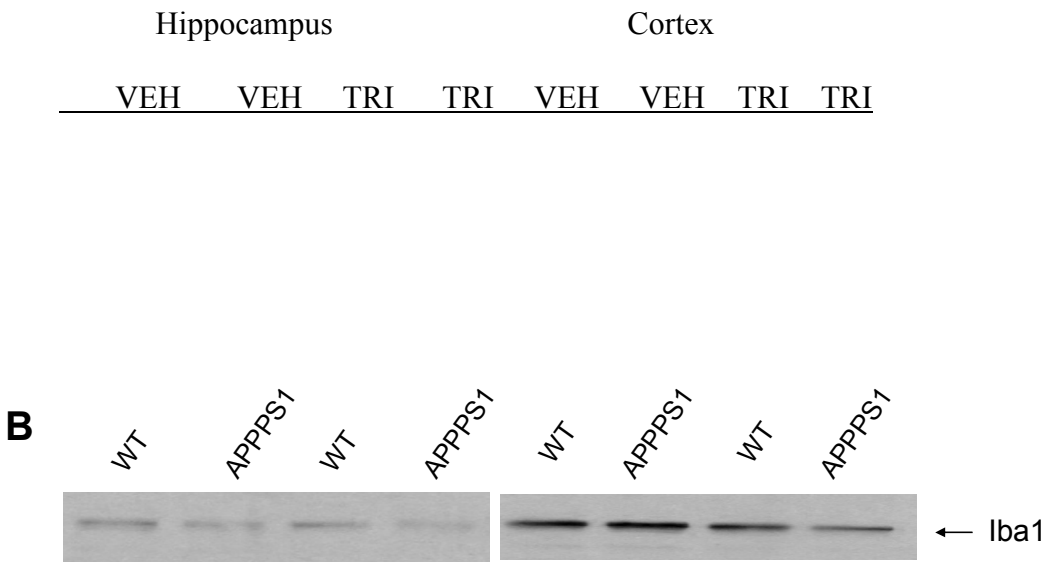
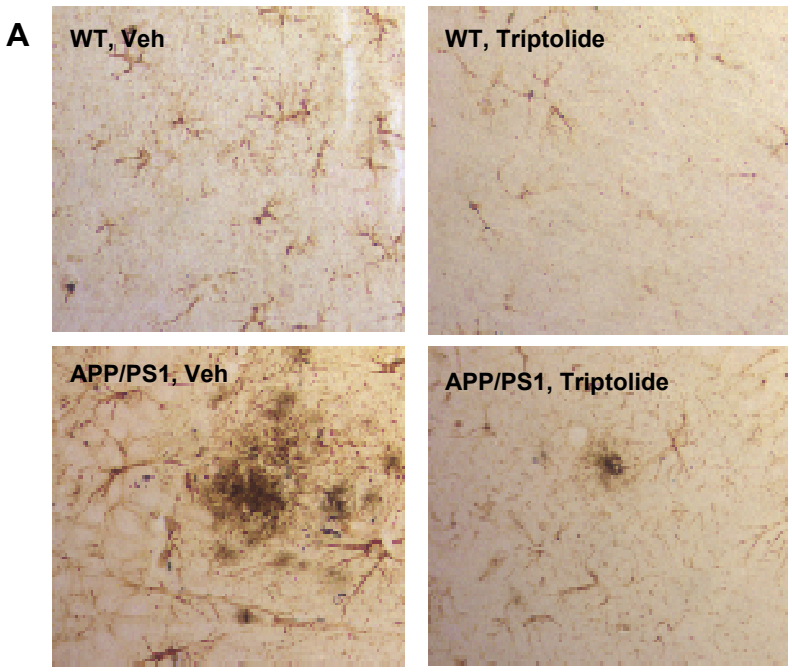


Figure 11 Triptolide treatment appears to cause a reduction in microglial reactivity in the APP/PS1 mice brains. **A** represents Campbell-Switzer staining for Aβ and immunostaining of Iba-1 in the hippocampal CA3 region. Wild-type (WT) and APP/PS1 mice with vehicle or triptolide are shown. There are less activated microglia in the triptolide treated APP/PS1 mouse brain than in the vehicle treated APP/PS1 mouse brain. **B** represents Western blot analysis of Iba-1 expression among the four genotypes. A slight reduction can be seen in the triptolide treated versus vehicle.



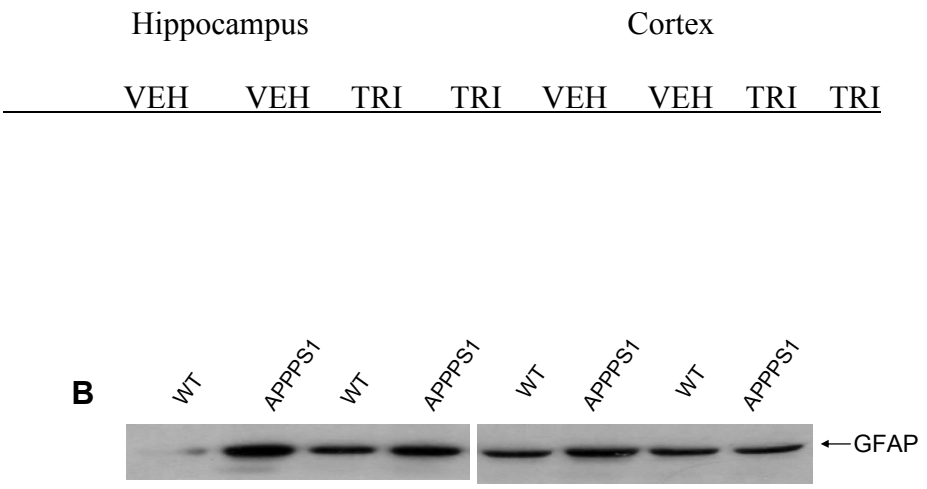


Figure 12 Triptolide treatment appears to cause a reduction in astrocyte reactivity in the APP/PS1 mice brains. A represents Campbell-Switzer staining for Aβ and immunostaining of GFAP in the hippocampal region. Wild-type (WT) and APP/PS1 mice with vehicle or triptolide are shown. There is less activated astrocytes in the triptolide treated APP/PS1 mouse brain than in the vehicle treated APP/PS1 mouse brain. B represents Western blot analysis of GFAP expression among the four genotypes. There appears to be no significant reduction in the triptolide treated brain versus the vehicle treated brain.

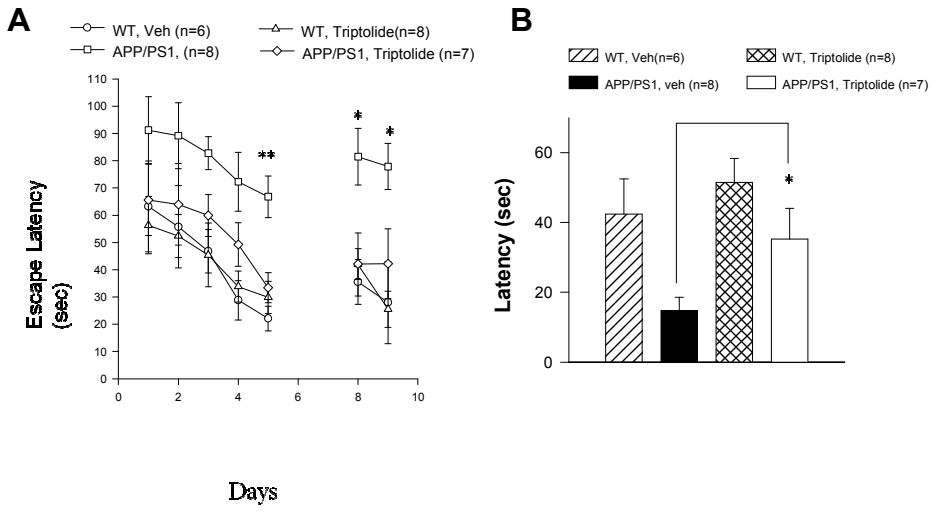


Figure 13 Treatment of APP/PS1 mice with triptolide results in attenuation of AD-type spatial memory deterioration. **A** represents acquisition of spatial learning in the Morris water maze hidden platform task. APP/PS1 mice treated with triptolide displayed learning similar to wild-type mice; while vehicle treated APP/PS1 mice displayed learning deficits. Latency score represents the time taken to escape to the platform from the water. Lines represent mean ± SEM from six to eight mice (indicated) per group. **B** represents the memory test in the Morris water maze probe trial without the platform. The deficits in the vehicle treated APP/PS1 mice were improved in the triptolide treated APP/PS1 mice. Error bars represent mean ± SEM from six to eight mice (indicated) per group. *p< 0.05, **p< 0.01 versus vehicle treated control APP/PS1 mice.

Discussion

As stated above, triptolide's therapeutic potential has been exploited for the use in rheumatoid arthritis and certain cancers. Its structure has similar homology to corticosteroids and thus is a potent immunosuppressant. This aspect of immunosuppression is believed to play a beneficial role in the inflammatory mechanisms of AD and help bring the patient's immune system to a state of effectively clearing amyloid plaques from the CNS. This is further supported by several epidemiological studies suggesting a potential benefit from using anti-inflammatory drugs on AD patients (Akiyama, Barger et al. 2000). Inflammatory processes in the CNS become detrimental to the AD patient. This is believed to be caused by activated glial cells that target senile amyloid plaques for degradation but in the process can damage neurons in and around the plaque deposits. Therefore, a possible solution for treatment in conjunction with inhibiting plaque formation would be to inhibit glial cell activation. Certain drugs have shown promise as treatments for AD and clinical trials are currently being done to study the effectiveness of such anti-inflammatory treatment. However, the mechanisms of action for this avenue of therapy still remains elusive since cytokines released from glial cells have neurotrophic as well as neurotoxic effects (Richartz, Stransky et al. 2005). This study has indicated that treatment of APP/PS1 double transgenic mice with triptolide can attenuate amyloid plaque deposition which concordantly will halt deficits in learning and memory. It has been reported that the effects

of anti-inflammatory drugs may not act via inhibition of prostaglandins but actually reducing the plaque burden on the CNS (Cirrito and Holtzman 2003). The aspect of cognitive deficits is the chief clinical manifestation of AD and arguably the central target of any drug therapy. Again, triptolide's effect on the immune system is a possible explanation for the halt of neurodegeneration and rescue of spatial learning and memory exhibited in treated mice. Of the two chief protein aggregates, only amyloid plaque deposition was observed to have been attenuated by treatment with triptolide. The Campbell-Switzer stain is a reliable silver stain for amyloid plaques in brain sections. Expression of the APP protein was not changed among the triptolide treated and vehicle treated APP/PS1 mice which indicates that triptolide had no effects on APP gene transcription. However, there was an elevated presence of CTFs, the cleavage products of alpha, beta, and gamma secretases. This clearly indicates that cleavage of the APP protein is not hindered and in fact may be enhanced at a specific point along the processing. The preclusion of plaques from the histological staining of the brain sections of APP/PS1 mice treated with triptolide can be explained by an enhanced clearance and degradation of plaque deposits. However, the activity of gamma secretase can also be part of the explanation since this enzyme is still poorly understood in its function. An enhanced level of inflammatory mediators in the APP/PS1 mice have been shown to increase BACE1 activity in the hippocampus and result in an increase in CTFs (Blasko, Beer et al. 2004). Also, gamma secretase has been shown to increase in expression in response to activated glial cells from brain injury (Nadler, Alexandrovich et al. 2008). In keeping with the idea that inflammation is heavily involved in the pathogenesis of AD, it should be noted that

certain benefits can arise from inflammatory mechanisms. This depends on which mediators are involved, the time the disease develops, and whether the response is chronic or acute inflammation (Sastre, Walter et al. 2008). Microglia can clear amyloid plaques through phagocytosis and release elements of neuroprotection such as glia-dervied neurotrophic factor (GDNF) (Liu and Hong 2003). Interestingly, it has been reported that microglia already activated from newly formed amyloid plaques can restrict the growth of the plaques if further activation of microglia is inhibited (Meyer-Luehmann, Spires-Jones et al. 2008). Since triptolide has been shown to inhibit inflammatory cytokine release, it is certainly possible that the clearance of amyloid by glial cells and the formation of new plaques keep each other in "balance" and reach a steady state of formation and clearance. Further testing would be needed to support the hypothesis of a reduction in microglia activation with triptolide treatment. With the gamma secretase complex, the situation becomes even more unclear. A recent study has shown that some anti-inflammatory drugs can directly modulate the activity of gamma secretase (Czirr and Weggen 2006). The gamma secretase is composed of four essential membrane proteins: aph-1, pen-2, nicastrin, and presnilin (PS) (Sastre, Walter et al. 2008). It has been reported that activated glial cells have an increase in expression of presenilin (Nadler, Alexandrovich et al. 2008) but it is still unknown whether this can affect the glial cell function. Further, in cases of familial AD (FAD), where the patient displays amyloid plaques earlier than usual, there is a positive correlation with an increase in inflammatory processes. Again, it is unclear whether the increase in presenilin promotes neuroinflammation through other pathways or whether it promotes neuroinflammation through a substantial increase in amyloid plaque

deposition (Sastre, Walter et al. 2008). Using figure 7 again, and based on the results of the experiment, there are a few mechanisms that triptolide could be exploiting in exerting its beneficial aspects in the APP/PS1 mice. Triptolide is very much possibly interacting at the specific receptors for microglia activation. It has been shown to inhibit specific cytokine production and NO production, thereby inhibiting microglial receptor activation. However, no evidence has shown that superoxide formation is inhibited and this is believed to be of paramount importance in glial cell activation. The reduction in cytokine production will also inhibit glutamate and Ca^{2+} excitotoxicity, as stated in the introduction, and can therefore reduce cell death. While these mechanisms are plausible for explaining an inhibition of neuronal cell death, thereby enhancing learning and memory in the APP/PS1 mice, it does not provide an adequate explanation for a reduction in amyloid plaque deposition.

This study also attempted to investigate the prevalence of tau hyperphosphorylation among the four genotype mice tested. Unfortunately, the western blot analysis of brain homogenates yielded conflicting and inconsistent data (data not shown). Therefore, it was determined that the study would forego any further attempt to assess the reason for this as it seemed beyond the scope of this project. The absence of any tau hyperphosphorylation data appears to be inconsequential when measuring the cognitive deficits in the Morris water maze although many believe that it is closely linked with neuronal damage. It should be further explored as to why this pathological hallmark of AD did not manifest itself in these groups of mice or some technological error occurred that is responsible for the conflicting data. When it comes to the major kinases involved with tau

hyperphosphorylation (Lovestone and Reynolds 1997), we see that there appears to be no change between the triptolide treated and vehicle treated APP/PS1 mice. The expression of total CDK5 remained constant throughout the four genotypes, indicating no change in expression of the inactivated form of CDK5, phosphorylated CDK5. GSK3 also showed no change.

Since triptolide has been shown to inhibit inflammatory cytokines and chemokines, it was expected that glial activation might be reduced in the diseased CNS to a neurotrophic level. And indeed the APP/PS1 mice treated with triptolide did appear to exhibit a decrease in glial number in cortical and hippocampal regions. It is unclear whether the decrease in glial activation is the result of a decrease in Aβ plaque deposits or whether triptolide's anti-inflammatory properties inhibited the activation directly. Microglia change has so many parameters including morphology, number, and cytokine production. But, the appearance of a decrease in glial number supports an anti-inflammatory effect and thus can be beneficial and effective in neurodegenerative disorders, such as AD.

This study demonstrates that administration of triptolide can reverse or inhibit spatial learning and memory deficits associated with APP/PS1 mice. The cognitive dysfunction associated with these mice is a result of an accelerated production of amyloid plaques and loss of functional synapses (Chen, McPhie et al. 2000). Although it is clear that triptolide attenuated the amyloid plaque accumulation in brains of APP/PS1 mice relative to vehicle treated APP/PS1 mice, it cannot be definitively ruled out that triptolide exerts its neuroprotective effects through other mechanisms. However, a possible solution

rests with the evidence that triptolide has neuroprotective effects on dopaminergic neurons which can increase and/or keep intact the ability to release cholinergic neurotransmitters that can protect against the cytotoxic effects of Aβ (Wang, Liang et al. 2008).

This study has provided evidence that triptolide can inhibit amyloid plaque deposit and other neuropathologies associated with AD. Also, cognitive deficits associated with APP/PS1 double transgenic mice were ameliorated. The long historical use of this Chinese herbal derivative has now been characterized in an AD mouse model and hopefully will lead to clinical trials where the effects can be further explored in human AD patients.

Future Studies

The present study has done a lot to answer the question of whether or not triptolide can be a beneficial therapeutic for treating Alzheimer's disease. There is a substantial decrease in amyloid plaque deposits and glial activation. Memory and learning abilities appeared to be rescued in the drug treated APP/PS1 mice relative to vehicle treated APP/PS1 mice. Although these are exciting results, the exact mechanism of triptolide's effects have not been elucidated. A more extensive study of gamma secretase could possibly reveal much in how the anti-inflammatory property of triptolide affects its activities. A more extensive study of the phagocytic ability of microglia in the dystrophic brain treated with triptolide may provide an answer to how the brain can inhibit the accumulation of Aβ. This would involve phagocytosis assays and electron microscopy studies of brain tissue. A thorough assessment of changes in synaptic integrity using drebrin and synaptophysin markers can provide direct evidence for an inhibition of neurodegeneration in triptolide treated mice. Further studies of iNOS changes and cytokine and super-oxide release in relation to AD and triptolide treatment can explain glial activation mechanisms and anti-inflammatory effects of triptolide. And finally, administration of triptolide in a much older APP/PS1 mouse model could fully answer the question of whether triptolide can attenuate and decrease Aβ accumulation. Complications would likely arise when trying to establish a rescue in memory deficits since neuronal

death cannot be reversed. Nevertheless, much work remains in fully characterizing triptolide's therapeutic affects and the results presented in this study will perhaps further the study of anti-inflammatory drug therapy, an avenue that has proven to be neuroprotective and inhibit hallmark pathological symptoms.

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VITA

Matthew James Allsbrook was born on October 7, 1984 in Norfolk, Virginia. After graduating with Honors from Salem High School in June 2003, he attended Virginia Commonwealth University from August 2003 until August 2007. Matthew earned his Bachelor of Science in Chemistry in 2007. While an undergrad, he worked in the Atomic Engineering Laboratory headed by Dr. Everett Carpenter in the Department of Chemistry at VCU. While there, he learned to synthesize magnetic nanoparticles via the reverse micelle process and achieved two publications with fellow graduate students in the lab. As a Master's student in the lab of his advisor, Dr. Guo-Huang Fan, Matthew collaborated on several projects within the lab, culminating in another publication in the Journal of Neuroscience in 2008. His research was devoted to elucidating and attempting to find treatments that could one day be beneficial to patients suffering from Alzheimer's disease.

In addition to his graduate studies, Matthew is also a 4 Palm Eagle Scout and a Brotherhood member of the Order of the Arrow National Honor Society. Matthew is also a classically trained violinist for over fifteen years, studying under several teachers from Europe to South Korea. He often performs with accompanying pianists in a variety of venues.