

**Molecular Mechanisms of Neuroprotection and Cognitive Enhancement by an Alternative
drug *Centella asiatica***

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 7, 2013

Keywords: *Centella asiatica*, Alzheimer's disease,
Synaptic plasticity, Inflammation

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Abstract

Complementary and Alternative medicine (CAM) is increasingly gaining enormous importance as a part of current day therapeutic regimen for various incurable, chronic neurological disorders, majorly because of the palliative treatment offered by them. *Centella asiatica*, is one of the herbal plants reported in Ayurveda as “**Medhya Rasayana**” attributing to its CNS related beneficial effects especially on intellect, learning and memory. Previous reports have found the whole plant extract of *Centella asiatica* (CA) to beneficially modulate the cognition and enhance the quality of life in healthy elderly volunteer. Recent reports on pharmacological investigation of *Centella asiatica* extract (CaE) in animal studies have established the cognitive enhancing effects of CaE in battery of behavioral tests. However, there is limited information regarding the molecular mechanism of action of *Centella asiatica*. In the current study, we studied the neuroprotective and mnemonic mechanisms of CaE in both *in vitro* and *in vivo* experimental settings. CaE was commercially obtained and tested for its bioactive chemical constituents through various biochemical tests. CaE was found to be an effective inhibitor of 15-LOX and Caspase-3 activity, thus exhibiting potent anti-inflammatory and anti-apoptotic capability. CaE, in immortalized hippocampal cell (H19-7 cell) line, induced activation of CAMKII/CREB/BDNF pathway subsequently resulting in modulation of NMDA receptors subunits expression. Against hydrogen peroxide (H₂O₂), a potent inducer of oxidative stress, CaE demonstrated neuroprotective effects and effectively neutralized the ROS generation. In addition, CaE in 5 and 10 µg/ml concentrations efficaciously attenuated the H₂O₂ induced increase in

caspase-3 levels. Taken together, our study for the first time demonstrated CaE to possess multi-target approach entailing to its anti-oxidative, anti-inflammatory and anti-apoptotic effects. Also, CaE induces the CREB activity through PKA/CAMKII pathway which possibly via modulating the excitatory transmission enhances the synaptic plasticity in the CNS.

Our previous report has established the CaE effect in lowering of amyloid load in double transgenic mouse model of Alzheimer's disease (AD). However, the mechanism of lowering β -amyloid load by CaE in the transgenic mice remains unexplored. Therefore, in the present study we studied the effect of CaE on two major pathophysiological pathways underlying the progression of cognitive deficits in the double transgenic APP_{swe}+PS1_{dE9} model. Firstly, the effect of CaE on the synaptic plasticity in hippocampus was assessed. We found a significant increase in the CREB phosphorylation accompanied with the changes in NMDA receptors subunits in the CaE treated transgenic mice. Also, we found the enhancement in long term potentiation (LTP) process in the hippocampus of the CaE treated transgenic mice along with the better performance in battery of behavioral tests analyzing learning and memory. Secondly, we studied the effect of CaE on the T-cell induced inflammation in the APP_{swe}+PS1_{dE9} mouse model. Moreover, CaE attenuated the activation of T-cell helper cells and increased the activity of T-regulatory cells to combat the β -amyloid induced neuro-inflammation. Thus, the current study established the protective role of CaE against β -amyloid induced neurotoxicity and shows that this alternative herbal drug has significant unexploited therapeutic potential in cognitive neurological disorders.

Acknowledgements

I praise and thank the Almighty, the most merciful for blessing me the strength and support, which helped me to finish this work. I would like to dedicate my work to my Guru Ji, parents and my sister. It's with their encouragement, love and inspiration, I have been able to quench my desire for higher studies and strive for excellence in research.

I would like to express my deep appreciation and sincere thanks to Dr. Muralikrishnan Dhanasekaran, for his continuous guidance, encouragement, support and patience throughout my doctoral studies at Auburn. I express my sincere gratitude to my committee members Dr. Vishnu Suppiramaniam, Dr. Rajesh Amin, Dr. Jianzhong Shen, Dr. Jaychandra Ramapuram and Dr. Kevin Huggins for keen supervision and contributions of resources, time and technical assistance, making this project possible. I would also like to thank Dr. Bruce F. Smith for helping me with real time PCR technique and for generously letting me use his laboratory resources. I would like to express my special thanks to Dr. Robert Judd, for serving in my graduate committee as an official reviewer of this dissertation. My thanks also go to my colleagues, Dr. Senthil Karuppagounder, Dr. Parameshwaran, Dr. Engy Ali, Manal Buabeid, Wanshu Ma, Ranjith Munigunti, Gayani Nannayakara, Mengyuan Zheng, Thomas Wieckowski, Dr. Sateesh Sathigiri, Dr. Vani Sree and Dr. Kariharan Thiruchelvam, for their support and good-spirited discussions during this study. I would especially like to thank Madhukar, my colleague and friend for his assistance in various avenues of this work. I would like to extend my appreciation and gratitude to all the faculty members, staff and fellow students at Dept. of Pharmacal Sciences, Dept. of Animal Health, Auburn University. I would like to offer my sincere thanks to

Dr. Lee Evans, Dean of HSOP and to the previous chair of the Pharmacal Sciences Department Dr. Charlene A. McQueen and Dr. Tahir Hussain, who have always supported and encouraged graduate research and education. I personally thank Dr. Rajesh Nair, Dr. Maninder Sandhey, and Dr. Subramanian Uthayathas for their scientific help and guidance.

I would like to personally thank my dearest friends, Sapana Shrivastava, Gurkishan Singh Chadha, Anadi Mahajan, Snadeep Gupta, Inderpreet Kaur, Mandeep Singh Mittal, Yogesh Sharma, Maninder Sandhey, Manjul Gupta, Ahmed Faraz, Mosavverul Hassan, Praveen Venkatramani, Vivek Ahuja, Murali, Jaspreet Aulakh, and Anurag Srivastava. They made my stay in auburn, the one of the most cherished memories of all. Finally, I would like to express my heartiest thanks to two great pillars of my life my Guru Ji and my parents, Mr. Sudershan Kumar Ahuja and Smt. Sudesh Ahuja for their understanding, unwavering love and help. Last but not least, I am also greatly thankful for my lovely sister Anishi Ahuja, who always been a ray of hope and happiness in my life.

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1. Introduction

Increased life expectancy in the United States from 55 years of age to over 75 has been, unfortunately, shown to have strong correlation with an epidemic of age-related cognitive disorders, especially Alzheimer's disease (AD). AD is a progressive, fatal neurodegenerative disorder that being among the most common causes of dementia, impairs basic bodily functions such as walking and swallowing and eventually, results in death. AD is the sixth-leading cause of death with 5.4 million affected people just in the United States alone (Jadidi-Niaragh et al., 2012). According to World Alzheimer's report of 2009, 35.6 million people were diagnosed with dementia in 2010 worldwide, a number which will double every 20 years and rise to 65.7 million in 2030 and 115.4 million in 2050 (Jadidi-Niaragh et al., 2012). AD is characterized by extracellular β -amyloid ($A\beta$) plaques (known as senile plaques, SPs) and intracellular neurofibrillary tangles (NFTs) together with severe atrophy in the cortical and hippocampal regions of brain (Schwab and McGeer, 2008). AD patients also shows prominent neuro-inflammation in the most affected areas of the brain such as cortex and hippocampus (Chu, 2012). Treatment of AD is very difficult pertaining to limitations to diagnosis and treatment options available. There are only very few marketed therapeutic interventions available for AD and even, those only provide palliative treatment (Atri, 2011; Chu, 2012). Thus, there is utmost need to develop novel effective and safer therapeutic interventions for AD.

Several lines of evidence suggests increase in rate of studies investigating herbal medicines either as a novel treatment option or as a resource for obtaining a therapeutic effective lead compound (Kumar et al., 2012). Complementary Alternative medicine (CAM) is now usually incorporated with approved therapeutic regimen for treating various illness (Alvari et al., 2012). *Centella asiatica* is one of the herbal plants that are traditionally used in Ayurvedic and

Chinese medicine for treating various ailments. *Centella asiatica* (Umbelliferae), an herbaceous creeping plant indigenous to almost all over the worlds including countries like China, Malaysia, Viet Nam, India, and southeastern United States. In India, the plant is found throughout the country and is commonly known as Brahmi in Sanskrit. Recently, pharmacological studies have demonstrated beneficial therapeutic effect of the Titrated Extract of *Centella asiatica* (TECA) in wound healing, microangiopathy, and gastric ulcers (Shukla et al., 1999; Cesarone et al., 2001a; Cesarone et al., 2001b; De Sanctis et al., 2001; Incandela et al., 2001; Paocharoen, 2010; Belcaro et al., 2011). Over the past few decades, investigational studies has shown the herb to possess antitumor activity (Babu et al., 1995), anti-inflammatory, CNS depressant activity (Sakina and Dandiya, 1990), anti-apoptotic, antioxidant properties and neuroprotective activity. Animal studies exhibits the outstanding performances of *Centella asiatica* treated animals in various memory tasks such as active and passive avoidance, object recognition, and water maze. Clinical studies performed with healthy elderly volunteers have shown positive modulation of cognition and mood along with improvement in physical performance and health-related quality of life (Wattanathorn et al., 2008; Mato et al., 2011). Moreover, in case of mentally retarded children it is reported to improve general mental ability (Appa Rao et al., 1973). Though, there is great deal of clinical as well as animal experimental evidence, but there is still paucity of studies shedding light on the specific mechanism of *Centella asiatica* with respect to the most common form of dementia i.e. AD. Hence, the current study aimed to establish the therapeutic potential of *Centella asiatica* extract (CaE) as well as decipher the underlying cellular mechanisms of action of CaE against AD in a very well established double transgenic AD mice model. Results from the current study, indeed establish the significant neuroprotective, cognition enhancing effects of CaE, which attribute to its modulatory effects on various inflammatory, apoptotic, oxidative and

synaptic plasticity pathways. Thus, CaE acts as a cocktail of therapeutic compounds capable of relieving amyloid beta deposition and improving cognitive function of brain.

The current study also employed double transgenic mice harboring familial AD linked Swedish mutant human APP (APP^{swe}) and mutant human presenilin (PS1-dE9) gene. Commonly referred as APP^{swe}+PS1dE9 mice, they display senile plaques (SPs) in hippocampus and cortex at six to seven months along with spatial learning deficit at seven months of age, (Savonenko et al., 2005; Garcia-Alloza et al., 2006). The amyloid beta plaques, in these mice, accumulate in the regions of the brain largely resembling the pattern of accumulation of senile plaques in human AD (Garcia-Alloza et al., 2006; Kim et al., 2012). At nine months of age they exhibit dystrophic neurites with very subtle neuronal cell loss along with relatively milder cerebral amyloid angiopathy (CAA) (Garcia-Alloza et al., 2006; Bonardi et al., 2011; Darvesh et al., 2012). In the current study we investigated the therapeutic potential of the concentrated herbal extract of *Centella asiatica* (CaE) against the amyloid beta induced myriad of pathophysiological alterations leading to cognitive deficits. CaE, assigning to its anti-oxidative, anti-apoptotic and anti-inflammatory effects protected APP^{swe}+PS1dE9 mice against the pathophysiological alterations. Also, ascribing to its direct effect on synaptic strengthening, CaE ameliorated the impaired LTP formation in these mice most probably by activation of the transcriptional factors related with LTP formation and modulating the glutamatergic transmission in the hippocampus.

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2. LITERATURE REVIEW

2.1. Dementia:

Two of the most fundamental processes of the brain are learning and memory. However, there are other important functional aspects of brain such as thinking, feeling, wanting, perceiving, curiosity, and behavior, but without learning and memory an organism will be capable of nothing, except simple reflexes and stereotyped behavior. In reality, it won't be wrong to state that both these processes define a human being by modulating his experiences and responses. Though, learning and memory seems similar and highly interconnected processes, psychologists define them separately. Definitively, learning is a process of acquiring information and memory is defined as the capacity to encode, store and retrieve that information and influence ones behavior. There are various forms of memory. Memory has been categorized, depending on time for which the acquired information is stored, into three major types, which further have sub-categories. Different types and sub-types of memories are described in the figure 2.1.

Dementia, an umbrella term is used to describe a syndrome or disorder characterized by remarkable decline in memory and loss of cognitive abilities in a previously unimpaired person. Physician's definition given in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)*, states symptoms including decline of memory accompanied with at least loss of one of the cognitive abilities such as coherent speech/ language, perception, judgment, execution of motor functions and / or reasoning (Mori et al., 2012), which for a definitive diagnosis must be present for at least six months. An estimated 36 million people were living with some form of dementia worldwide in 2010, which if went unchecked is predicted to rise to 66 million by 2030 and 115 million by 2050 (Alzheimer's Disease International., 2009;

Label, 2009). Dementia is an age related disorder and affects 10-15% of adults >65 years old and its prevalence increases by 35-50% in adults of more than 85 years of age (Mori et al., 2012).

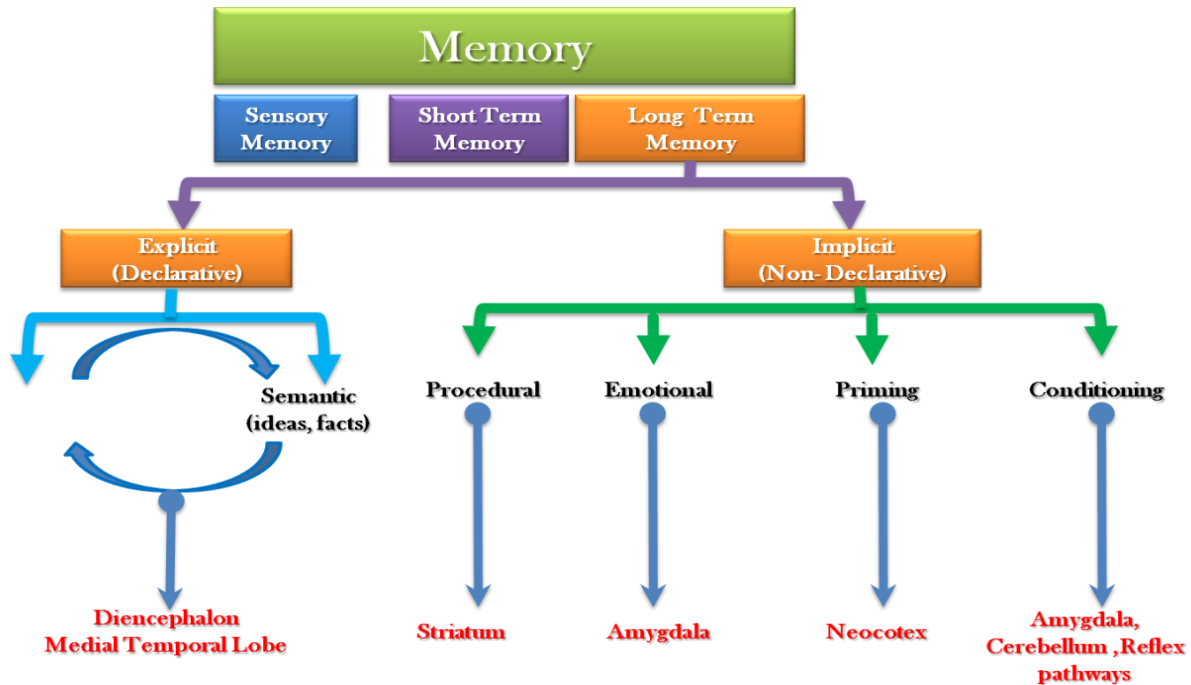


Figure 2. 1: Different types and subtypes of human memory. Memory is temporally of three types Sensory, Short-term and Long-term memory. Long-term memory is further subcategorized in to Explicit and Implicit memory which have further sub classifications. *J Cell Sci* 121(Pt 19):3121-3132.

Although, dementia is of many types but Alzheimer’s disease is the most common type of dementia. Other types of dementia includes Mild Cognitive Impairment (MCI), Vascular Dementia, Mixed Dementia, Dementia with Lewy Bodies, Parkinson's Disease, Fronto-temporal Dementia, Creutzfeldt-Jakob Disease (CJD), Normal Pressure Hydrocephalus, Huntington's Disease, Wernicke-Korsakoff Syndrome. The characteristic features of these types of dementia are described in the table 2.2.

Memory Type	Definition
Sensory memory	Ability to retain impressions of sensory information after the original stimuli through the five senses of sight, hearing, smell, taste and touch have ended Retained accurately, but very briefly
Short term memory	Kind of “scratch-pad” also referred to as "the brain's Post-it note". Holds a small amount of information (typically around 7 items or even less) in mind, for a short period of time (typically from 10 to 15 seconds)
Long term memory	Intended for storage of information over a long period of time. Can store a seemingly unlimited amount of information almost indefinitely Requires process of consolidation, involving rehearsal and meaningful association Process of physical changes involved in the structure of neurons, known as long-term potentiation
Explicit (Declarative)	Includes the memories of facts and events e.g. “Paris is the capital of France” Declarative memory refers to those memories that can be consciously recalled Sub-divided into episodic memory ad semantic memory.
Implicit (Non-Declarative)	Includes the types of memory systems that do not have a conscious component Memories for skills and habits e.g. riding a bicycle, driving a car, playing golf or tennis or a piano Also referred as procedural memory
Episodic	Memory of experiences and specific events in time in a serial form Also known as memory of autobiographical events One particular type of Explicit Memory
Semantic	More structured record of facts, meanings, concepts and knowledge General factual knowledge, shared with others and independent of personal experience Type of Explicit Memory
Procedural	Type of implicit memory Enables us to carry out commonly learned tasks without consciously thinking Even what we think of as "natural" tasks, such as walking, require procedural memory.
Priming	Priming is the effect in which exposure to a stimulus influences response to a subsequent stimulus For instance, if a person reads a list of words including the word “concert”, and is later asked to complete a word starting with “con”, there is a higher probability that they will answer “concert” than, say, “contact”, “connect”.

Table 2. 1: Classification of Memory into various types. (Alzheimer’s Disease International, 2009)

Types of Dementia	Symptoms	Therapy
Mild Cognitive Impairment	Loss of memory, language, or another mental function	<ul style="list-style-type: none"> • No therapy • Use risk reducers
Vascular Dementia	High blood pressure, high cholesterol, hardening of the arteries, diabetes-leads to impaired blood flow to brain	<ul style="list-style-type: none"> • Monitoring of blood pressure, weight, blood sugar and cholesterol
Mixed Dementia	Alzheimer's disease and vascular dementia occur at the same time	<ul style="list-style-type: none"> • Monitoring of blood pressure, weight, blood sugar and cholesterol
Lewy bodies Dementia (LBD)	Alpha-synuclein composed Lewy bodies deposition, cognitive symptoms, Movement symptoms, rapid eye movement (REM) sleep disorder	<ul style="list-style-type: none"> • Cholinesterase inhibitors • Do not use antipsychotics
Parkinson's disease (PD)	Lewy bodies deposition	<ul style="list-style-type: none"> • Cholinesterase inhibitors
Frontotemporal Dementia (FTD)	Changes in personality, Eat & gain weight	<ul style="list-style-type: none"> • None
Creutzfeldt-Jakob Disease (CJD)	Prion protein deposition, Problem with memory, movement, mood	<ul style="list-style-type: none"> • Antidepressant,
Normal pressure hydrocephalus	Fluid surrounding the brain and spinal cord is unable to drain normally, Problem with memory, movement, bladder	<ul style="list-style-type: none"> • Shunt: to drain the fluid
Huntington's Disease	Inherited changes in a single gene, Problem with memory, movement,	<ul style="list-style-type: none"> • Antidepressant, Anti-convulsants, Antipsychotics
Wernicke-Korsakoff Syndrome	B ₁ deficiency	<ul style="list-style-type: none"> • Stop drinking, B₁ supplement
<u>Others</u> Down syndrome Prenatal alcohol / nicotine exposure	Genetic impairment-chromosome 21	

Table 2. 2: Various forms of dementia and their characteristic features. Most of these dementias are age related. AD is the most common form of dementia related to age. (Alzheimer's Disease International, 2009)

2.2. Alzheimer's disease (AD):

Alzheimer's disease (AD), the most common type of senile dementia, is a progressive, fatal, irreversible neurodegenerative disorder characterized by early decline in learning and memory gradually progressing to global debilitation of higher cognitive abilities (Alzheimer's Disease International., 2009). On the basis of time of onset of disease, AD has been categorized in two categories, a) familial AD (FAD) which has well defined pathological genetic cause including genetic mutations in the amyloid precursor protein (APP) and presenilin (PS) genes that accounts for almost 3% of all AD cases and affects people at an early age of less than 60 (Mori et al., 2012), and b) sporadic AD, which has no clearly defined pathological factors, represents 97% of AD cases and affects people of age 65 or above (has late onset of disease) (Label, 2009). Early signs of AD include memory loss, which is also known as amnesic mild cognitive impairment (MCI). This mild memory loss is often accompanied by decline in other cognitive abilities such as word finding, vision/spatial issues and impaired reasoning. As the disease progress, AD depending on severity of symptoms is known to enter three distinct phases, which are Mild AD, Moderate AD, and Severe AD.

Stages of AD	Symptoms and signs
Mild Alzheimer's Disease	<ul style="list-style-type: none"> • Worsening of memory loss • Getting lost • Trouble handling money and paying bills • Repeating questions • Mood and personality changes with poor judgment • Diagnosis takes place generally at this stage
Moderate Alzheimer's Disease	<ul style="list-style-type: none"> • Problem recognizing family and friends • Inability to learn new things • Inability to carry out complex tasks • Hallucinations, delirium and paranoia
Severe Alzheimer's Disease	<ul style="list-style-type: none"> • Severe impairment of communication • Complete reliance on caregiver for every activity • Complete bed ridden state • Pneumonia and other bed ridden ailments

Table 2. 3: Stages of Alzheimer’s disease ranging from mild to severe AD.

2.3. Prevalence and economic impact of AD:

According to World Alzheimer’s report of 2009, 35.6 million people were diagnosed with dementia in 2010, a number which will double every 20 years and rise to 65.7 million in 2030 and 115.4 million in 2050. The report stated that there is 10% increases in the estimated figure of 2010 in comparison to the Lancet report of 2005. The increase is due to the inclusion of several new studies from the low income and developing countries, enabling to estimate better and robust figures for 2010. A forecast of 40% increase in dementia cases in Europe, 63% in North America, 77% in the southern Latin America and 89% in the developed Asia Pacific countries, which when compared with 117% growth in East Asia, 107% in South Asia, 134-146% in the rest of Latin America, and 125% in North Africa and the Middle East represents a steeper increase rate in the low income and developing countries. AD is the sixth-leading cause

of death, which affects 5.4 million people of all ages diagnosed in the United States alone in the year 2012 and projected to rise to 15 million by the year 2050 (Mori et al., 2012). In the US, one in eight people of age 65 and older and nearly half of people of age 85 and older have Alzheimer's disease. Men have fewer incidences of AD and other dementia than women with almost two-thirds of Alzheimer's diagnosed American population being women. Each year there are about 7.7 million new cases of dementia, which imply an incidence rate of new case every four seconds, in the world.

AD has risen to being a worldwide catastrophe, affecting human health as well as economy globally. The worldwide cost of AD and other dementia was estimated to be US\$604 billion in 2010 (Alzheimer's Disease International., 2009). This figure comprised about 70% of the costs occurring in Western Europe and North America as there was limited figures regarding the developing countries where accessibility of information was difficult. Total cost of AD includes direct cost of medical treatment, social care (provided by community care professionals, and in residential home settings) and indirect costs pertaining to informal care (unpaid care provided by family and others). In the US alone, 15 million Americans provide unpaid care for AD or other dementias who usually are primarily family members, relatives and friends. An estimated 17.4 billion hour of unpaid care, which is approximately valued at over \$210 billion has been spent on AD in the year of 2011 (Alzheimer's disease facts and figures, 2012).

2.4. Etiology and Pathophysiology of AD:

The etiology of AD is very complex and is still the subject of continuous research. Although, AD is a century old disease, yet the etiological causes of this disease lie under promiscuity. Morphologically and histologically, brain atrophy and enlargement of cerebral

ventricles along with deposition of amyloid plaques and neurofibrillary tangles in the cortex and hippocampus followed by neuronal and synaptic loss are the important hallmarks of the disease (Khachaturian, 1985; Cummings et al., 1998; Imbimbo et al., 2005). The neuritic plaques, also known as senile plaques (SP) are extracellular lesions composed of the 40 to 42 amino acid long peptide A β fragments derived from amyloid precursor protein (APP), whereas the neurofibrillary tangles (NFTs) are intracellular lesions composed of twisted filaments of tau protein, a cytoskeleton protein (Gomez-Isla et al., 1996; Selkoe, 1998). The diagnosis of AD can only be confirmed after post-mortem brain autopsy exhibiting SP, NFTs and atrophy of the cortical and temporal lobes of the brain. Almost all cases of genetically inherited form of AD i.e. FAD is accounted by the mutations in either the gene encoding the APP or in genes that encode APP processing complex cellular machinery [e.g., presenilin-(PSEN)-1 and PSEN-2] (Imbimbo et al., 2005; Piaceri et al., 2013). However, the sporadic form of AD has been linked to several genetic and environmental factors. Despite a wealth of knowledge gained from many years of research, the etiology of sporadic AD remains elusive (Piaceri et al., 2013). Several theories have been put forth to account for pathogenesis of AD. Out of these theories, only amyloid-beta and tau hypothesis has gained wide acceptance from scientific community, though these theories still needs to be formally tested.

2.5. Amyloid-Beta (A β) hypothesis:

According to the amyloid-beta or amyloid hypothesis, A β peptides, a 40–42 amino-acid polypeptide β (A β ₄₀ and A β ₄₂), are considered the primary causative agent for AD (Cummings et al., 1998; Farlow, 1998; Imbimbo et al., 2005; Karantzoulis and Galvin, 2011; Piaceri et al., 2013). Amyloid hypothesis is based on the characteristic extracellular senile plaques, one of the histological hallmarks of AD, found in the brain of most of the AD patients (Imbimbo et al.,

2005). These A β peptides are derived by proteolysis from the amyloid precursor protein (APP), a type I transmembrane glycoprotein produced in many cells. Briefly, APP is physiologically processed by two competing pathways, one non-amyloidogenic and other is amyloidogenic. Non-amyloidogenic pathway involved initial proteolysis with α -secretase enzyme that generates sAPP α and carboxy terminal 83-aa fragment, C83 (also known as carboxy terminal fragments, CTF α), which is followed by further proteolysis of C83 fragment with γ -secretase resulting in formation of N-terminal peptides, p3 and APP intracellular domain (AICD). On the other hand, in amyloidogenic pathway, the initial proteolysis of APP is performed by β -secretase generating sAPP β and carboxy terminal 99-aa fragment, C99 (also known as CTF β). The C99 fragment is further processed by γ -secretase to secrete A β peptides and APP intracellular domain (AICD). These A β peptides of varying length, according to amyloid AD theory, oligomerize to form oligomers and accumulate in the brain, which is the key initiating event in the development of AD, although the precise species of A β that drives neurotoxicity is one of the many uncertainties that remain to be resolved. β -Secretase activity is linked to a single protein, Beta-site APP Cleaving Enzyme (BACE) and reconstitution of γ -secretase activity in yeast has revealed the presence of four components: presenilin, nicastrin, APH-1 and PEN-2 (Gotz et al., 2004). The genetic framework to this theory was obtained from the discovery of FAD patients with mutations at APP, PSEN-1 and BACE protein resulting in robust development of cognitive decline along with pathological hallmarks of A β -deposition in the brain of these patients along with at an age less than 65 years (Citron et al., 1992; Piaceri et al., 2013). Sporadic AD patients have shown increased BACE activity and linkage to the APOE polymorphism as the best documented risk factor for developing late onset AD (Bales et al., 1997; Farlow, 1998). The finding that knockout of APOE 4 in APP transgenic mice rescues A β deposition and associated

cognitive decline strengthens the A β -hypothesis (Bales et al., 1997). Several biochemical studies also fortify the A β -theory. The most arguable are the precedence of A β deposition before formation of NFTs, neuronal loss and clinical symptoms of AD (Lewis et al., 2001); exacerbation of tau fibril formation in presence of A β (Hurtado et al., 2010) and the presence of higher plasma levels of A β in the incipient AD (Mayeux et al., 2003) and MCI patients (Chiu et al., 2012).

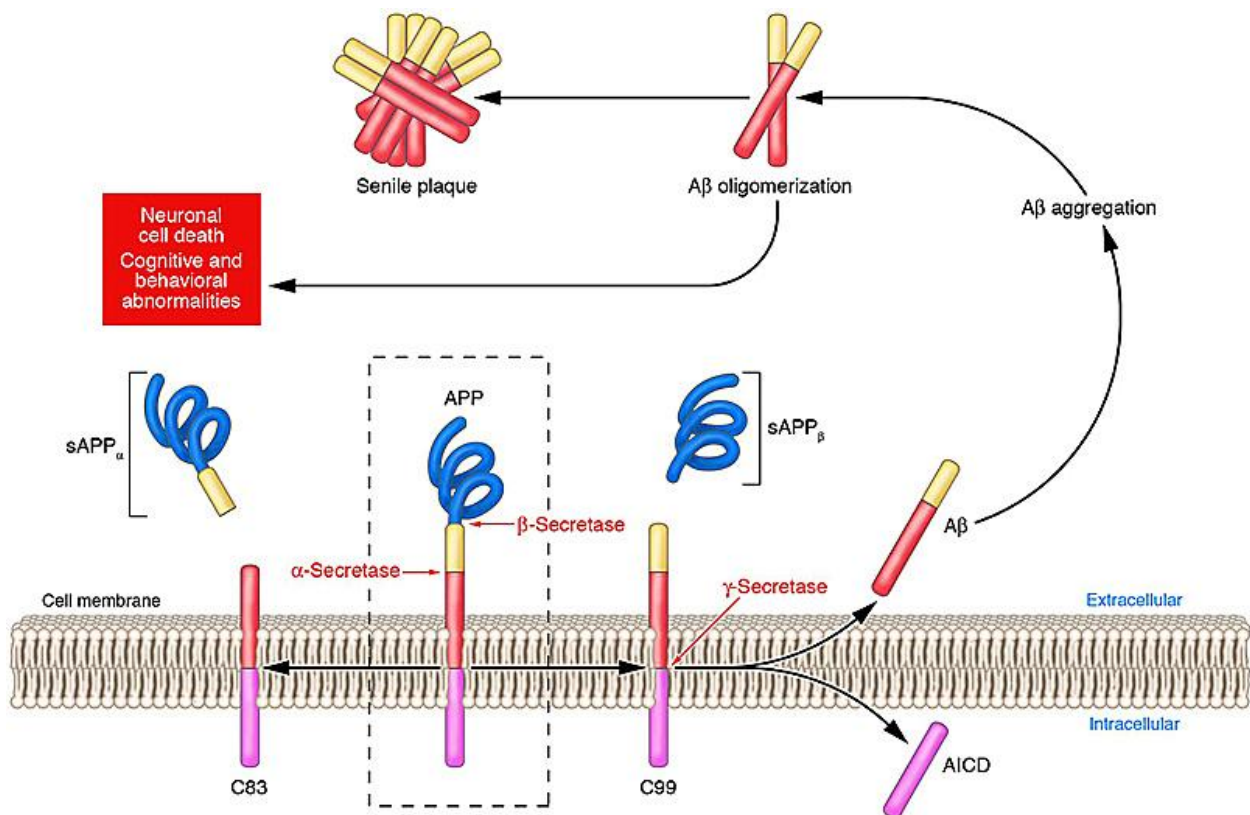


Figure 2. 2: The non-amyloidogenic and amyloidogenic pathways of APP processing. APP is cleaved by either alpha- or beta-secretase. Cleavage by alpha-secretase (the non-amyloidogenic pathway) generates sAPP-alpha and C83 (left). Cleavage by beta-secretase (the amyloidogenic pathway) generates sAPP-beta and C99 (right). C83 is cleaved by gamma-secretase, generating AICD and p3 (not shown). C99 is also cleaved by gamma-secretase, generating AICD and A-beta (right). *Frontiers in bioscience: a journal and virtual library* 17:2024-2034.

2.6. A β neurotoxicity:

A β induces neuronal toxicity via various mechanisms resulting in a multifaceted attack on the neuronal cell integrity and CNS function. These mechanisms includes A β induced oxidative stress (Butterfield et al., 2001), disruption of mitochondrial activity (Schapira and Reichmann, 1995), energy imbalance (Carvalho et al., 2012), stimulation of neuroinflammation (Verri et al., 2012), perturbation of Ca²⁺ homeostasis (Resende et al., 2007), disruption of axonal transport (Decker et al., 2010) and activation of apoptotic signaling (Imaizumi et al., 1999; Kudo et al., 2012); all resulting in the neuronal cell death and AD pathogenesis. Perturbation of Ca²⁺ homeostasis mediated via slow N-methyl-D-aspartate receptors (NMDARs) ion channels results in stimulation of various oxidative pathways that eventually leads to excitotoxicity. Thus, excitotoxicity plays a central role in neuronal cell death, which is prominently evident in various chronic progressive neurodegenerative disorders (Thellung et al., 2012). In addition, several *in-vivo* and *in-vitro* studies showed that A β peptides can induce synaptic deficits including inhibition of LTP in the hippocampal perforant path (Rowan et al., 2004; Parameshwaran et al., 2008; Ferreira et al., 2012). A β peptides attenuate 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptors (AMPA) and N-methyl-D-aspartate receptors (NMDARs) surface expression and function, resulting in impairment of glutamatergic transmission (Parameshwaran et al., 2008). Thus, A β deposition through induction of these detrimental effects can lead to neurotoxicity resulting in progression of AD.

2.7. Oxidative stress in AD:

Oxidative stress is one of the well investigated and crucial players of AD (Moreira et al., 2008). Autopsy studies from the AD patients revealed significant reactive oxygen species (ROS) in the early stages of AD, which declines with the disease progression (Nunomura et al., 2001).

Oxidative stress has been studied both as a secondary as well as primary event regarding AD pathogenesis (Markesbery, 1997; Butterfield et al., 2001). According to the Amyloid theory, A β deposition is considered as the key initiating factor for ROS generation through various mechanisms including the interaction with metals such as Fe²⁺ and Cu⁺ resulting in release of hydrogen peroxide, which further induces the lipid peroxidation with consequent production of very reactive neurotoxic aldehyde, 4-hydroxynonenal (Abdul et al., 2008). Mitochondrial dysfunction leading to altered activity of important enzyme complexes involved in energy metabolism and electron transport chain consequently resulting in superoxide radical (O²⁻) formation is another mechanism of A β induced oxidative stress. Recent study in transgenic mice validates the mitochondrial dysfunction and reduced SOD activity as one of the mediators of ROS formation in AD, as superoxide radical was not being metabolized in to less reactive form of hydrogen peroxide (Abdul et al., 2008). These reactive species then oxidize other molecules including Nitric oxide (NO) to form highly reactive peroxynitrite radical and other hydroxyl radical. Free radicals thus generated attacks DNA resulting in DNA cleavage and damage, which is a specific feature of neuronal cell death found in AD (Sultana et al., 2006; Abdul et al., 2008; Moreira et al., 2008; Verri et al., 2012)

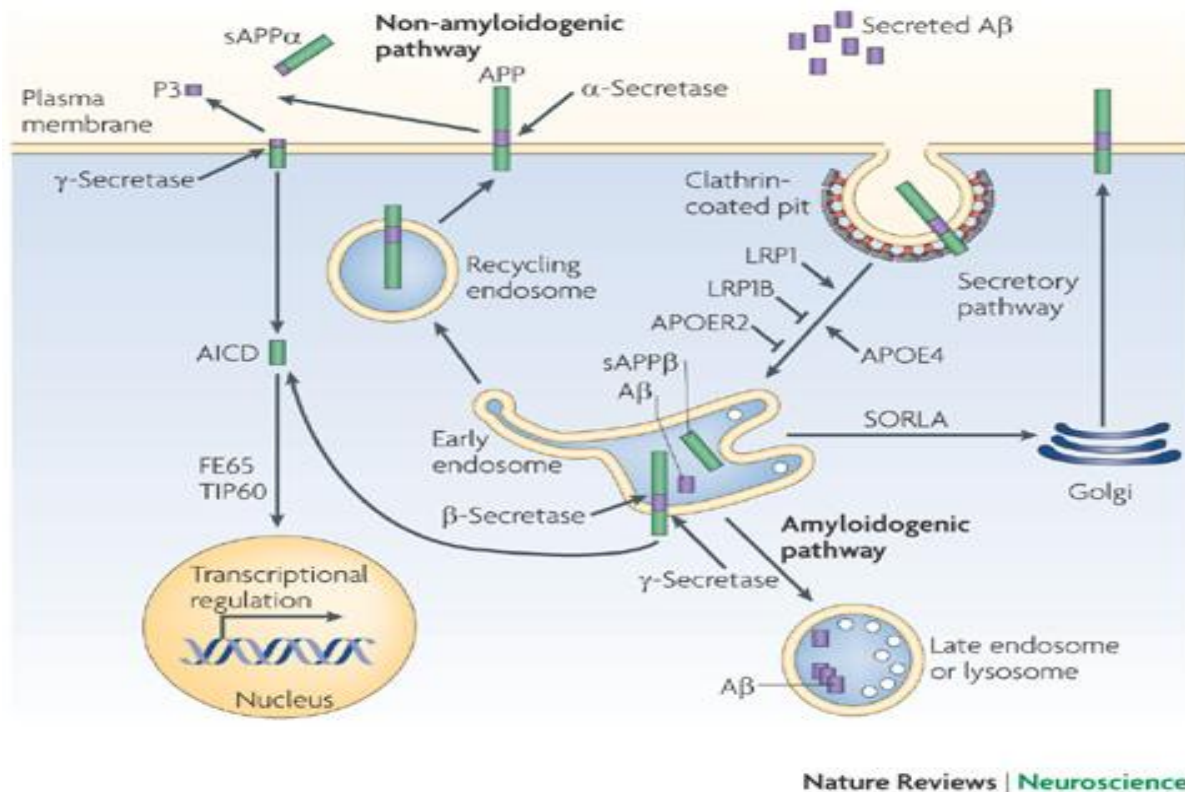


Figure 2. 3: Different pathway of processing of β -Amyloid. Newly synthesized amyloid precursor protein (APP) traffics through the secretory pathway to the plasma membrane, where it is primarily cleaved by the α -secretase in the non-amyloidogenic pathway to produce a minimally toxic peptide called P3. *Nature Reviews Neuroscience* 10, 333-344

Interestingly, just like the oxidative stress theory of aging, it is speculated that free radical damage and oxidative stress play a major role in the pathogenesis of Alzheimer disease (AD) (Markesbery, 1997; Choi et al., 2012). Supporting this theory are the direct evidences including increased brain metal content such as Al^{3+} and Mn^{2+} capable of stimulating free radical generation (Leskovjan et al., 2011), the presence of increased lipid peroxidation and its product 4-hydroxynonenal in the ventricular fluids of AD patients as well as transgenic animals (Siegel et al., 2007), increased levels of damaged proteins and DNA in the AD brain (Markesbery, 1997; Moreira et al., 2008), disrupted mitochondrial dysfunction and energy metabolism, and most

importantly is the exacerbation in the A β release and deposition due to oxidative stress (Siegel et al., 2007; Abdul et al., 2008; Chami and Checler, 2012; Verri et al., 2012). The indirect evidence strengthening this hypothesis would be the various *in-vitro* and *in-vivo* studies showing that free radicals mediate the neuronal cell death (Papa and Rockwell, 2008), which could be prevented and rescued by the blocking the free radicals either by sequestration and inhibiting their generation (Dumont et al., 2009; Chami and Checler, 2012; Choi et al., 2012). In addition, the extensive research and effort spent toward developing the antioxidative therapy for the treatment of AD represents additional indirect evidence for oxidative theory of AD (Aliev et al., 2008). Thus, it can be reasonably stated that the oxidative stress being the earliest as well as the most deleterious event of AD, presents itself as one of the best candidates for developing therapeutic interventions for AD.

2.8. Neurochemical alterations in AD:

Significant changes in the levels of various neurotransmitters have been found in AD patients, which closely correlate with its different cognitive and non-cognitive symptoms (Reinikainen et al., 1990). Initial histological and pathological studies revealed significant degeneration of cholinergic nucleus basalis of Meynert revealing substantial deficits in the cholinergic neurotransmitter system in the neocortical and hippocampal regions of AD patients (Bowen et al., 1976; Francis et al., 1999; Gottwald and Rozanski, 1999). Remarkable improvement in the cognitive function of AD patients after administration of cholinergic based therapies supported the pathogenic role of Acetylcholine deficiency (Gottwald and Rozanski, 1999; Atri, 2011). Variations in nor-epinephrine (NE) and serotonin (5-HT) levels were also detected in the cortical regions of the brain AD patients resulting in the secondary symptoms of depression, aggression and psychosis observed in AD patients (Engelborghs and De Deyn,

1997). Similarly, levels of glutamate and GABA, the most abundant excitatory and inhibitory neurotransmitters of the brain respectively, were found to be altered in AD, though perturbations in glutamatergic system were more severe and prominent than GABAergic system (Hyman et al., 1987; Reinikainen et al., 1990; Engelborghs and De Deyn, 1997; Butterfield and Pocernich, 2003; Schwab et al., 2012). Due to their limited pathogenic potential and only palliative therapeutic implications, these neurotransmitter alterations are considered to be manifestations of collateral damage occurring as severe neuronal degeneration in AD (Robichaud, 2006). However, cholinergic and glutamatergic alterations are the cornerstone of the current AD therapies.

Glutamatergic transmission in neocortical and hippocampal regions is known to be severely perturbed in AD (Hyman et al., 1987; Butterfield and Pocernich, 2003). Glutamate levels have been shown to vary in different regions of brain in AD patients, with remarkable increase in the occipital lobe and significant decrease in frontal and temporal lobes (Ernst et al., 1997; Fayed et al., 2011). During neurodegeneration, most importantly it is the synaptic levels of glutamate that is viewed as crucial for neuronal physiology and survival. In support, a recent study has observed significant increase in extracellular glutamate levels in APP transgenic mice model (Schallier et al., 2011). Glutamate induced excitotoxicity is majorly mediated by NMDA subtypes of glutamate receptors, which also mediates the glutamate induced processing of APP resulting in enhanced secretion of A β . Elevated A β levels in the brain leads to oxidative stress resulting in oxidation of various lipids, sugars, and protein molecules in the neuronal cells including glutamine synthetase enzyme, an enzyme involved in conversion of glutamate to glutamine. Investigative studies have found oxidized form along with reduced activity of this enzyme in the brain of AD patients (Bowen et al., 1976). In addition, reduction in activity and

protein expression of GLT-1, glial glutamate transporter has been reported in both clinical and experimental cases of AD (Sheldon and Robinson, 2007). Coupled with decreased glutamine synthetase activity and reduced GLT-1 protein levels, in AD patients there is remarkable decrease in synaptic clearance of glutamate in AD patients consequently resulting in NMDA mediated neuronal injury. Physiologically, glutamate is converted to GABA in CNS by glutamate decarboxylase enzyme (GAD). Recent reports demonstrates an almost 50% increase in the mRNA of GAD enzyme in the AD patients suggestive of increased GABAergic stimulation in dorsal striatum (Reinikainen et al., 1990; Schwab et al., 2012). Increased GABA levels via presynaptic GABA_A receptors results in chronic inhibition of CNS neurons leading to neuronal dystrophy, deafferentation and increased degeneration (Schwab et al., 2012). All these evidence are suggestive of altered glutamate levels playing a crucial role in pathogenesis of AD.

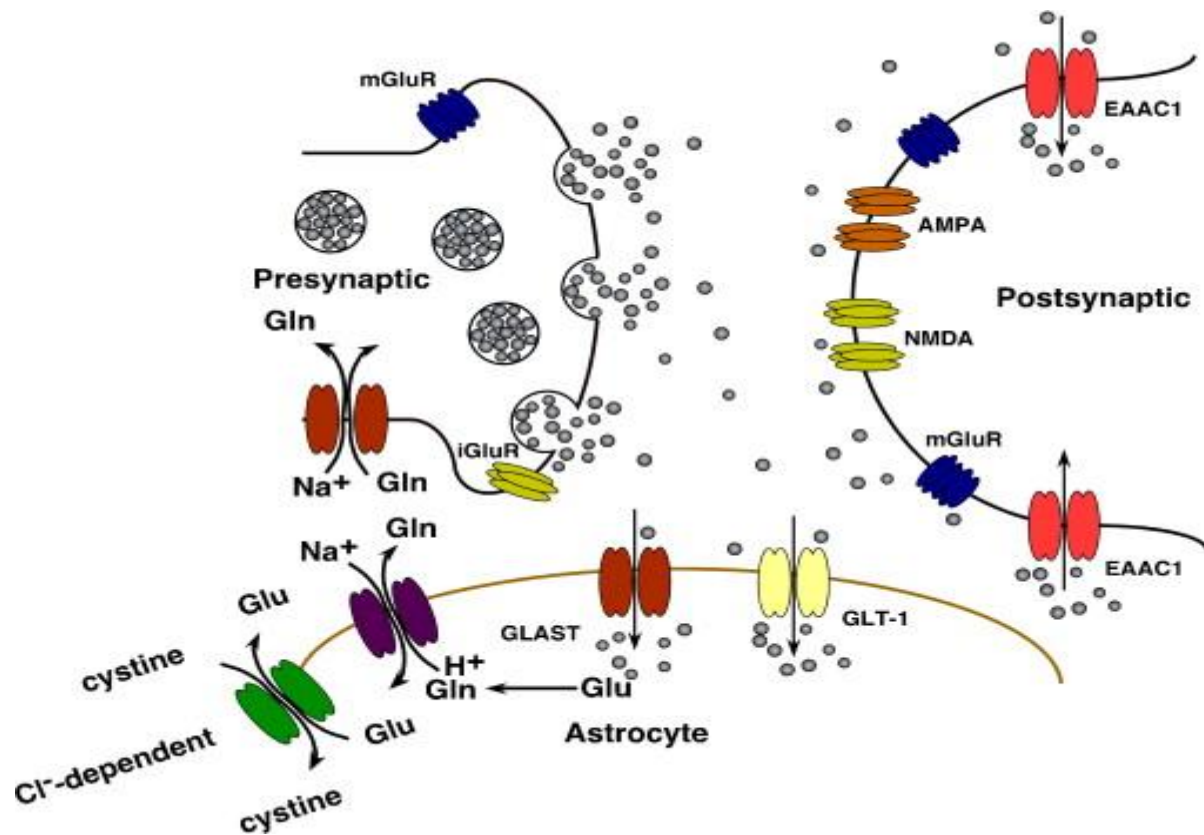


Figure 2.4: Schematic of an excitatory synapse. Glutamate released from pre-synaptic terminals activates ionotropic and metabotropic glutamate receptors. Glutamate uptake is mediated by a family of Na⁺-dependent glutamate transporters. Glial cells possess glutamine synthetase, enabling them to convert transported glutamate into glutamine, which can then be shuttled to neurons via glutamine transporters and converted to glutamate (reviewed in 67 and 128). System exchanges glutamate for cystine, providing cysteine as a precursor for glutathione synthesis. *Neurochemistry international* 51:333-355 (Sheldon and Robinson, 2007)

2.9. Role of inflammation in Alzheimer's disease:

Brain inflammation is an early pathological hallmark of AD (Eikelenboom et al., 2010), coexisting with A β plaques and NFTs (McGeer et al., 1989). Specific to AD, neuroinflammation demonstrates chronic and self sustaining pathogenic process, immensely capable of neuronal injury and neurodegeneration. Inflammatory hypothesis of AD states that chronic inflammation is set in motion by A β , which through various inflammatory mechanisms play a crucial role in

neurodegeneration in AD. Numerous tangential and direct evidences establish the pathophysiological relevance of neuroinflammation in AD (reviewed in Akiyama et al., 2000b). The first evidence would be the co-localization of activated microglia, astroglia and monocytes around the A β plaques and dystrophic neurites and that too only in the brain regions highly affected by AD pathology such as frontal neocortex, limbic cortex with no signs of inflammation in unaffected areas like cerebellum (Dickson et al., 1988; Rogers et al., 1988). Secondly, though modest but significant inflammation is found in the patients with low Braak scores for AD pathology (i.e. patients without history of dementia but sufficient A β and neurofibrillary tangles at autopsy) along with upregulation of the inflammatory genes including gene encoding for complement factors, major histocompatibility complex proteins II, cell adhesion molecules and enzymes involved in prostaglandin synthesis (Lue et al., 1996; Blalock et al., 2004; Parachikova et al., 2007). Third, A β through various *in-vitro* and *in-vivo* studies, has shown to activate the complement cascade directly, stimulate secretion of inflammatory cytokines, chemokines and other markers from the activated monocytes (O'Barr and Cooper, 2000), microglia (Del Bo et al., 1995; Chong, 1997), astrocytes (Hu et al., 1998), endothelial cells (Suo et al., 1998) and neurons (Del Bo et al., 1995; Du Yan et al., 1997). Additionally, A β also induces ROS generation through NADPH oxidase and myeloperoxidase pathway in stimulated monocytes, microglia, and neurons, which is conceived to have major implications in pathogenic process of AD (Bianca et al., 1999). Fourth, recent reports establish that these pro-inflammatory mediators such as IL-1, IL-6 and IFN- γ induces APP secretion and processing as well as inhibit the production of soluble APP (sAPP, a well known neuroprotectant), consequently resulting in enhanced A β deposition and neurotoxicity (Blasko et al., 1999b). Fifth, multiple line of evidence has strongly suggested that anti-inflammatory therapies are effective in delaying the onset or slowing the progression of

AD, indirectly implying the pathogenic importance of inflammation in AD (reviewed in Jaturapatporn et al., 2012). Sixth, is the direct evidence of the beneficial effects of the passive immunization therapy with A β antibodies (reviewed in Aisen and Vellas, 2013). All these evidences establish the pathogenic relevance of chronic neuro-inflammatory processes in pathophysiology and progression of AD.

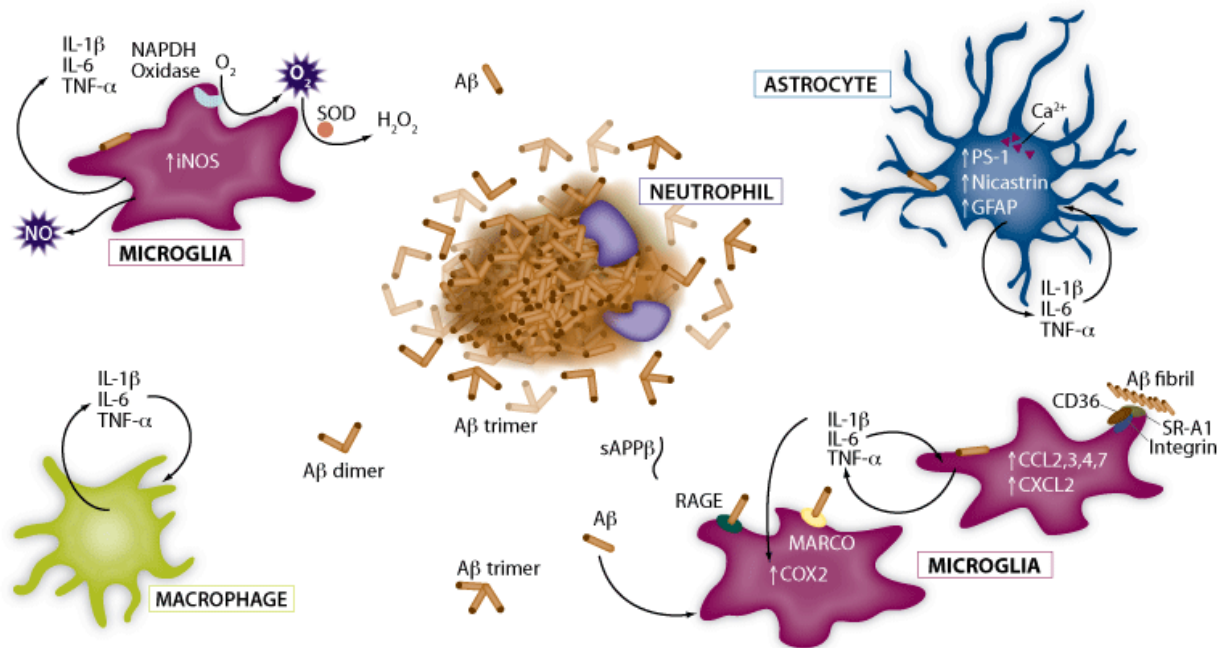


Figure 2.5: Accumulation of A β as mature plaques and associated inflammation. During Alzheimer's disease (AD), A-beta molecules accumulate as mature A-beta plaques. In response to the deposition of A β and the release of chemoattractants from damaged neurons, activated astrocytes, microglia, and macrophages release pro-inflammatory cytokines such as IL-1beta, IL-6, and TNF-alpha. Although this initial response is an attempt to protect the brain, the prolonged state of chronic inflammation is believed to be a major detrimental factor during AD. View a complete list of products related to neuroinflammation.

(http://www.rndsystems.com/rnd_page_objectname_molecular_balance_in_the_brain.aspx)

2.10. T-helper cells in AD:

Role of T-cells in AD is a dichotomous and controversial one, reported to have none to significant involvement in AD, and is associated with both neuroprotection and neurotoxicity. Early as well as recent reports indicate the T-cell regulated inflammatory response is involved in either pathophysiology, progression or both of the most common dementia related neurodegenerative disorder (Togo et al., 2002; Panossian et al., 2003; Magnus et al., 2005). The brain, earlier thought to be an immune privileged organ, has now shown evidence of T-cell infiltration and cytotoxic responses by cytotoxic T-cell lymphocytes (CTLs), reflecting immune preference as a function of immune response regulation at other cellular levels. The most considerable factor for this change of view point is the demonstration of BBB permeability to activated T-cells, which increases dramatically during systemic infection or other neurodegenerative diseases (Ransohoff et al., 2003). Togo and colleagues recently reported infiltration of T-cells in AD brain as compared to non-AD degenerative dementias, and controls, but due to absence of expression of proliferation markers on these cells, suggested that these were most likely not effector T-cells (Togo et al., 2002). However, this conclusion is thought to be based on underestimation of total number T-cells in AD brain attributable to difficulty of detecting T-cells because of their rather entrance to Virchow-Robbins space, a region between cerebral vessels and the brain parenchyma (Archambault et al., 2005). Despite this, the T-cell number surveying AD affected brain is yet less as compared to other neurodegenerative disorders such as multiple sclerosis and requires further investigation to determine whether T-cell has any etiopathogenic involvement in AD.

Recent investigations are indicative of involvement of dendritic and microglia cells in orchestrating the T-cell response in AD (Rogers et al., 1988; Magnus et al., 2005; Fisher et al.,

2011). During CNS inflammation in neurodegenerative disorders, activated microglia express costimulatory molecules such as CD80, CD86, and CD40 in order to interact with T-cells and modulates T-cell mediated response by promoting either their proliferation, T-effector functions (e.g., cytokine secretion), or both (Aloisi, 2001; Magnus et al., 2005; Wirenfeldt et al., 2011). Microglia activation and T-cell induced cytotoxic inflammatory response is interceded by augmented secretion and release of pro-inflammatory cytokines that induces several cellular pathways leading to heightened ROS formation and cytotoxicity. Moreover, the cytokines and other secreted cellular products also play a crucial role in orchestrating the neuronal immune response by regulating the neuronal cell viability or BBB permeability, examples of those would be cytokines such as IL-1, IL-2 and IL-17a (Huppert et al., 2010). Furthermore, these cytokines are also required for the maturation and differentiation of T-cell and governs which type of T-cell mediated response will predominate in a given disease state (reviewed in Rubio-Perez and Morillas-Ruiz, 2012). Generally, the T-cells are differentiated into T-helper cells ($CD4^+$) or T-effector cells ($CD8^+$) cell types. T-helper cells then divide into two major subtypes, T-helper 1 (Th-1) and T-helper 2 (Th-2), though recently a new subtype of T-helper cells, Th-17 cells were found that secretes IL-17 and does not share the general characteristics of Th-1 and Th-2 response. Th-17 cells induced IL-17a release is a predominant response in various autoimmune related disorders including inflammatory bowel syndrome (IBS) and multiple sclerosis (MS). In case of CNS autoimmune disorder i.e. MS, IL-17 producing T-helper cells impairs the blood brain barrier (BBB) integrity and increase its permeability to other lymphocytes. Recent investigation has shown that IL-17a, a subtype of IL-17 cytokines, induces NADPH oxidase dependent ROS generation resulting in disruption of endothelial tight junctions that consequently leads to impaired BBB integrity. Evidences like these demonstrate the robust pro-inflammatory

nature of Th-17 and IL-17 in propagating pathogenic mechanisms in various auto-immune or chronic immune system mediated disorders. However, the presence of Th-17 and their involvement in case of chronic inflammation related neurodegenerative disorders has not yet been shown.

2.11. Synaptic deficits and cognitive dysfunction in Alzheimer's disease:

Loss of synapses is an early and invariant characteristic feature of AD that strongly correlates with the severity of dementia, a relationship which is not exhibited by the amyloid plaque deposition (Masliah et al., 2001; Scheff et al., 2007). Significant decrease in synaptic density and loss of pre-synaptic and post-synaptic markers in cortical and hippocampal areas of AD brain were revealed by electron microscopy and immunohistochemical staining (Masliah et al., 2001; Reddy et al., 2005). A growing deal of literature suggests that A β peptides and hyperphosphorylated tau leads to synaptic dysfunction and neuronal loss, respectively (Frautschy and Cole, 2010). The main perpetrator is soluble non-fibrillar A β assemblies, commonly known by different names such as A β -derived diffusible ligands (ADDLs), oligomers, paranuclei, and protofibrils, which emerge long before the A β deposits. Three basic mechanisms has been proposed to account for the A β induced synaptic deficits, which are a toxic gain of function (due to novel interactions by new conformations of A β), loss of normal physiological function and precipitation of physiological dysfunction by excessive A β .

Physiologically, A β peptides play a crucial role in homoeostatic plasticity by depressing the excitatory transmission through AMPA and NMDA subtypes of glutamatergic receptors. In addition, A β can inhibit LTP in the hippocampal perforant path (Chen et al., 2000; Wang et al., 2002; Rowan et al., 2004) and can also attenuate AMPARs and NMDARs surface expression and function. Toxic gain of function has been reported through various *in-vitro* and *in-vivo*

studies, where ADDLs have been shown to avidly bind dendritic arbors of certain cultured neurons (Lacor et al., 2004; Lauren et al., 2009), mediate dendritic spine loss (Lacor et al., 2007), increased generation of ROS, disrupting the balance of excitatory and inhibitory neurotransmission leading to epileptic behavior in transgenic mice. A β mediated dysfunction involves internalization of NMDA receptors through a calcineurin dependent pathway (Dewachter et al., 2009) and impairment of synaptic transmission and plasticity through activation of group I mGluRs with p38 MAP kinase and calcineurin as downstream effectors (Hsieh et al., 2006; Li et al., 2009). Also, A β has shown to interact with NMDA receptors and disrupt the calcium balance inside the cell resulting in excitotoxicity and resultant cell death. Synaptic deficits are a common phenomenon in various transgenic animal disorders that are employed for studying different pathophysiological aspects of AD. Thus, loss of synaptic connections and impairment of synaptic plasticity is rudimentary in pathogenesis of memory impairment in AD and future research in understanding the underlying mechanisms of synaptic toxicity may help in development of novel therapeutic interventions.

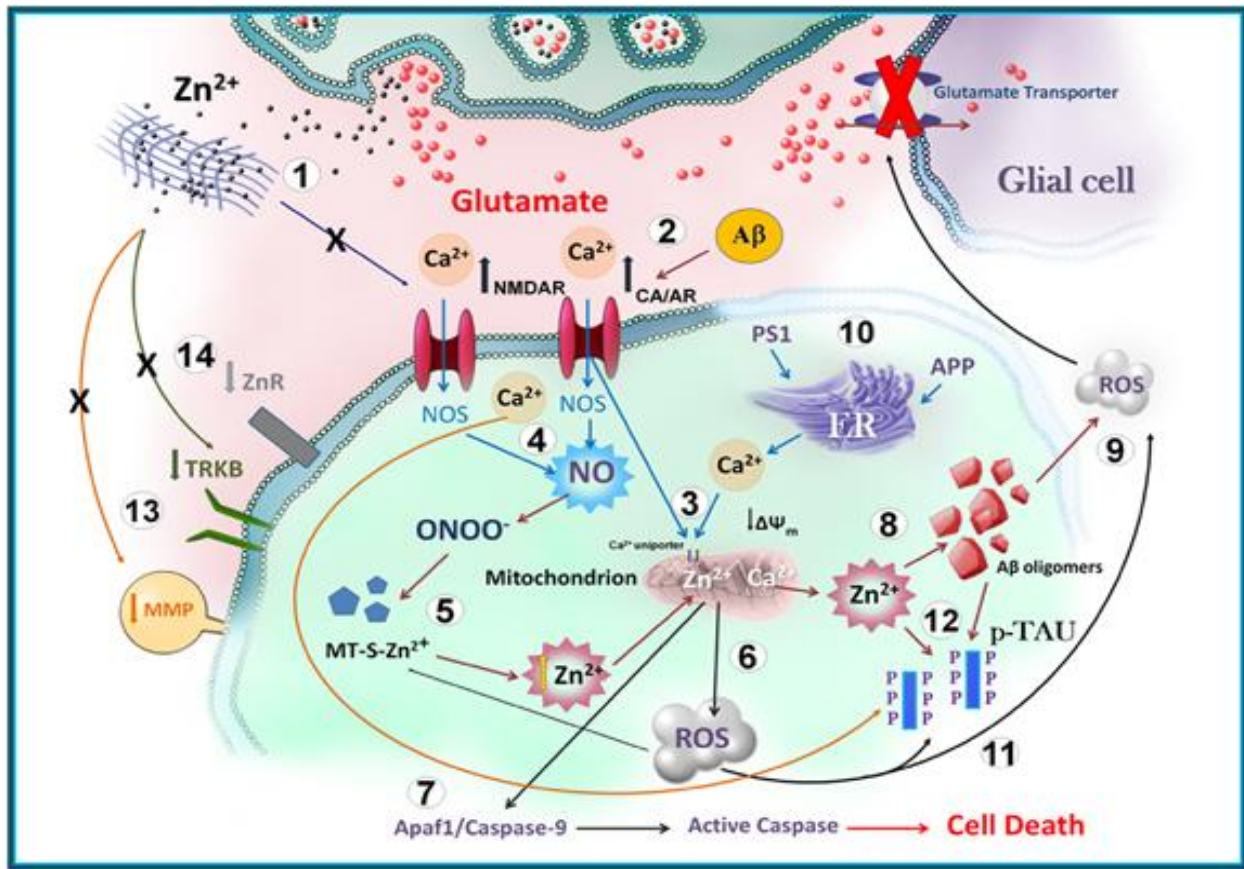


Figure 2.6: A β , oxidative stress, tau, glutamate receptor overactivation, Ca $^{2+}$, and Zn $^{2+}$ dyshomeostasis may synergistically promote AD-related synaptic and neuronal loss. The sequestration of Zn $^{2+}$ by the amyloid contained in extracellular plaques (1) may remove the cation from the synaptic cleft and release the Zn $^{2+}$ -dependent NMDAR blockade (1). A β also directly activates Ca-ARs (2). Both phenomena promote Ca $^{2+}$ overload, increased superoxide generation from mitochondria (3), as well as nitric oxide (NO) production from Ca $^{2+}$ -dependent activation of NO synthase (NOS) (4). Reactive oxygen and nitrosative species (ROS and RNS) then mobilize Zn $^{2+}$ from MTs leading to toxic Zn $^{2+}$ concentrations (5) that further impair mitochondrial function, promote more ROS generation (6), and release pro-apoptotic factors (7). ROS-driven Zn $^{2+}$ mobilization may also facilitate intra-neuronal A β aggregation (8). A β may amplify this vicious loop by increasing the generation of ROS that when extracellularly released reduce glutamate reuptake (9) and overactivate NMDARs and Ca-ARs. Mutant PS1 and APP enhance Ca $^{2+}$ dyshomeostasis by altering the ER-mitochondrial network (10). NMDAR-mediated [Ca $^{2+}$] $_i$ rises and oxidative stress may also accelerate tau hyperphosphorylation (11) and recent evidence indicates that oligomers from AD patients are directly promoting tau

hyperphosphorylation as well (12). Zn²⁺ sequestration also impairs BDNF signaling (13) (by blocking TrkB and or MMPs), and also reduces the modulating effects exerted by the activation of its own ZnR (14). All these processes, set in motion at the level of synaptic spines, may be the *primus movens* of synaptic dysfunction, neuronal deafferentation, and death. *Cell death & disease* 2:e176 (Corona et al., 2011)

2.12. Experimental models of Alzheimer's disease:

Alzheimer's disease, a 100 years old neurological disorder, is modeled in laboratory environment through various *in-vitro* and *in-vivo* experiments. Experimental modeling of AD in *in-vitro* as well as *in-vivo* conditions is done based on the various hypothesis/theories put forth to account for its pathogenesis. Mostly these hypotheses includes ageing, cholinergic, glutamatergic, amyloid, tau and inflammatory hypothesis but the most prominent and well accepted of them are amyloid and tau hypothesis. The reason is their coexistence in almost all of clinical cases and their strong correlation with cognitive impairment and neuronal dystrophy in AD. As explained earlier, the A β oligomers strongly proportionate with synaptic deficits and memory impairment and tau neurofibrillary tangles colligate very well with the neuronal injury and cell death. Among these animal models, the models which are currently widely used are the double and triple transgenic models that utilizes both amyloid and tau hypothesis. Although, these transgenic models represents only less than 10% of AD cases (that have hereditary genetic mutations as pathological causes), but they shed light on various pathogenic events initiated by A β and tau insult in the brain.

There are not as much *in-vitro* models as there are *in-vivo* models for AD. This might be due to the chronic nature and cognitive symptoms of AD, which are difficult to be modeled through *in-vitro* experiments. Only to illustrate the molecular mechanisms of toxic insult of A β

and to establish the preliminary protective effect of an agent by acting on those molecular mechanisms and that too in the selective neuronal populations, the *in-vitro* experiments on hippocampal and cortical cell types and tissue cultures are performed. For instance, to describe the neurophysiologic effects of A β insult on the glutamatergic neurotransmission, the hippocampal slice cultures are commonly used. These *in-vitro* models are very helpful in answering the specific questions and thus there are no specific models but they are tailor made. Thus for collecting the preliminary neuroprotective data for our experimental herbal drug we chose H19-7 rat hippocampal cell line. The H19-7 is a rat immortalized hippocampal cell line, which in the *in-vitro* conditions is able to form synaptic connections in certain specific conditions that results in their differentiation. The toxic insult in this cell line has shown to cause neuronal cell death.

2.13. Bigenic Transgenic APP^{swe}+PS1 mice:

In the current project we employed a double transgenic APP^{swe}+PS1^{dE9} mice, which harbors familial AD linked Swedish mutant human APP and mutant human presenilin (PS1-dE9) gene and overproduce A β 1-42 in the brain of these mice resulting in development of Alzheimer's related pathology (Jankowsky et al., 2004). At six to seven months of age these mice exhibit senile plaques (SPs) in hippocampus and cortex along with spatial learning deficit at seven months of age and at nine months of age dystrophic neurites with very subtle neuronal cell loss appears (Savonenko et al., 2005; Garcia-Alloza et al., 2006). Furthermore, robust inflammation is also prevalent in this animal model, with elevated hippocampal levels of GFAP, clustered microglia and astrocytes at an early age of 4 and 6 months around the amyloid plaques deposits and elevated levels of inflammatory markers such as TNF-alpha, IL-b, 1 IL-6 and MCP-1 at 10 months of age in hippocampus and cortex (Garcia-Alloza et al., 2006; Ruan et al., 2009). Thus

this animal model simulated both synaptic deficits and inflammatory alterations characteristic to AD and was the best candidate among other animal models to serve our specific aim.

2.14. *Centella asiatica*:

Centella asiatica is a slender, green to reddish-green in color, creeping perennial herb that belongs to Umbelliferae (also known as Apiaceae) family and is common inhabitant of tropical swampy areas. Commonly, indigenous to the countries like Africa, Australia, Cambodia, Central America, China, Indonesia, Madagascar, the Pacific Islands, South America, Thailand, southern United States of America, India, Malaysia and Viet Nam. *Centella asiatica* has several common names depending on the geographical location such as gotu kola in Sinhala, Mandukaparni in Sanskrit, Kodakan in Malayalam, pegoga in Malayasia and botanically it is known as *Hydrocotyle asiatica* L. and *Trisanthus cochinchinensis*. The herb is well known for its therapeutic values in Asian countries and thus is a part of their folklore medicine system such as India, China and Malaysia. Especially, Indian medicine system i.e. Ayurveda consider *Centella asiatica* a vital herb, which is used for treating various ailments including wound healing, albinism, anaemia, asthma, bronchitis, cellulite, cholera, measles, constipation, dermatitis, diarrhoea, dizziness, dysentery, dysmenorrhoea, dysuria, epistaxis, epilepsy, haematemesis, haemorrhoids, hepatitis, hypertension, jaundice, leukorrhoea, nephritis, nervous disorders, neuralgia, rheumatism, smallpox, syphilis, toothache, urethritis, and varices; and as an antipyretic, analgesic, anti-inflammatory, and “brain tonic” agent. Therapeutically, the whole aerial part of the herb is beneficial and hence, is used to prepare extract.

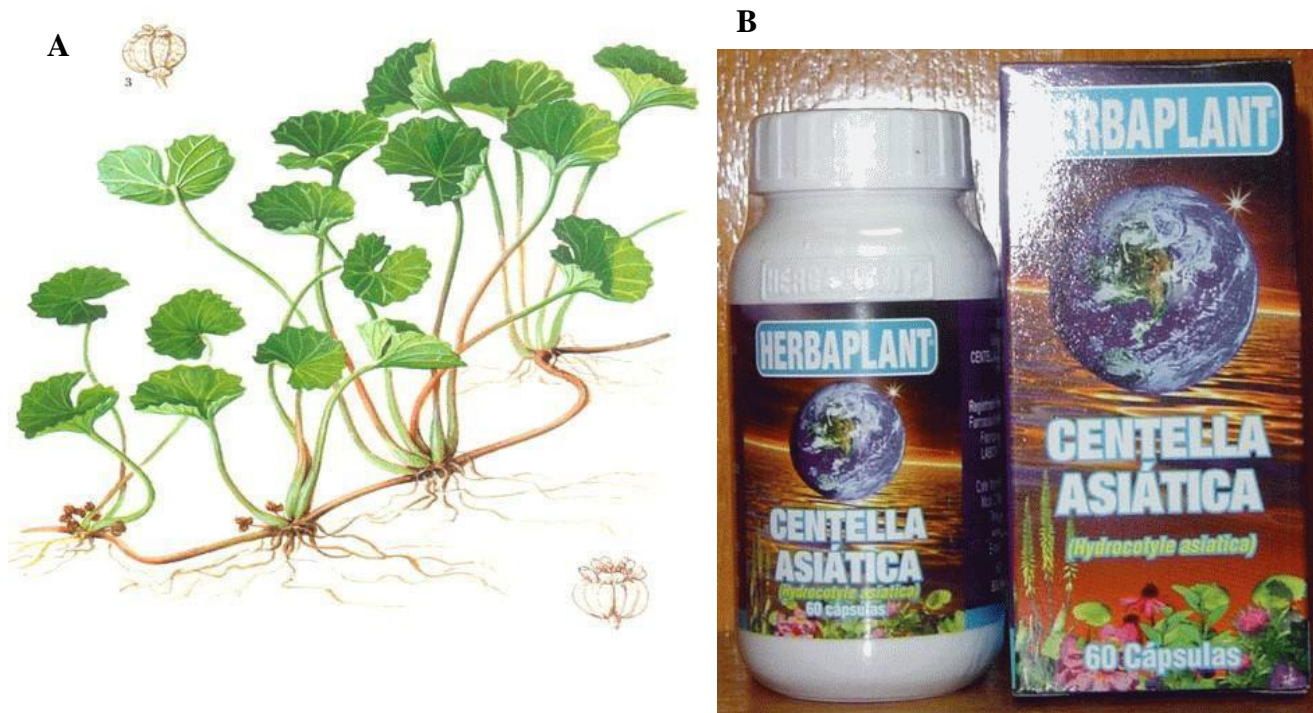


Figure 2.7: Representative illustration of *Centella asiatica*, a perennial herbal plant inhabitant of different parts of Asian countries. A) The picture depicts the creeping stolons, green to reddish-green in color, connecting plants to each other. It has long-stalked, green, reniform leaves with rounded apices which have smooth texture with palmately netted veins.

(<http://www.helpfulhealthtips.com/wp-content/uploads/2010/07/Centella-Asiatica-300x300.jpg>)

B) Commercial preparation of *Centella asiatica*, which is commonly used as food supplement. There are various such marketed preparations. (<http://www.pharmacywall.com/2010/09/30/centella-asiaticabrahmi/>)

Rigorous experimental studies are undertaken to extract and identify different chemical constituents from the herbal extract of *Centella asiatica*. Till date triterpenes, essential oils, amino acids and other compounds have been identified as its chemical constituents. The triterpenoids exists in forms of saponins and glycosides that includes asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, centellose, brahmic, centellic, madecasic acids and asiatic acid (A-A) (Singh and Rastogi, 1969). Medicinal value of the herbal

extract as well as the individual components of *Centella asiatica* is extensively studied in preliminary as well as clinical studies. Moreover, the molecular mechanism rudimentary in therapeutic effects of this drug is also being investigated. Experimentally, it has been approved for treatment of wounds, burns, and ulcerous skin ailments, and prevention of keloid and hypertrophic scars (Brinkhaus et al., 2000; Gohil et al., 2010; Paocharoen, 2010; Belcaro et al., 2011), to treat second- and third-degree burns and also topically to accelerate healing, particularly in cases of chronic postsurgical and post-trauma wounds (Belcaro et al., 2011). Recent investigations have implicated its effects in improving cognitive performance in rat models (Veerendra Kumar and Gupta, 2002; Wattanathorn et al., 2008; Gohil et al., 2010; Shinomol et al., 2011; Xu et al., 2012). Moreover, couple of human studies has reported significant improvement in general mental ability of mentally retarded children (Appa Rao et al., 1973) on treatment with *Centella asiatica* extract (CaE). *Centella asiatica* extract (CaE) improved performance of rats in passive avoidance task, which is also demonstrated by asiatic acid in another study (Nasir et al., 2010), implicating the asiatic acid as one of the cognitive enhancers present in the extract. Furthermore, *Centella asiatica* is considered to possess neuroprotective properties against amyloid beta toxicity (Xu et al., 2008) and reactive oxygen generator, hydrogen peroxide. Mechanistically, bioactive compounds are very strong antioxidant, free radical scavengers and mitochondrial energizers. *Centella asiatica* extract (CaE) is well known potent antioxidant and anti-apoptotic capabilities as it has been shown to decrease caspase expression. *Centella* has also shown to strongly inhibit subtypes of phospholipase 2, implicating the significant anti-inflammatory activities (Defillipo et al., 2012). In addition it is reported to affect the dendritic arborizations by stimulating the dendrites of neuronal cells of rat brains, neurite elongations in human SH-SY5Y cells and accelerate axonal regeneration in rats

suggesting neuronal regeneration as another beneficial effect. Recent report has indicted multifaceted approach targeting Amyloid beta formation and deposition, which includes inhibiting BACE1 and up-regulating ADAM10 in primary cortical neurons (Patil et al., 2010). There is a dearth of knowledge regarding mechanisms that are involved in neuroprotection and cognitive enhancement by *Centella asiatica* against amyloid beta induced neurotoxicity.

2.15. References

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3. *Centella asiatica* extract (CaE) enhances synaptic plasticity and exhibit neuroprotection in neuronal cells against oxidative stress

Abstract

Centella asiatica (CA) is a well known medicinal herbal drug traditionally used in folklore Indian Ayurvedic medicinal system and in other parts of Asia for more than hundred years to treat a plethora of ailments. The whole aerial portion of the plant is highly rich in various biologically active compounds. Several lines of evidence report antioxidant and neuroprotective actions of Centella but the mechanism of action is unknown. Moreover, recent reports as well as ancient texts mentions cognition enhancement as one of the beneficial medical properties of Centella. In the current study we commercially obtained a concentrated extract of *Centella asiatica* containing 20% asiaticoside and used it to elucidate the neuroprotective and antioxidant action against well known pro-oxidants and neurotoxin in two neuronal cell lines. In addition, we also investigated the molecular mechanisms related to neuroprotection as well as mnemonic activities in the hippocampal neuronal cell line H19-7. In addition to preliminary characterization of the current extract, the neuroprotective action of *Centella* against hydrogen peroxide was also studied in hippocampal cell line. These results were found to be reproducible in the other neuronal cell line PC12. Reactive oxygen species were found to be effectively reduced in in-situ as well as homogenized samples of H19-7 cells. Caspase activity was also found to be greatly reduced in presence of Centella extract. We also found Centella to increase the phosphorylation of ERK1/2 and CREB by itself at different doses and affect the NMDA receptors in differentiated and un-differentiated hippocampal neuronal cells. Similar observations were found when cells were treated with hydrogen peroxide in presence or absence of two

concentrations of Centella. Thus, the current study describes anti-caspase, antioxidant actions of Centella namely attributing to its asiaticoside concentration. Modulation of glutamatergic receptors and stimulation of ERK1/2-CREB-BDNF pathway was found to be one of the putative mechanisms involved in synaptic strengthening shown by the Centella.

3.1. Introduction

Phytomedicine is increasingly gaining importance in current day medical practice due to their therapeutic value as well as reduced side effects. An herbal extract is considered to be nature's own handpicked cocktail of various bioactive compounds capable of affecting various physiologic pathways in synergetic and/ or antagonistic manner. This provides an effective mean of reinforcing body's defense against free radicals and render neuroprotection. *Centella asiatica* (CA), also known as Gotu Kala, is a creeping perennial herb that grows wildly in Asia, especially in India, China and Thailand. It belongs to family Apiaceae (Umbelliferae) and is a psychoactive medicinal plant that had been traditionally used since centuries in Ayurvedic system for treating various ailments. Recently, pharmacological studies have demonstrated beneficial therapeutic effect of the Titrated Extract of *Centella asiatica* (TECA) in wound healing, microangiopathy, and gastric ulcers (Shukla et al., 1999; Cesarone et al., 2001a; Cesarone et al., 2001b; De Sanctis et al., 2001; Incandela et al., 2001; Paocharoen, 2010; Belcaro et al., 2011). Over the past few decades, investigational studies has shown the herb to possess antitumor activity (Babu et al., 1995), anti-inflammatory, CNS depressant activity (Sakina and Dandiya, 1990), anti-apoptotic, antioxidant properties and neuroprotective activity. Recent reports have indicated various pharmacological effects of *Centella* on CNS such as stimulatory-nervine tonic, rejuvenant, sedative, anxiolytic, and intelligence promoting property. The whole

plant extract extract exhibits stimulating activity on the dendrites of neuronal cells of rat brains (Mohandas Rao et al., 2009) and improves cognitive performance in rat models (Kumar et al., 2011; Prakash and Kumar, 2012; Xu et al., 2012). In clinical studies *Centella* has shown to possess positive modulation of cognition, mood and improves physical performance in healthy elderly volunteer (Wattanathorn et al., 2008; Mato et al., 2011).

Chemically, CA is composed of many constituents such as flavanoids, tri-terpenoids, such as asiatic acid (AA), mecadessic acid (MA), asiaticoside (James and Dubery, 2009) scentellin, asiaticin and centellicin (Zhang et al., 2008). Investigations involving single constituents and their derivatives had been undertaken to determine their therapeutic efficacy. One of such studies has established the cognitive performance enhancing effect of three of the asiatic acid derivatives (Soumyanath et al., 2005). Tri-terpenoids such as asiaticoside had been shown to possess many of the characteristic qualities related to cognitive performance. However, the mechanism of the extract on cognition and memory has not been examined.

Therefore, the present study was designed to investigate the possible *in-vitro* neuroprotective mechanisms of *Centella asiatica* extract (CaE) *per se* in H19-7 cells. Neuroprotection of *Centella asiatica* against acute oxidative stress was also determined in two different neuronal cell populations. The possible mechanism of neuroprotection by CaE in presence of oxidative stress in form of acute hydrogen peroxide (H₂O₂) exposure was also evaluated in H19-7 cell line. We found CaE to be an effective inhibitor of Caspase-3 enzyme and 15-LOX. In addition we demonstrated that the CaE itself possess the capability to modulate the NMDA receptors possibly via activating the CAMKII-CREB-BDNF pathway. In accordance with the *per se* effects of CaE, we found CaE to modulate NMDA subtypes of glutamatergic receptors and exert neuroprotection when challenged with H₂O₂ induced oxidative stress.

3.2. Materials and Methods:

Centella asiatica Extract (CaE): Dried *Centella asiatica* extract (CaE) was obtained from Ambe Phytoextracts Pvt Ltd, an ISO certified company from India. The dried CaE was specifically obtained as 20% concentrated extract of asiaticoside. Certification of analysis was also obtained along with the dried extract which showed HPLC-UV estimation of extract to be 20%.

Preparation of aqueous solution of *Centella asiatica* extract: Dried *Centella* extract was dissolved in three different concentrations, 100mg/ml, 10 mg/ml and 1mg/ml in 15 ml polyvinyl tubes in Millipore purified water. The solution was kept overnight with mild agitation on a shaker at room temperature. After 14-18 hrs of incubation, the resulting solution is taken out which contained both dissolved and un-dissolved fraction of the dried extract. This solution was centrifuged at 1,500 rpm for 15 min and supernatant was removed. The supernatant was then filtered with 0.22 µm Millipore filter in the pyrogen free environment. The obtained solution was stored at -80°C for future use.

Analysis of total protein, sulphhydryl, phenolic and flavanoids content: Dried CaE obtained above was assessed for its physical properties as well as chemical constituents. We assessed the pH of the obtained solution as well as the specific gravity. Protein assay was performed using the Thermo Scientific Pierce 660nm Protein Assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as standards for obtaining the standard curve for protein measurement. For total sulphhydryl content, glutathione (0.01-1µg) was used as external standard. The sulphhydryl content was measured fluorimetrically, by a condensation reaction between O-phthalaldehyde (OPT) with sulfhydryl group to yield a fluorescent product at pH 8.0, at excitation and emission wavelengths of 340 and 420 nm, respectively (Dhanasekaran et al., 2007). The total

polyphenol content (TPC) was determined by the reaction with Folin and Ciocalteu's phenol reagent (Swain and Hillis, 1959). Pyrogallol acid was used to prepare standard curve for estimation of TPC.

The total flavonoid content was determined using the method of Quettier et al. (2000) with minor modifications. Briefly, 1ml of 2% aluminium trichloride was mixed with one part of different concentrations of aqueous extract solution and absorbance readings were taken at 430 nm after 10 min. The total flavonoid content was determined using a standard curve of quercetin at 0.039-0.125 mg/ml.

Effect of CaE on 15-Lipoxygenase enzyme activity: The 15-Lipoxygenase inhibitory activity of reconstituted aqueous CaE was examined using a Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI). Linolenic acid was used as a substrate for soy 15 LOX enzyme. The assay detects the formation of hydroperoxides by activity of 15-LOX on linolenic acid through chromagen consisting of reagent 1 and reagent 2 (FeSO₄·7H₂O and NH₄SCN, respectively) which changes to bright yellow and was read at 490 nm.

Protease inhibition assays: The ability of CaE solution to inhibit the activity of human recombinant caspases was determined using a standard fluorometric microplate assay (Thornberry, 1994). Briefly, caspase enzyme (PharMingen, San Diego, CA, U.S.A.) was incubated at 37°C with 5 µM of the fluorogenic caspase-3 substrate z-DEVD-AFC (BioMol, Plymouth Meeting, PA, U.S.A.) in a buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT. At the end of the incubation period, the amount of cleaved AFC was measured on a fluorescent microplate reader, using an excitation wavelength of 380-400nm and an emission wavelength of 505 nm. The percentage inhibition

was compared with the well known Caspase-3 inhibitor, z-DEVD-FMK, which was used in 500 nM range. Results are expressed as percentage inhibition as compared to z-DEVD-FMK.

Embryonic Hippocampal neuronal cell line (H19-7): The H19-7 cell line, originally derived from hippocampi dissected from embryonic day 17 (E17) Holtzman rat embryos and immortalized by retroviral transduction of temperature-sensitive tsA58 SV40 large T antigen (Morrione et al., 2000). H19-7 cells were maintained in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 10% fetal bovine serum, 200 µg/ml G418, and 1µg/ml puromycin in flasks coated with 15 µg/ml poly-L-lysine. Cells were propagated in 75 cm² poly-L-lysine coated tissue culture flasks, harvested by trypsinization after reaching 80% confluence (3-4 d). They were equilibrated in a humidified atmosphere of 5% CO₂/95% O₂ at an air temperature of 34°C. The cultures were used within 20 passages after the cells were received. For differentiation of H19-7 cells, the propagating cells were incubated at 39°C in N2 medium, which consists of DMEM supplemented with 1% fetal bovine serum, 50 units/ml penicillin, 1µg/ml puromycin, 200 µg/ml G418, 2 mM glutamine, 31.5 ng/ml progesterone, 100 µg/ml transferrin, and 30 µg/ml sodium selenate. Basic fibroblast growth factor (BFGF) was added in this media to attain a concentration of 50 ng/ml and the cells were allowed to differentiate.

Cell survival assays: Estimation of cytotoxic concentrations of CaE: H19-7 cells were grown in 10 cm² culture plates in propagating DMEM medium at permissive temperature and conditions (mentioned above). After harvesting in trypsinized media they were plated in into poly-L-lysine coated 96 well plates at a density of 2×10^5 cells/ml. After 24 hours, cells were incubated with different concentrations of CaE (0, 2.5, 5, 10, 15, 20, 25, 50, 100, 150, 200, and 250 µg/ml) for

24 and 48 hours in serum-fed and serum-free conditions to assess non-toxic concentrations of CaE. Non-cytotoxic concentrations of CaE were also estimated in both un-differentiated and differentiated H19-7 cells.

MTT analysis and CCK-8 kit analysis: After incubating cells with the appropriate treatment cell survival was analyzed with either MTT analysis or CCK-8 cell counting kit. The MTT assay was performed as described earlier (Dhanasekaran et al., 2006). Briefly, MTT was added at a final concentration of 1 mg/ml. The medium was discarded after the incubation for 4 hrs at 37 °C and the insoluble dark blue formazan crystals were dissolved in 100 µl of DMSO. Absorbance was subsequently measured at 570 nm with a reference wavelength of 630 nm using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA).

CCK-8 cell counting kit utilizes highly water-soluble tetrazolium salt, WST-8, which is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye. Briefly, CCK-8 reagent was added at a final concentration of 1 mg/ml and kept for incubation at 37°C for 4 hours. Absorbance was subsequently measured at 450 nm using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA).

Effect of CaE on NMDA receptor and transcription factors: H19-7 cells were grown at a density of 2×10^5 cells/ml in poly-L-lysine coated 10 cm² cell culture dishes. The cells were maintained in high glucose DMEM supplemented with 10% FBS in 5% CO₂/95% O₂ at an air temperature of 34°C. After achieving 60% confluence, cells were incubated with various concentrations of CaE (0, 10, 20, and 50µg/ml) for 24 hours. After 24 hours cells were lysed in cell lysis buffer and the whole cell extract was prepared.

Immortalized hippocampal cells were differentiated while incubated with CaE at three different concentrations (CaE 10, 20 and 50 μ g/ml) for 18 hrs in 10 cm² cell culture dishes.

Neuroprotection against Hydrogen Peroxide (H₂O₂): After finding the appropriate non-cytotoxic concentrations range of CaE, 4 to 5 different concentrations ranging from 5 to 200 ug/ml were used to assess the cytoprotective effects of CaE against H₂O₂. For finding neuroprotective effects of CaE against hydrogen peroxide, cells were grown at a density of 2 \times 10⁵ cells/ml for CaE for 12 hours followed by addition of 300 μ M H₂O₂ for another 12 hours. This was followed by MTT assay or CCK-8 assay as described below. In case of assessing the neuroprotective effects of CaE in differentiated H19-7 cells, the H19-7 cells after attaining 80-90% confluence in 96 well plates were incubated in non-permissive temperature of 39°C in N2 differentiation medium as described above. CaE at different concentrations (concentrations ranging from 5 to 200 ug/ml) was added at the same time for 12 hours, which was followed by the addition of 300 μ M H₂O₂ in the wells. The cells were incubated for other 12 to 6 hours and cell proliferation assay was performed with either MTT or CCK-8 reagent.

Estimation of Reactive oxygen species (ROS) generation: PC12 Cells (10⁵/well) were plated into 96-well poly-L-lysine -coated plates in propagating conditions. Cells, after reaching a confluence of 60-70% were washed three times with PBS. Cells were then incubated with differentiation medium supplemented with 1% FBS and 50 ng/ml NGF for 48 hours in 5% CO₂/95% air at 37°C. After 48 hours of incubation, the medium was removed and the cells in the plates were washed with PBS buffer and then incubated with 10 μ M DCFH-DA in the differentiation medium in 5% CO₂/95% air at 37°C for 60 min. After DCFH-DA was removed, the cells were washed with PBS and incubated with differentiation medium containing different concentrations

of CaE with or without H₂O₂ (100 μM) for a total of 12-14 hours. The fluorescence of the cells from each well was measured and recorded in a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) at an excitation wavelength of 485 nm and the emission wavelength of 530 nm with temperature maintained at 37°C. The fluorescence from each well was captured, digitized, and stored on a computer using KC4 software. Data points were taken every 30 min for 2 hours and the data were exported to Excel (Microsoft, Seattle, WA, USA) spreadsheet software for analysis.

Western Blot: At the end of the incubation period, the cells were harvested by centrifugation, washed with PBS and lysed in a buffer containing 50mM Tris-Cl, pH 7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Complete Protease Inhibitor tablets, Roche Molecular Systems). Protein concentration of each sample was measured using the Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Protein samples were aliquoted into 50 μg fractions and were stored in -70 °C for storage. On the day of experiment samples were boiled with sample loading buffer for 5 minutes and then loaded onto a gradient 4-12% NuPAGE Bis-Tris Gel (Life Technologies, Grand Island, NY) and electrophoretically transferred onto PVDF membrane through wet transfer method. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline plus 0.1% Tween-20 at pH 7.4 (TBST). The membranes were incubated overnight at 4 °C with specific antibody constituted in 5% BSA in TBST. The membranes were probed with antibodies anti NR2A (Cell Signaling, USA), anti NR2B (Cell Signaling, USA), anti-NR1 (Cell Signaling, USA), GluR2 (Santacruz, USA), p-CAMKII, CAMKII, CREB, p-CREB, BDNF, anti-caspase-3 (Santacruz, USA), BAX, bad, p- along with anti β-Actin and anti-GAPDH, which were used as a loading control. Membranes were then washed with TBST (3 X,

each for 10 min) and incubated with specific species dependent HRP-conjugated secondary antibodies for 60 min at room temperature. Membranes were again washed three times for 10 min with TBST after incubation with each antibody. After washing membranes were incubated with chemiluminescent reagent (Enhanced ECL- substrate, Pierce, Rockford, IL) for 5 minutes. Membranes were wrapped in plastic wrap and placed in the x-ray film cassette with protein side oriented up. Chemiluminescence signal was measured as band density obtained on the X-ray film through utilization of Quantity One software (Bio-Rad, Hercules, CA). Band densities for each sample was normalized to its β -actin or GAPDH signal and reported as percentage control.

Statistical analysis: Results are given as mean \pm SD. Statistical analysis was performed by using One Way ANOVA with Dunnet test as an appropriate post-hoc test at the significance level $p < 0.05$. All statistical analysis was done using Prism-V software.

3.3. Results:

Chemical constituents of CaE solution: We commercially obtained the *Centella asiatica* extract, which had 20% asiaticoside content from Ambe Phytoextracts Pvt Ltd Company (Delhi, Haryana, India). Though we received certificate of analysis with the extract, yet to check against batch to batch variation we performed the usual biochemical analysis of the reconstituted aqueous extract. The table 3.1 describes the results we obtained for the physical as well as biochemical properties of CaE aqueous solution. To avoid the batch to batch variation we also performed the fingerprinting with respect to triterpenoid content by HPLC-UV detection following the method described by Zhang *et al* (Zhang et al., 2008). Figure 3.2 show the fingerprint obtained from the HPLC-UV analysis.

CaE extract inhibits 15-Lipoxygenase (15-LOX) enzyme activity: To assess the anti-inflammatory potential of CaE, we measured the *in-vitro* inhibitory action of CaE on 15-LOX enzyme. The Lipoxygenase (LOX) enzymes are non-heme iron containing dioxygenases that catalyzes the hydroperoxydation of polyunsaturated fatty acids containing *cis,cis*-1,4- pentadiene systems. There are three members of LOX family designated 5-LOX, 12-LOX and 15-LOX based on the position of the addition of molecular oxygen to fatty acid chain. The 15-LOX enzyme catalyses the peroxidation of arachidonic acid skeleton at carbon 15 position to produce 15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15(S)-HETE), or on linoleic acid at 13 position to produce 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13(S)-HPODE). It also produces lipoxins that are generated from the conversion of leukotrienes such as LTA₄ released by surrounding cells such as alveolar macrophages and leukocytes during inflammation. The CaE at different concentrations demonstrated the dose dependent inhibitory activity on the enzymatic action of the 15-LOX enzyme (Fig. 3.3, n=6). We employed concentrations ranging from The maximum inhibition of 30 % was observed at a concentration of 1000 µg/ml, which is very high if compared to IC₅₀ value of 38.6 µg/ml reported for methanolic extract of *Centella asiatica* (Akula and Odhav, 2008). The discrepancy between our and reported results can be ascribed to the differences in the enzyme isoform under investigation as well as the type of extract studied. In the current study we have used the aqueous fraction of the titrated extract of *Centella asiatica* compared to the methanolic extract used in the above cited study. In addition, we employed the 15-LOX as our enzyme of choice compared to 5-LOX employed by the previous reports.

CaE is a potent caspase-3 inhibitor: In order to characterize the basic *in-vitro* anti-apoptotic properties of CaE, we examined its ability to inhibit the activity of recombinant human caspase-

3, the principal effector caspase of the apoptosis pathway. CaE efficiently inhibited recombinant human caspase-3 with an 80% inhibition attained at 250µg/ml (Fig. 3.4, n=6). When compared to a well known selective caspase-3 inhibitor, z-DEVD-FMK, CaE at 2.5 µg/ml was equivalent to 20 µM inhibitor. To best of our knowledge, this is for the first time that CaE is shown to possess direct caspase-3 inhibitory activities. Though *in-vivo* effect of CaE on caspase activity has been previously reported, where *in-vivo* administration of Asiaticoside (pure tri-terpenoid constituent of *Centella asiatica*) protected the liver cells against LPS induced hepatotoxicity via inhibiting the activity of caspase-3 enzyme.

Cytotoxicity studies: Immortalized hippocampal H19-7 neuronal cells, both in un-differentiated and differentiated states, were incubated with titrated aqueous solution of CaE at various concentrations for 24 hours. In case of non-differentiated H19-7 cells, incubation of CaE at various concentrations resulted in concentration dependent reduction in cell viability (Fig. 3.5, n=6). Toxicity of CaE in the undifferentiated H19-7 cells was found to be at ≥ 100 µg/ml concentration. The maximum percentage cell viability of undifferentiated H19-7 cells was found to be $\geq 80\%$, even with the 1000 µg/ml concentration of CaE. On the other hand, CaE in differentiated H19-7 cells exhibits a LD 50 of 700µg/ml (Fig. 3.5, n=6). Unlike undifferentiated cells, differentiated H19-7 cells exhibits robust apoptosis due to the stressful differentiating conditions, which may be the reason for CaE to demonstrate excessive toxicity.

Centella asiatica modulates NMDA subtypes of glutamatergic receptors via modulating transcription factors: *Centella asiatica* is traditionally employed as a brain tonic for enhancing the cognitive abilities of the human brain. Few clinical studies have established the clinical efficacy of CaE among mentally challenged and compromised children. These results reasonably

suggest the possible effect of CaE on the excitatory glutamatergic neurotransmitter pathways, which plays prominent role in modulating learning and memory in mammalian CNS. In order to evaluate the possible modulation of glutamatergic system, we studied the *in-vitro* effect of CaE on the NMDA receptors in the immortalized un-differentiated H19-7 cells, the most important subtype of glutamate receptors involved in memory formation. CaE dose dependently increased NR2b and NR1 subunits of NMDA receptors, though NR2a receptor's expression was found to be down regulated (Fig. 3.6, n=6). Concordantly, phosphorylation of Ca²⁺/calmodulin-dependent protein kinases II (CAMKII) at Threonine 286 residue was found to be elevated in the hippocampal cells, with CaE 20 µg/ml exhibiting the highest response (Fig. 3.6, n=6). Similarly, the phosphorylation induced activation of transcription factor, cAMP response element-binding protein (CREB), enhanced significantly with CaE (Fig. 3.6, n=6). Both CAMKII and CREB activation was found to increase dose dependently with CaE, though the effect started to decline at 50 µg/ml concentration. CREB activation in the nucleus is known to regulate the transcription of BDNF and c-fos genes, which have shown to be crucial for maintaining the learning and memory function of the hippocampus. On the same lines, we found substantial increase in the expression of BDNF protein in the H19-7 cells on CaE incubation for 12 hours, with the maximum response exhibited by CaE 20 µg/ml concentration (Fig. 3.6, n=6). The increase in BDNF was accompanied by decrease in the level of pro-BDNF, which acts as a precursor for BDNF. The decrease in pro-BDNF proportionate very well with the dose-dependent increase in the BDNF levels.

CaE inhibits apoptotic pathway in *in-vitro* conditions in H19-7 cells: Neurodegeneration associated with chronic neurological disorders involves apoptosis as one of the crucial mechanisms of aberrant cell death. Though, a normal physiological response when becomes

erratic leads to neuronal cell death, converting into hand of chronic disorders leading to progression of disease. Therefore, to find the anti-apoptotic capability of CaE, we evaluated the *in-vitro* effect of CaE *per se* on the apoptotic machinery known to execute the cell death in neurons in differentiated H19-7 cells. Selection of differentiated H19-7 cells is based on the fact that on differentiation the H19-7 cells undergo extensive apoptosis. As expected, CaE effectively reduced the caspase-3 levels in all concentrations, though CaE 10 μ g/ml showed insignificant increase (Fig. 3.7., n=6). We did not find any significant change in the BAX expression, though Bcl-xL levels were found to vary with different concentrations of CaE. Lower doses decreased Bcl-xL levels, whereas CaE 50 μ g/ml increased it dramatically (Fig. 3.7, n=6). Reasons for this erratic response in Bcl-xL levels were unknown. Overall the caspase-3 levels depict the anti-apoptotic activity of CaE on the differentiated H19-7 cells.

Neuroprotection by CaE against H₂O₂ induced neuronal cell death: In order to establish the neuroprotective effects of CaE attributing to its antioxidant activity, cell survival in CaE pre-treated differentiated H19-7 cells exposed to H₂O₂ was determined through CCK-8 cell survival assay. H19-7 cells were differentiated for 12 hours and then pre-treated with CaE at different concentrations for 2 hours. After completion of 2 hours of incubation with different concentrations of CaE, H₂O₂ was added for a total of 12-16 hours along with different concentrations of CaE. N-acetylcysteine at 60 μ M concentration was used as positive control attributing to its antioxidant properties. Our results showed decreased cell survival due to H₂O₂ exposure for 12 hours. CaE treatment at lower concentration of 5 μ g/mL (Fig. 3.8 A, n=6, p<0.05) significantly protected differentiated hippocampal neurons against H₂O₂ induced neuronal cell death. Neuroprotection by CaE was equivalent to positive control N-acetylcysteine administration (Fig. 3.8 A, n=6, p<0.05). At higher concentrations, CaE was unable to protect

neuron against oxidative stress. This is in consistent with the *per se* effect of CaE, where CaE at higher concentrations was found to be cytotoxic.

We evaluated the CaE mediated strengthening of neuronal cell resistance to oxidative stress in another neuronal cell line, PC-12 cell line. Similar to H19-7 cells, PC12 cells after differentiation were pre-incubated with CaE for 2 hours and after 2 hours incubation H₂O₂ was added for a total of 12 hours. Oxidative stress induced by H₂O₂ (100uM) administration caused significant neuronal cell death, almost a 60% cell death (Fig. 3.8 B, n=6, p<0.005). CaE pre-treatment protected PC12 cells from oxidative stress induced cell death at CaE 5 µg/mL (Fig. 3.8 B, n=6, p<0.05) and CaE 50 µg/mL (Fig. 3.8 B, n=6, p<0.05) concentrations. Though there was increase in the neuronal cell population in CaE 10 and 20 µg/mL groups but the increase was not significant. Neuroprotection of CaE against does not exhibit a dose dependent response and vary with the neuronal cell type used in evaluation. In case of PC12 neuronal cells, CaE 50 µg/mL exhibited significant neuroprotection and prevented neuronal cell death, though in H19-7 cells CaE 50 µg/mL was not able to protect the cells from H₂O₂ induced cell death. A possible reason could be the difference in the concentration of H₂O₂ used in PC12 cell line, which was three times lower than that has been used in case of H19-7 cells. Another possibility could be the fact that *Centella asiatica* extract is a mixture of bioactive constituents rather than a single compound and at certain concentrations one component overshadows the other component.

CaE ablated H₂O₂ induced ROS generation in neuronal cells: Oxidative stress in the cells leads to induction of apoptotic pathway, which eventually causes cell death. In order to evaluate the antioxidant capability of CaE, we incubated differentiated H19-7 cells with CaE for 12-16 hours and then exposed it to oxidative stress by adding 100µM H₂O₂. H19-7 cells on differentiation undergo apoptosis and have relatively more oxidative stress than undifferentiated cells. On

exposure to H₂O₂ for 2 hours only, the oxidative stress increases tremendously (Fig. 3.9 A, an increase of almost 7 times, n=6, p<0.005). CaE exposure at 5 µg/mL (Fig. 3.9 A, n=6, p<0.005) and 10 µg/mL (Fig. 3.9 A, n=6, p< 0.005) concentrations for 12-16 hours protected the cells from generation of ROS species induced by acute H₂O₂ exposure. CaE at higher concentrations was unable to prevent the building up of ROS species in neuronal cells. At higher concentrations such as CaE 50µg/mL, even higher levels of ROS species were observed implying the toxic effects of CaE. This is in concordance to our previous results, where CaE at higher concentrations exerted cytotoxicity in differentiated H19-7 cells.

In order to firmly establish the *in-vitro* antioxidant activity of CaE, we utilized PC12 cells, a neuronal cell line. Similar to H19-7 cells, PC12 cells were differentiated and after differentiation exposed to CaE pre-treatment at different concentrations for 12 hours. After 12 hours of incubation, PC-12 cells were exposed to a relatively lower concentration of H₂O₂ (100 µM) for 2 hours only. H₂O₂ exposure for 2 hours caused generation of ROS species in PC12 cells as compared to PBS treated control group (Fig. 3.9 B, n=6, p<0.005). CaE at lower concentrations prevented ROS generation in differentiated PC12 cells. CaE 5 µg/mL (Fig. 3.8 B, n=6, p<0.005) and 20 µg/mL (Fig. 3.9 B, n=6, p<0.005) were found to be most effective in protecting PC12 cells from H₂O₂ oxidative stress. Alike in case of H19-7 cells, CaE 50 µg/mL was found to be ineffective in preventing the H₂O₂ induced oxidative stress. Reason for that might be the cytotoxic potential of CaE at higher concentrations as observed in cell survival experiments. Also, it has been well proved that CaE at higher concentrations possess anti-cancer property attributing to its stimulation of apoptotic pathway. Thus, there might be the possibility that at higher concentrations CaE stimulates apoptotic pathway and do not exhibit cytoprotective actions.

CaE modulates NMDA receptors and ameliorates H₂O₂ induced toxicity: CaE per se in H19-7 cells has shown to modulate NMDA subtypes of glutamatergic receptors via PKA/CAMKII/CREB/BDNF pathway. Oxidative stress is known to induce synaptic deficits and to mediate excitotoxicity through glutamatergic transmitter system. We set to determine the effect of CaE in presence of oxidative stress on NMDA subtypes of glutamatergic receptors in differentiated H19-7 cells. H19-7 cells were cultured in 10 cm² culture dishes in propagating media and after 24 hours shifted to differentiation conditions as explained earlier. After 12 hours of differentiation cells were incubated with two concentration of CaE 5 µg/mL and 10 µg/mL for a 12 hours followed by H₂O₂ (100 µM) challenge for another 2 hours in presence of CaE. The cells were then extracted and protein was subjected to western blot for assessing NR2a and NR2b receptor levels in different treatment groups. We found significant increase in the NR2b subtype of NMDA receptors in H₂O₂ challenged group (Fig. 3.10 A, n=5, p<0.05). CaE pretreatment prevented the increase in the NR2b receptor levels at CaE 10 µg/mL concentration (Fig. 3.10 A, n=6, p<0.05), though CaE 5 µg/mL also did show decrease in the receptor level but it was insignificant as compared to H₂O₂ challenged group. In case of NR2a levels, acute H₂O₂ challenge lead to a significant decline in the receptor expression as compared to control PBS administered group (Fig. 3.10 B, n=6, p<0.05). Decline in NR2a receptor expression by acute H₂O₂ exposure was effectively counteracted by CaE 10 µg/mL pretreatment of H19-7 cells (Fig. 3.10 B, n=6, p<0.05). Alike NR2b, NR2a level were not recovered significantly by CaE 5 µg/mL pretreatment implying the ineffectiveness of this particular concentration to modulate NMDA receptors. An increase in the NR2b receptor level induced by acute H₂O₂ challenge signifies the possibility of augmented excitotoxicity mediated by these Ca²⁺ permeable NMDA subtypes of glutamatergic ion channels. Previous reports have depicted the involvement of NR2b receptors in

mediating excitotoxicity in the neuronal cells and NR2a receptors are known to mediate the neuronal survival (Liu et al., 2007). Thus, CaE by decreasing the NR2b receptor expression and increasing the NR2a level prevented the ROS generation and neuronal cell death.

Figures and Figure Legends

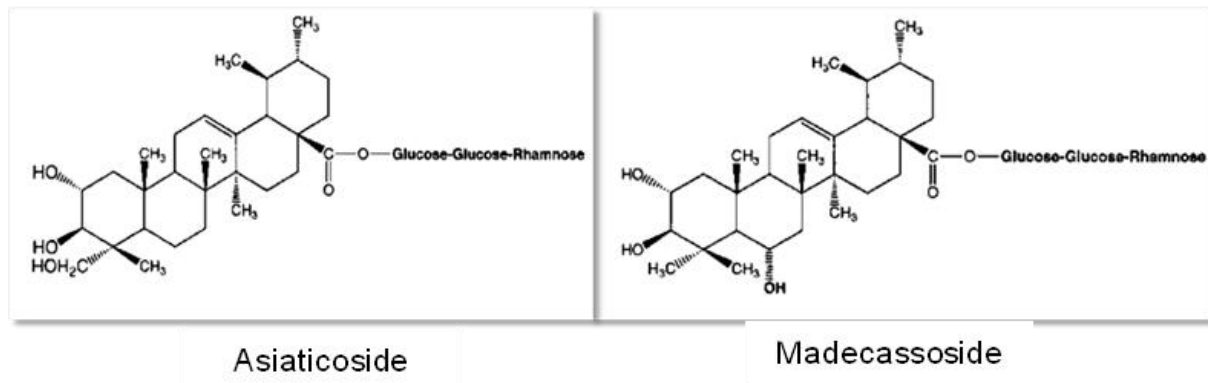


Figure 3.1. The figure represents the chemical structure of two major tri-terpenoid glycosides present in the *Centella asiatica* extract (CaE).

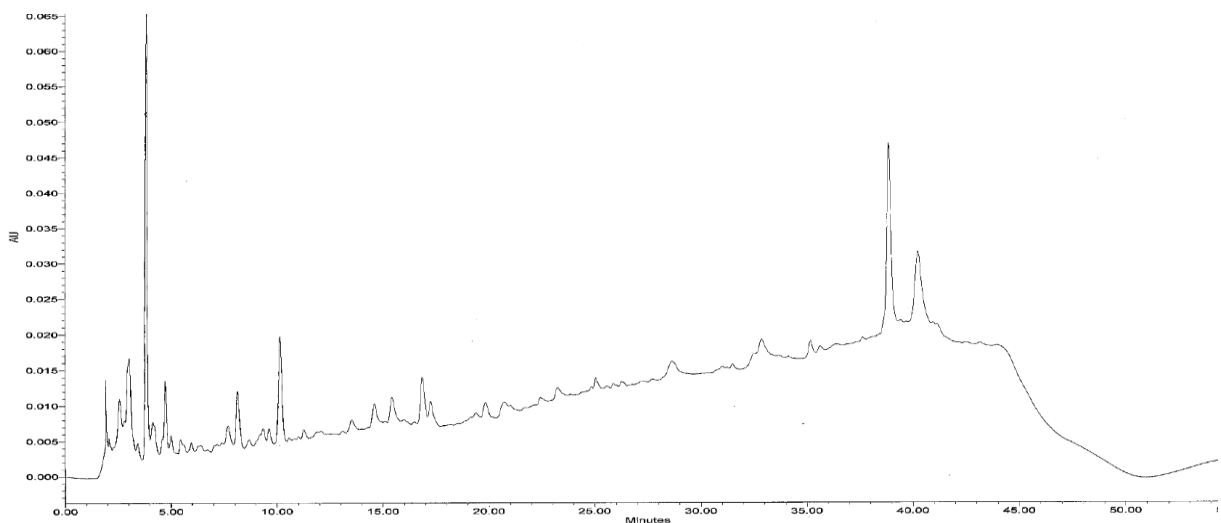


Figure 3.2. The figure represents the fingerprinting by HPLC-UV detection of the *Centella asiatica* extract (CaE).

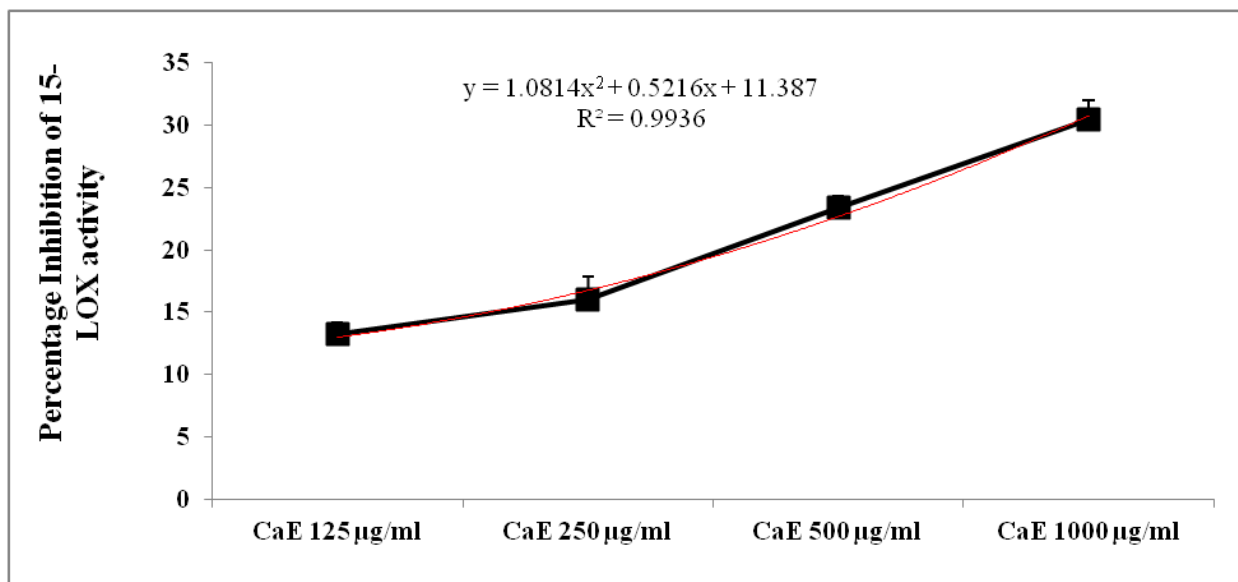


Figure 3.3. Effect of CaE on soybean 15-Lipoxygenase (15-LOX) activity. A representative dose–response curve of 15-LOX inhibition by CaE. Each concentration of drug was tested in triplicate in a standard 15-LOX colorimetric assay, using arachidonic acid as the substrate. The percent inhibition was calculated using control samples in which activity was measured in the absence of drug. Each data point represents the mean, and the error bars represent the SEM.

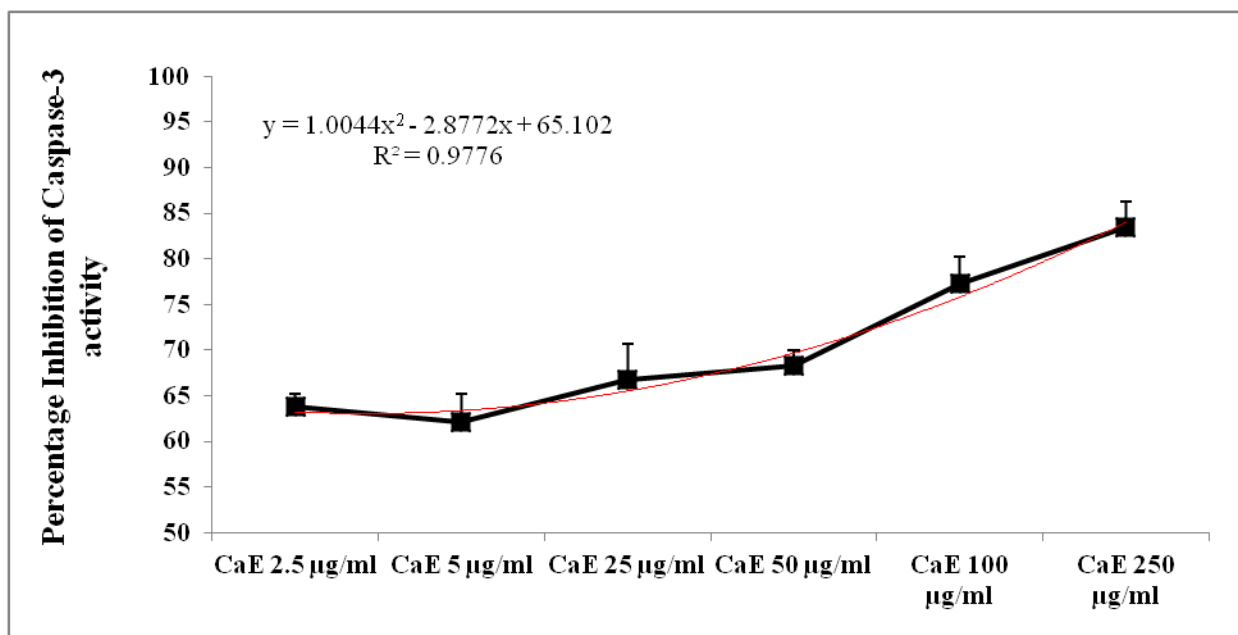


Figure 3.4. Effect of CaE on recombinant human caspase-3 activity. (a) A representative dose–response curve of caspase-3 inhibition by CaE. Each concentration of drug was tested in

triplicate in a standard caspase-3 enzyme fluorometric assay, using z-DEVD-AFC as the substrate. The percent inhibition was calculated using control samples in which activity was measured in the absence of drug. Each data point represents the mean, and the error bars represent the SEM.

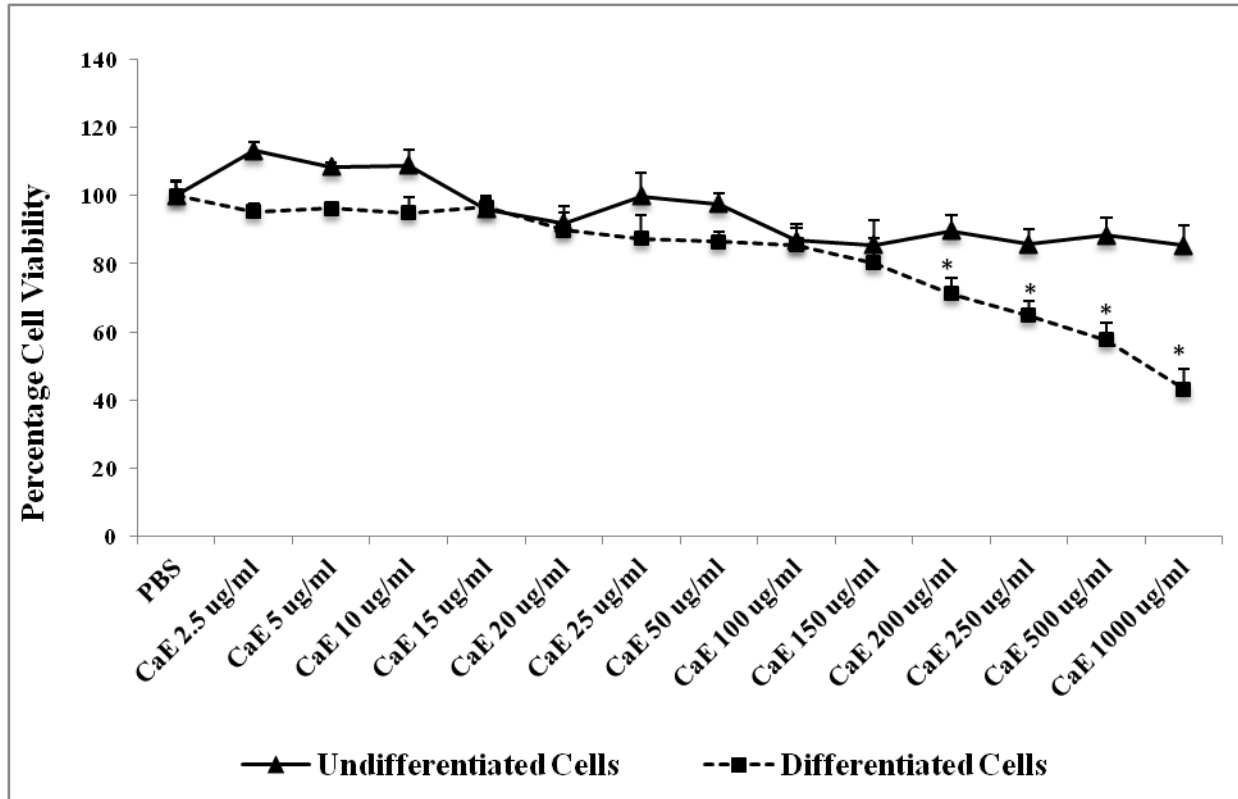


Figure 3.5. Cytotoxicity of CaE on immortalized hippocampal H19-7 cells both in undifferentiated and differentiated states by MTT Cell Proliferation assay. Percentage of viable cells is proportional to MTT reduction. Un-differentiated H19-7 cells were incubated with CaE at 2.5 to 1000 $\mu\text{g/ml}$ concentrations for 24 h at 34°C. CaE is toxic to neuronal cell culture at concentration ≥ 100 $\mu\text{g/ml}$. CaE was incubated with differentiated H19-7 cells at non-permissive temperatures 39°C in N2 differentiation medium supplemented with 1% FBS and 50 ng/ml bFGF. Each data point represents the mean, and the error bars represent the SEM obtained from the 3 different independent experiments running each sample in duplicates.* is significant compared to control (n=6, $p < 0.05$).

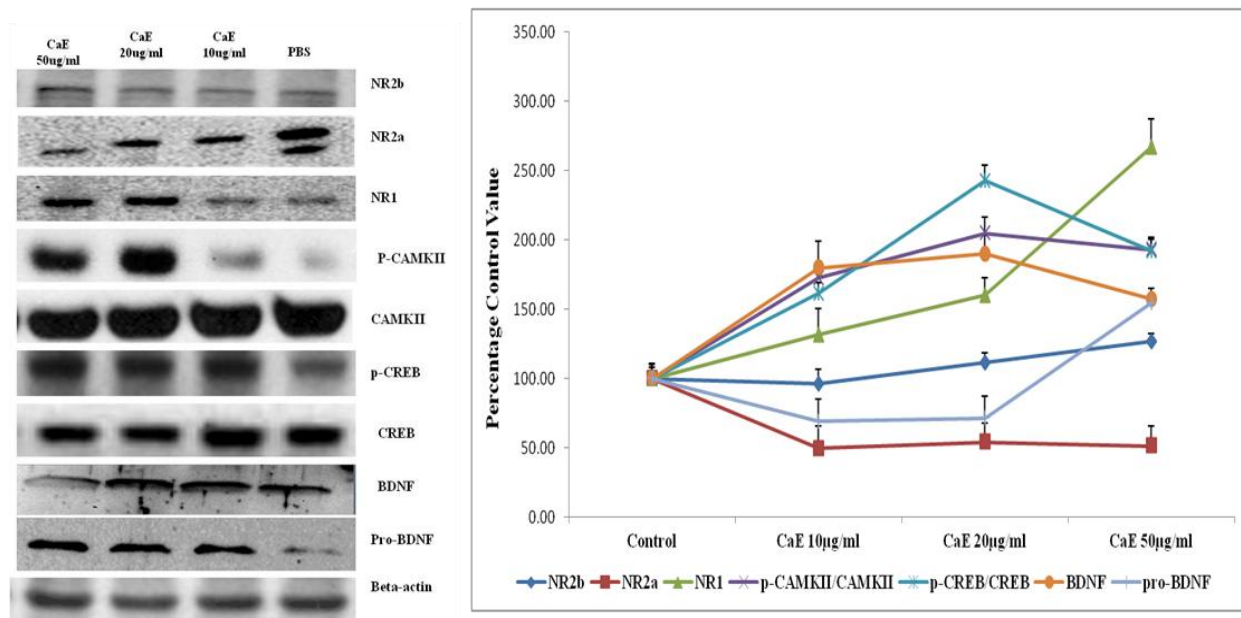


Figure 3.6. CaE alters NMDA subtypes of glutamate receptors via CAMKII/CREB/BDNF pathway. H19-7 cells in 10% FBS supplemented DMEM were grown in 5% CO₂/95% O₂ at an air temperature of 34°C for 24 hours. Undifferentiated H19-7 cells were then incubated with three different concentrations of CaE (10, 20 and 50 µg/ml) for 24 hours. Cell lysate was extracted after incubation and analyzed for different markers with western blot technique. Representative western blot of NR1, NR2a, NR2b, p-CREB, CREB, p-CAMKII, CAMKII, BDNF, and pro-BDNF treated with CaE for 24 hours illustrated on the right side. Quantitative densitometry analysis of different protein markers are presented on the left side. Changes in the different proteins were expressed as percentage of control after normalization with β-actin bands. Results are mean ± SE from 3 independent determinations.

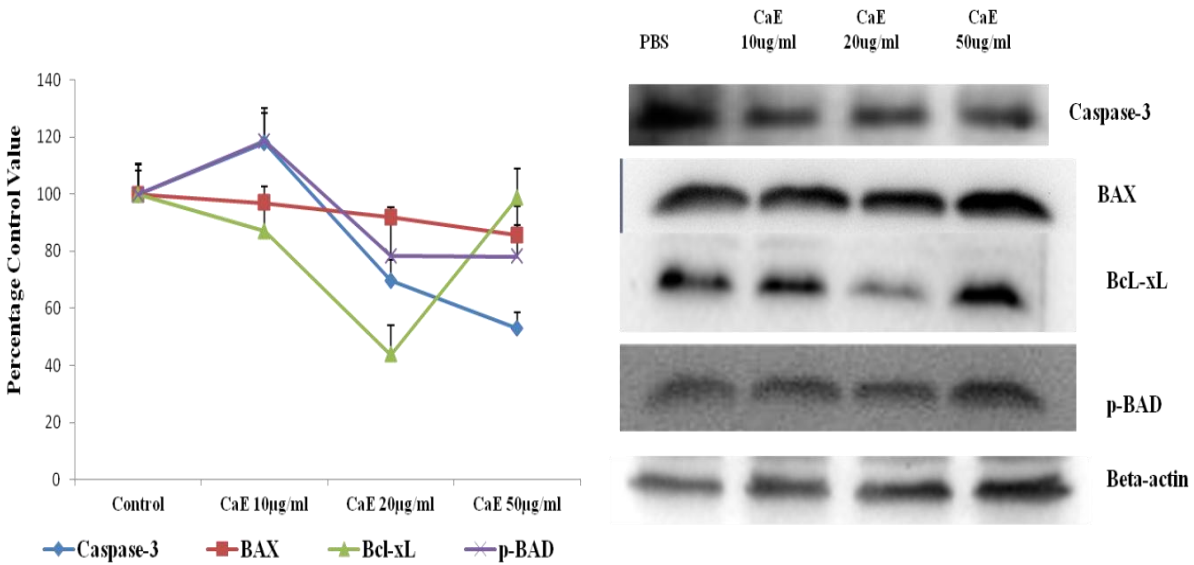


Figure 3.7. Anti-apoptotic properties of CaE. H19-7 cells in 10% FBS supplemented DMEM were grown in 5% CO₂/95% O₂ at an air temperature of 34°C for 24 hours. After 24 hours they were transferred to differentiation N2 media supplemented with 1% FBS and 50 ng/ml bFGF to accelerate the differentiation process. CaE at 10, 20 and 50µg/ml concentrations was added to the differentiating H19-7 cells for a total of 16-18 hours. Cell lysate was extracted after incubation and analyzed for pro-apoptotic and anti-apoptotic markers with western blot technique. Membranes were probed with anti-caspase-3, anti-BAX, anti-Bcl-xL and anti-p-BAD antibodies. The intensity of each marker bands was normalized with their respective beta-actin bands and mean ± SE from 3 independent determinations were determined. P < 0.05 was considered to be statistically significant. Results were expressed as percentage control values.

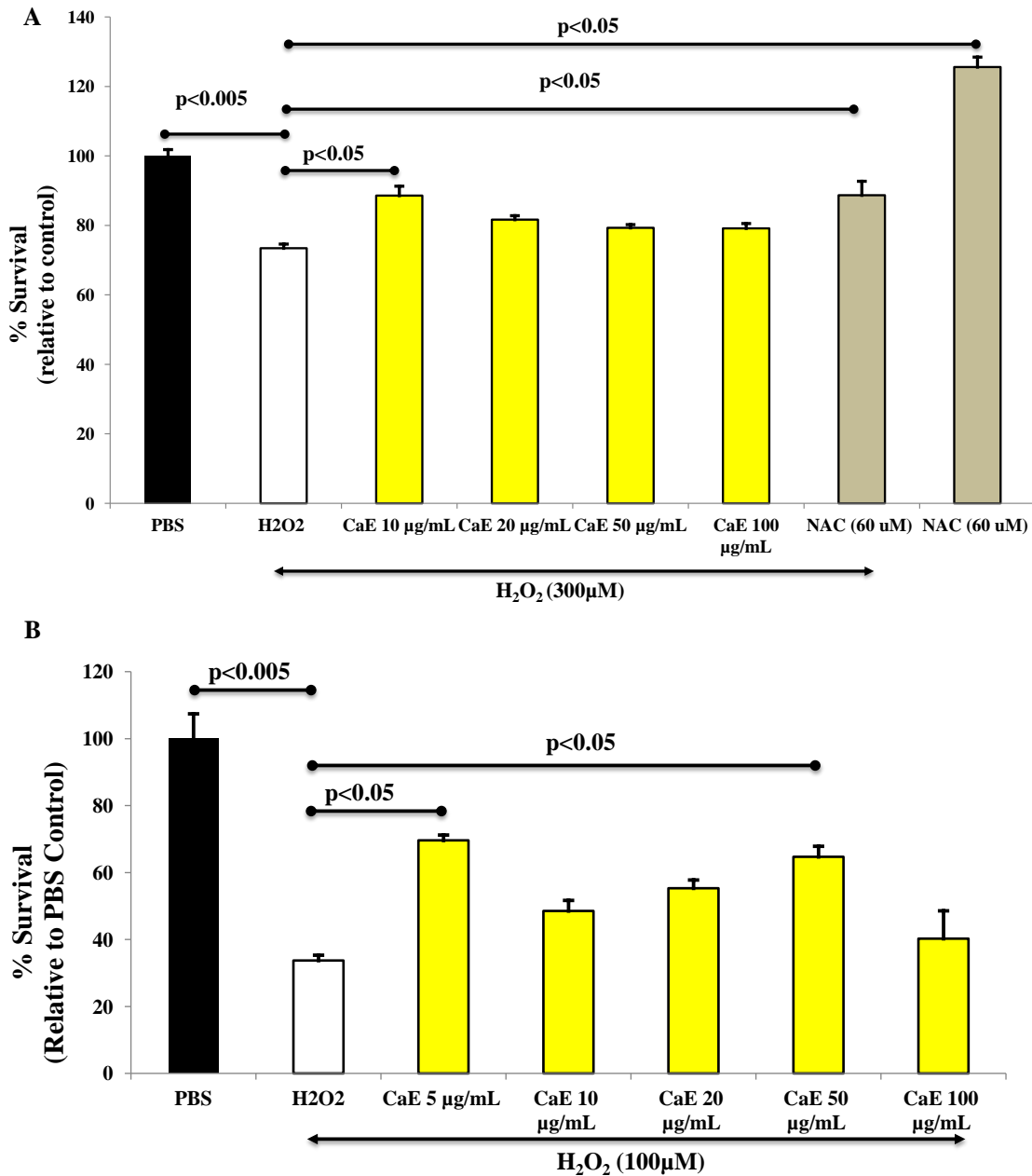


Figure 3.8. Neuroprotection by CaE against H₂O₂ induced oxidative stress. Percentage of viable cells is proportional to MTT or WST-8 reduction. A) H19-7 cells were differentiated at non-permissive temperatures 39°C in N2 differentiation medium supplemented with 1% FBS and 50 ng/ml bFGF. Differentiated H19-7 cells were incubated with five different concentrations of CaE ranging from 5 to 100 μg/mL for 2 hours followed by 12 -16 hours of H₂O₂ (300 μM) challenge in presence of CaE at 39°C. CaE at 10 μg/mL concentration protected H19-7 neuronal cells against H₂O₂ (300 μM) exposure for 12 -16 hours. Neuroprotection by CaE was equivalent

to positive control, which was N-acetylcysteine (60 μ M), a well known antioxidant. B) PC12 cells were differentiated by replacing the propagating medium with the differentiation media containing 1% FBS and 50 ng/ml of NGF for 48 hrs. After 48 hours incubation in the differentiation medium, fresh differentiation medium was added along with the CaE in concentrations ranging from 5 to 100 μ g/mL followed after 15 min by H₂O₂ (100 μ M) challenge for a total of 24 hours in presence of CaE at 37°C. CaE at 5 μ g/mL and 10 μ g/mL concentration effectively protected differentiated neuronal PC12 cells from H₂O₂ induced cell death. Data is presented as means \pm SEM, n = 6.

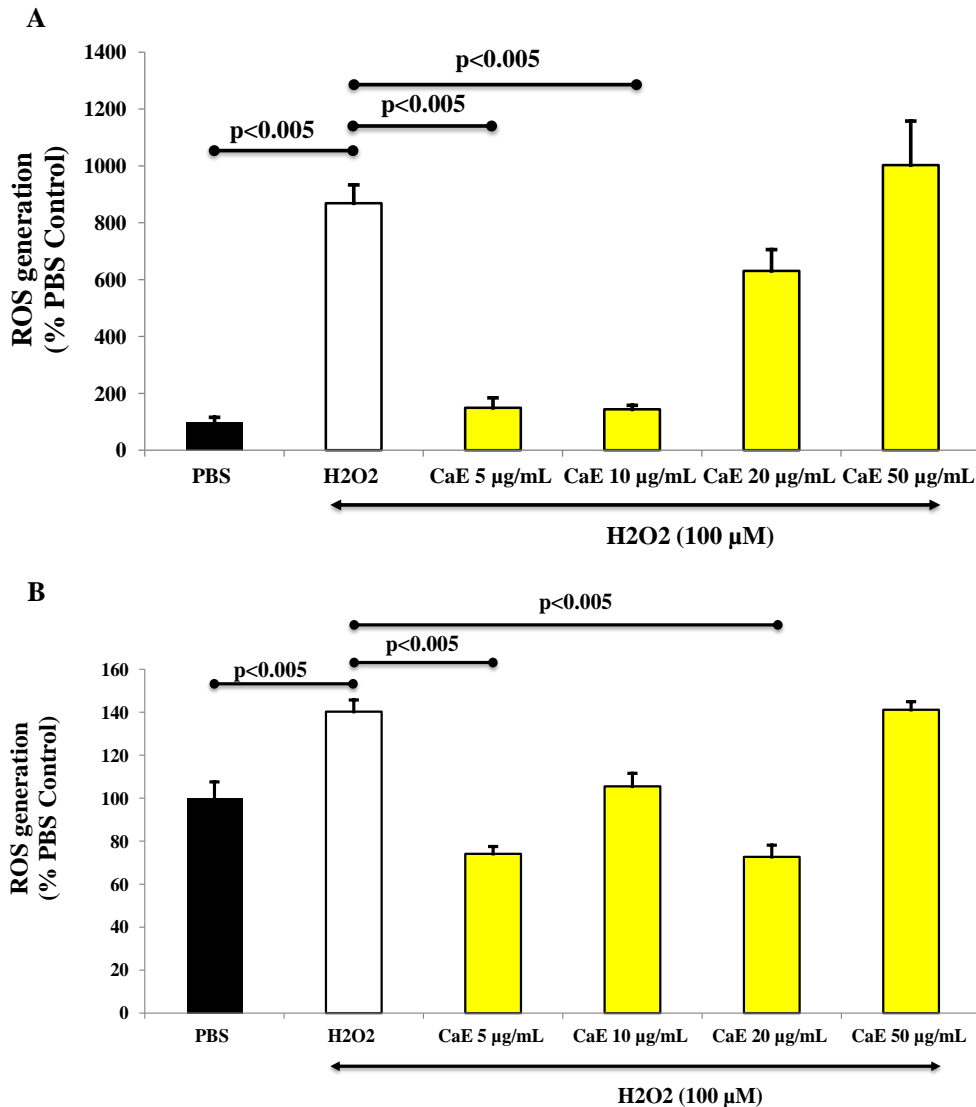


Figure 3.9. CaE prevented ROS generation against acute H₂O₂ exposure. A) H19-7 cells were differentiated at non-permissive temperatures 39°C in N2 differentiation medium supplemented with 1% FBS and 50 ng/ml bFGF. Differentiated H19-7 cells were incubated with 10 µM DCF-DA for 60 minutes at 39°C. DCF-DA incubation was followed by washing three times with PBS. H19-7 cells were then incubated with five different concentrations of CaE ranging from 5 to 100 µg/mL for 12 hours followed by an acute H₂O₂ (100 µM) challenge for two hours in presence of CaE in the differentiation conditions. Fluorescence was read for 2 hours in a fluorescent plate reader at 480nm excitation and 530 nm emission wavelengths with readings taken at every 30 minute interval. CaE at 5 µg/mL and 10 µg/mL concentration prevented generation of ROS species in H19-7 neuronal cells against H₂O₂ (100 µM) exposure 2 hours. B)

PC12 cells were differentiated by replacing the propagating medium with the differentiation media containing 1% FBS and 50 ng/ml of NGF for 48 hrs. After 48 hours of differentiation PC12 cells were incubated with 10 μ M DCF-DA for 60 minutes at 37°C followed by washing three times with PBS. Fresh differentiation medium was added along with the CaE in concentrations ranging from 5 to 100 μ g/mL for 16-18 hours followed by H₂O₂ (100 μ M) challenge for another 2 hours in presence of CaE at 37°C. Fluorescence was read for 2 hours in a fluorescent plate reader at 480nm excitation and 530 nm emission wavelengths with readings taken at every 30 minute interval. CaE at 5 μ g/mL and 20 μ g/mL concentration prevented generation of ROS species against H₂O₂ (100 μ M) exposure 2 hours in differentiated PC12 neuronal cells. Data is presented as means \pm SEM, of observations obtained from three different independent experiments running each sample in duplicates, n = 6.

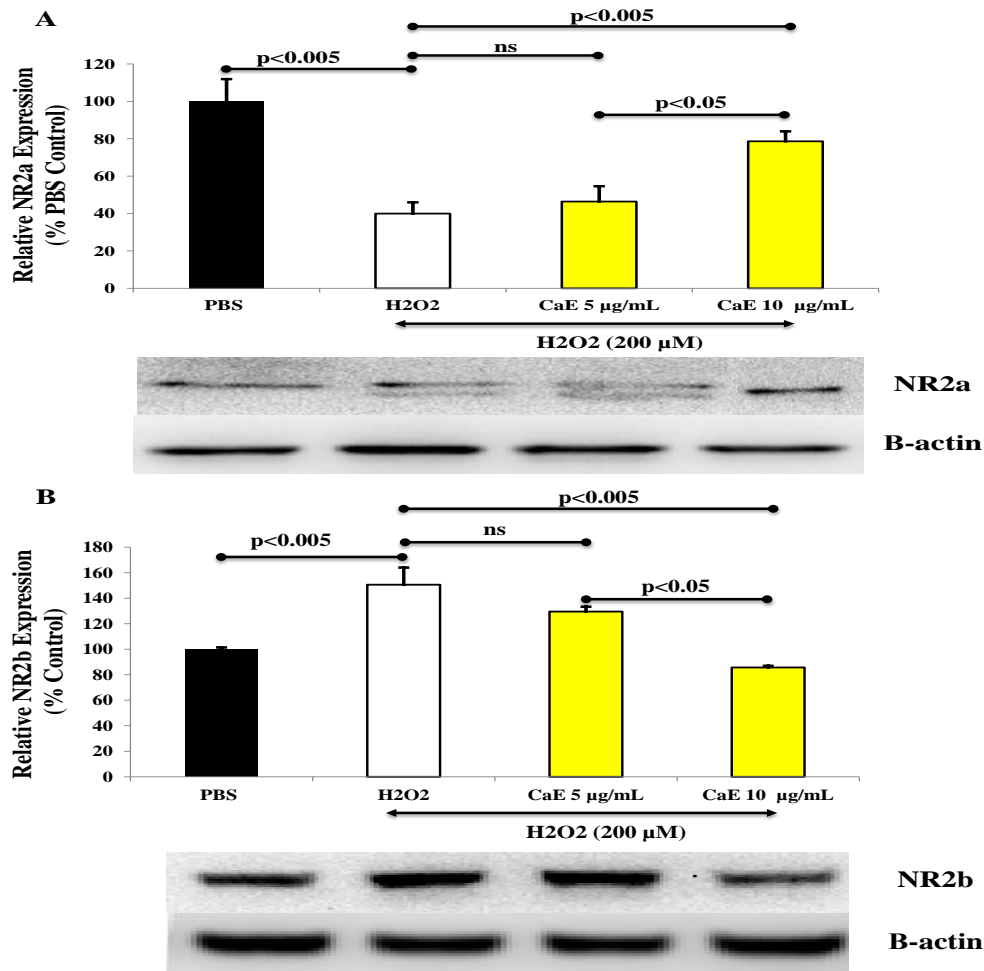


Figure 3.10. NMDA receptor mediated neuroprotection by CaE. H19-7 cells in 10% FBS supplemented DMEM were grown in 5% CO₂/95% O₂ at an air temperature of 34°C for 24 hours. After 24 hours they were transferred to differentiation N2 media supplemented with 1% FBS and 50 ng/ml bFGF to accelerate the differentiation process for 12 hours. Differentiated H19 cells were incubated with two different concentrations of CaE (5 and 10 µg/ml) for a total of 12 hours, which was followed by an acute exposure to H₂O₂ challenge for another 2 hours at 39°C. Cell lysate was extracted after incubation and analyzed for NMDA subtypes of receptors with western blot technique. Membranes were probed with anti-NR2a and anti-NR2b antibodies. The intensity of each marker bands was normalized with their respective beta-actin bands and mean ± SE from 3 independent determinations were determined. A) Bar plot represents quantitative densitometry analysis of NR2a protein expression. B) Bar plot represents quantitative densitometry analysis of NR2b protein expression. p < 0.05 was considered to be statistically significant. Results were expressed as percentage control values.

Characteristic feature	Value
Color of the powdered extract	Dark Green
Solubility in water	1mg/ml with no visible residue
Solubility in ethanol (99%)	Insoluble
Color of CaE aqueous solution (1 mg/mL)	Greenish brown
pH of the aqueous solution	5.7
Protein content	6.69 µg/mg of powdered extract
Flavonoid content (Equivalent to Quercetin)	22.76 µg equivalent of Quercetin/mg of powdered extract
Polyphenols content (Equivalent to Pyragallol)	21.7466 µg equivalent of Pyragallol/ mg of powdered extract

Table 3.1: Physical and biochemical features of CaE

3.4. Discussion:

Our current study revealed that the titrated concentrated extract of *Centella asiatica* (CaE) exhibits neuroprotective properties based on the strong anti-apoptotic and anti-inflammatory abilities. In addition CaE enhanced phosphorylation of CREB effectively in immortalized hippocampal H19-7 cell line through activation of CAMKII kinase and demonstrated the modulation of transcription factors involved in regulating early response elements associated with learning and memory in hippocampus. Moreover, the lowering of the caspase-3 levels in differentiating H19-7 cells establishes the effective anti-apoptotic and thus neuroprotective capabilities of CaE. Overall, the results from the current study reasonably render CaE a potential therapeutic intervention in neurological disorders compromising the cognitive performance.

Centella asiatica extract has already been shown to be an effective anti-inflammatory agent, with strong immunomodulatory properties (Punturee et al., 2005; Barbosa et al., 2008). We tested the anti-inflammatory potential of our commercial CaE preparation on 15-LOX and COX enzyme. The COX-2 and 15-LOX enzymes are important components of the arachidonic pathway that results in the formation of inflammatory prostaglandins, leukotrienes and other mediators, that are associated with numerous chronic neurological disorders (Hoozemans et al., 2004). Our results showed the direct inhibition of 15-LOX enzymes by our commercial CaE preparation (Fig. 3.3). The result is in concordance with the previous report of *Centella* extract significantly inhibiting the activity of Ca^{2+} -independent Phospholipase A₂ (iPLA₂) and cytosolic PLA₂ in the rat brain homogenate. Phospholipase A₂ is closely associated with COX-2 in neuronal cells (Kolko et al., 2002) and thus, drugs affecting either of them are going to inhibit the profound inflammatory reaction induced by either of the factors (Burnett et al., 2011). As our CaE preparations are highly concentrated with asiaticoside content, it can be reasonably

suggested that the COX-2 and 15-LOX inhibition is the effect of Asiaticoside. The flavonoid content is another candidate for the immunomodulatory effect of CaE on these enzymes.

Apoptosis is one of the major routes of neurodegeneration that is aberrantly initiated by the chronic disorders. In neurological disorders such as Alzheimer's disease (AD), and Parkinson's disease, apoptosis plays a significant role in causing neurodegeneration. Apoptosis can be triggered by various pathways (LeBlanc, 2005). Especially in AD, recent reports have suggested early induction of caspase-6 that leads to activation of downstream apoptotic mediators including the caspase-3. Induction of caspase-6 is responsible for the breakdown of the cytoskeletal structure of neurites and damages proper trafficking of proteins and organelles, resulting in neurodegeneration and synaptic loss in AD (LeBlanc, 2005). Our study demonstrated CaE to be an effective inhibitor of Caspase-3 activity (Fig. 3.4). The inhibitory potential of CaE 2.5µg/ml extract was found to be equipotent as the 20 µM selective peptide caspase-3 inhibitor, z-DEVD-FMK. In order to test the effect of CaE on the protein expression of Caspase-3, we determined the caspase-3 levels in the differentiated H19-7 cells. We found significant concentration-dependent decline in the caspase-3 levels in H19-7 cells after 16-18 hours of incubation with various concentrations of CaE. These effects were *per se* effect of CaE on the differentiated H19-7 cells, which are shown to undergo apoptosis during differentiation process. These results are in line with the previous report depicting the anti-apoptotic activity of CaE against aluminum induced neurotoxicity in rats (Prakash and Kumar, 2012), where caspase-3 activity induced by aluminum toxicity was shown to effectively controlled by CaE administration. In addition, the other anti-apoptotic markers such as p-BAD, Bcl-xL and BAX were found to be altered with CaE incubation. The effects of CaE on these markers was not

proportionate with the effect on caspase-3 levels, which depicts that CaE modulated the caspase-3 levels with mechanisms other than BAX, Bcl-xL pathway.

There was significant increase in CREB phosphorylation by CaE that can be possibly the molecular mechanism of synaptic strengthening and cognitive enhancement of CaE by facilitating the neuronal dendritic arborization and axonal regeneration in rats (Soumyanath et al., 2005; Mohandas Rao et al., 2006). CREB is a transcription factor, which is actively induced during long term potentiation, one of the physiological processes of memory formation in the brain. CREB regulates immediate early genes such as c-fos and proteins such as BDNF, which are critical for the neuronal survival and protein synthesis (Malinow et al., 2000). We observed elevated level of BDNF (Fig. 3.6) in H19-7 cells treated by various concentrations of CaE, which correlated very well with the proportionate decrease in pro-BDNF levels. The increase in BDNF accompanied the enhanced pCREB levels (Fig. 3.6). A great body of evidence demonstrates BDNF to strongly stimulate the dendrite outgrowth (Huang and Reichardt, 2003) by activating TrkB receptors resulting in stimulation of various proteins including the activation of CaMKII (Minichiello et al., 2002). Activation of CREB leads to activation of various protein kinases such as MAPK, CAMKII, and protein kinase C as well as other signaling factors such as Wnt, all of which plays important role in neuronal survival and arborization (Wayman et al., 2006). The present data suggests that the effect of CaE on cognitive enhancement might be mediated by increased CREB signaling. This is in accordance with a previous report where they have shown marked increase in CREB phosphorylation after *in-vitro* incubation of neuroblastoma cells with aqueous extract of Gotu Kala (Xu et al., 2008).

Moreover, CREB derived increase in BDNF levels have shown to positively modulate the NMDA receptor activity and expression on the pre- and post- synaptic sites (Madara and

Levine, 2008). In accordance with this, we found elevation in the levels of NR2b and NR1 receptors, which very well correlated with increase in BDNF levels. Additionally, BDNF through activation of TrkB receptor enhances the synaptic localization of other pre- and post-synaptic proteins such as synaptobrevin, which are very important for maintaining synaptic plasticity (Minichiello et al., 2002; Huang and Reichardt, 2003). Several pathways lead to the phosphorylation of CREB such as PKA, nitric oxide signaling and CAMKII activation. We have found in our study, the increased phosphorylation of CAMKII which suggests that the CaE induced induction of CREB may be mediated by the induction of PKA/CAMKII/CREB pathway in the hippocampal neurons. The previous study has pointed the role of ERK/MAPK in activation of CREB pathway due to aqueous extract of CaE. We through our results are suggesting that PKA/CAMKII pathway may be the other signaling mechanisms by which CaE is modulating the CREB activation and regulating the BDNF levels.

There are currently dearth of studies investigating the cellular and molecular mechanisms of cognitive enhancement by *Centella asiatica* and its individual chemical constituents. Asiatic acid and asiaticoside are proposed to be the major component eliciting the facilitation of learning and memory performance observed with CaE in animal studies (Gupta et al., 2003; Veerendra Kumar and Gupta, 2003; Rao et al., 2005). In our study, we have employed the concentrated extract of CaE, which is 20% composed of asiaticoside and the rest is of other bioactive compounds such as flavonoids, poly-phenols and tannins. It is widely believed that a natural herbal extract exhibits strong beneficial properties attributing to its diverse composition of various chemical components. Thus, the use of titrated extract of CaE rather than a single component seems more preferable as per the current study requirement, where our focus was to investigate the mechanisms underlying both neuroprotection as well as cognition enhancement.

Oxidative stress is the central pathogenic factor underlying various neurodegenerative disorders (Karpinska and Gromadzka, 2013). Hydrogen peroxide (H_2O_2) is an endogenous biochemical marker of oxidative stress. If provided exogenously to cellular system, H_2O_2 leads to oxidative stress and eventually causes cell death. In the current study, we employed H_2O_2 as the exogenous oxidant for our neuronal cellular model and tested CaE neuroprotective potential against H_2O_2 . Our results establish the neuroprotective activity of CaE against H_2O_2 in the cell survival assays in two completely different neuronal populations. In case of both hippocampal and pheochromocytoma cell line, CaE at lower concentrations proved to be more effective in preventing the cell death. The outcome of cell survival assay is in line with the *in-vitro per se* effect of CaE, where higher concentrations were found to be more toxic. As H_2O_2 is a well known pro-oxidant, we aimed to study the generation of ROS species in both of the neuronal cell population and evaluate the antioxidant activity of CaE against H_2O_2 . For that we pre-incubated the differentiated H19-7 as well as PC12 cells with different concentrations of CaE and challenged it with acute exposure to a lower concentration of H_2O_2 for 2 hours. Our results were in accordance with our observations obtained from the cell survival assay. CaE at lower concentration was found to be most effective in combating generation of ROS species mediated by acute H_2O_2 challenge. This is the direct evidence of antioxidant activity of CaE in neuronal population. We then wished to investigate the cellular mechanism associated with the neuroprotective activity of CaE against the H_2O_2 induced neurotoxicity. In order to evaluate the molecular mechanism we determined the NMDA receptor expression. We found a significant elevation in NR2b receptor in the H_2O_2 challenged group, which subdued due to CaE treatment. NR2b receptors are known to mediate the excitotoxicity associated with high levels of glutamate. On the other hand CaE treatment induced overexpression of NR2a receptors, which were found

to be significantly depleted in the H₂O₂ challenged group. Thus, CaE by modulating the expression of excitotoxic NMDA receptors entails the neuroprotective effect in the hippocampal cells.

In conclusion, our study demonstrates the strong evidence in favor of anti-inflammatory, anti-apoptotic as well as PKA/CAMKII/CREB/BDNF pathway. The direct evidence of neuroprotection and antioxidant action of CaE against H₂O₂ challenge further strengthen the use of CaE in various neurodegenerative disorders, which have oxidative stress as the central pathogenic factor. Based on the above results, in terms of the potential therapeutic benefits of CaE, we propose that CaE exhibits a greater potential for treating neurodegenerative disorders affecting cognitive abilities.

3.5. References

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4. Associative evidence of T-cells mediated inflammation in pathogenesis of synaptic and behavioral deficits in Bigenic mice model of Alzheimer's disease

Abstract

Alzheimer's disease (AD) is a progressive chronic neurodegenerative cognitive disorder, which deleteriously affects the elderly people. Limited information is known regarding the etiological factors responsible for AD. Although, inflammation is considered to be one of the pathological factors associated with AD, paucity of supportive experimental evidence still surrounds the fact. Therefore, to better understand and establish the pathophysiological involvement of chronic inflammation in double transgenic mice model of AD, the current study was carried out. Bigenic mutated APP^{swe}+PS1^{dE9} mice along with non-transgenic control C57/BL6 mice, belonging to 10 months of age, were procured and assessed for behavioral, biochemical and synaptic deficits. Amyloid-beta (A β) levels, BACE activity was assessed biochemically. Synaptic plasticity in terms of LTP formation was assessed by the extracellular field recordings and associated synaptic excitatory receptors were evaluated by western. We also analyzed the pro-inflammatory cytokines, chemokines and T-cells in different regions of the brain of both groups, through various immunological and biochemical techniques.

Substantial increase in amyloid beta levels together with increased BACE activity was observed in bigenic mice as compared to age matched control. Marked elevations in pro-inflammatory cytokines in cortical and whole brain lysates were observed. Flow cytometric evaluation revealed higher number of CD4⁺ IL-17a and IFN-gamma secreting T-cells, revealing the robust T-cell infiltration of brain of transgenic mice. Behavioral deficits in learning and memory tasks were also exhibited along with impaired LTP formation by APP^{swe}+PS1^{dE9}

mice. Synaptic deficits were also accompanied by decreased mRNA expression of NR2b and increased expression of NR1 subtypes of NMDA receptors as well as decreased phosphorylation of hippocampal protein kinases such as ERK1/2 and p70S6 kinases together with low levels of activated CREB transcriptional elements. Thus, the current study establishes the involvement of T-cell mediated neuroinflammation in APP^{swe}+Ps1 mice, which correlates very well with the cognitive decline in these mice.

4.1. Introduction

Alzheimer's disease (AD) is a progressive, fatal neurodegenerative disorder that being among the most common causes of dementia, impairs basic bodily functions such as walking and swallowing and eventually, results in death. AD is the sixth-leading cause of death with 5.4 million affected people just in the United States alone (reviewed in (Alzheimer's Disease International., 2009; Alzheimer's disease facts and figures, 2012)). AD is among one of the chronic debilitating neurological disorders that that cannot be prevented, cured or even slowed. AD has been categorized into two major types: a) familial AD (FAD) which has identified pathological cause as genetic mutations in the amyloid precursor protein (APP) and presenilin (PS) genes accounting for almost 3% of all AD cases and affects people at an early age of less than 60 (reviewed in (Bekris et al., 2010; Tanzi, 2012)); and b) sporadic AD, which has no clearly defined pathological factors, represents 97% of AD cases and affects people of age 65 or above (has late onset of disease) (Hampel and Lista, 2012; Sadigh-Eteghad et al., 2012). Clinically, both types of AD are associated with severe loss of memory, language, visuospatial skills, and emotion followed by inability to walk and swallow, though AD patients with early onset tend to have more of the brain anomalies than late onset AD patients. Pathologically,

presence of extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs) in the localized regions of the brain are the characteristic hallmarks of AD. Biochemically, the SPs are amyloid proteins deposits composed mainly of beta-convoluted sheet of amyloid peptide of length 1-40 and 1-42 ($A\beta_{1-40}$ and $A\beta_{1-42}$), which gets cleaved from Amyloid precursor protein (APP) present in the neuronal cell membranes. On the other hand, NFTs are structurally aggregates of hyperphosphorylated tau proteins, a microtubule associated cytoskeleton protein present in the neuronal cells. Though, amyloid-beta theory is considered as the cornerstone of AD pathogenesis, it does not explain all the aspects of AD (Rozemuller et al., 2005; Duyckaerts et al., 2008) and thus, exact cause of AD still remains elusive. Moreover, there is plethora of increasing evidence that support the involvement of inflammation in the pathogenesis of AD (Rozemuller et al., 2005; Schwab and McGeer, 2008; Amor et al., 2010; Choi et al., 2010). To thoroughly address this issue, we examined the inflammation and its markers in a well established transgenic model of AD and correlated them with cognitive deficits observed in these animals.

Inflammation plays a crucial role in various neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), traumatic brain injury and Multiple sclerosis (MS) (Blasko et al., 2004). The importance of inflammation can be evaluated from the emergence of inflammation hypothesis, a recent new hypothesis proposed by Zhang et al., (Zhang et al., 2004) and extensively supported by several investigators (van Groen et al., 2011), for Alzheimer's disease and several other neurodegenerative disorders (Schwab et al., 2010). For instance, AD patients have shown to have elevated levels of gliosis and pro-inflammatory markers such as IL-1 and prominent complement activation in postmortem brain (Rozemuller et al., 2005). These reports, along with recent established role of immune response in pathological

regeneration and clearance of protein aggregates such as A β (Zhang et al., 2004; Mizwicki et al., 2012), has generated a mounted interest in studying the exact mechanism and ways of modulating of immune responses associated with AD in order to develop a therapeutic potential (Schwab and McGeer, 2008). Furthermore, a number of reports have suggested potential role of T-cells the in the AD pathology and/or progression of disease (reviewed in (Town et al., 2005)). Various studies have demonstrated that In the AD patient's brain, activated but not fully differentiated T-cells were detected in higher propensity in the brain parenchyma than age-matched non-dementic control brains (Togo et al., 2002). Itagaki et al., in their study depicted altered trafficking of both CD4 (T-helper-inducer) and CD8 (T-cytotoxic-suppressor) T-cells in significantly large amounts in the hippocampus and temporal cortex of AD brain as compared to the normal brain tissues, indicating a prominent cell mediated immune response in AD (Itagaki et al., 1988). Recently, a study has documented shortening of T cell telomere length in AD patients, and for the first time displayed a direct correlation of MMSE scores with T-cell telomere length, suggesting a possible antigen-specific involvement at some point of the disease process (Panossian et al., 2003). However, there are other contradictory evidences that deny the involvement of T-cells in the AD pathophysiology and/or progression (Akiyama et al., 2000b). In order to clearly understand the involvement of T-cell in AD pathogenesis, we studied the T-cell infiltration and associated markers in the brain of well established transgenic mice AD model.

This conspicuity in the pathophysiological pathways of disease progression in AD has led to generation of various transgenic animal models, so as to study the pathophysiology, progression and development of therapeutic interventions for AD. AD linked mutations of human including APP (Hsiao et al., 1996a), presenilin 1 (PS1) (Mehta et al., 1998), presenilin 2

(PS2) (Herreman et al., 1999) and apolipoprotein E (ApoE 4) (Raber et al., 1998) proteins; either alone or in combination, has been extensively exploited in creating these transgenic mouse models. Transgenic mouse model depending on its altered genotype exhibits several characteristic features of AD, but in varied propensity and magnitude. The Tg2576 line that has APPSwe mutation displays increased Amyloid beta, both $A\beta_{1-40}$ and $A\beta_{1-42}$, expression and deposition between 9 and 12 months of age (Hsiao et al., 1996), whereas when crossed with PS1 mutant line showed accelerated deposition at an early age of 5-8 months (Holcomb et al., 1998; Dhanasekaran et al., 2009). Thus, due to their accelerated pathological alterations in amyloid deposition, it can be well argued that pathological changes attributing to immune response and inflammation in AD can be, on an acceptable basis, well investigated in double transgenic mouse. In the current study, we employed double transgenic APPSwe+PS1dE9 mice, which harbors familial AD linked Swedish mutant human APP and mutant human presenilin (PS1-dE9) gene and overproduce $A\beta_{1-42}$ in the brain of these mice resulting in development of Alzheimer's related pathology (Jankowsky et al., 2004). The APPSwe+PS1dE9 mice line exhibit SPs in hippocampus and cortex at six to seven months along with spatial learning deficit at seven months of age and at nine months of age they exhibit dystrophic neurites with very subtle neuronal cell loss (Savonenko et al., 2005; Garcia-Alloza et al., 2006). The APPswe/PS1dE9 mice displayed relatively milder cerebral amyloid angiopathy (CAA), contrary to what is normally observed in APP+PS1 transgenic mice, it also strongly shifted $A\beta$ expression to $A\beta_{42}$ without affecting $A\beta_{40}$ which has been proposed to be due to the extensive $A\beta$ deposition seen in APPswe/PS1dE9 mice (Garcia-Alloza et al., 2006; Bonardi et al., 2011). Furthermore, robust inflammation has been already reported in APPswe/PS1dE9 mice line, with elevated hippocampal levels of GFAP and GFAP-positive astrocytes around the amyloid plaques deposits

(Garcia-Alloza et al., 2006). Recently, Ruan et al., demonstrated appearance of clustered microglia and clustered astrocytes at an early age of 4 and 6 months concentrated around the amyloid deposits and that increases prominently with age (Ruan et al., 2009). The same study also detected elevated levels of inflammatory markers such as TNF-alpha, IL-1, IL-6 and MCP-1 at 10 months of age in hippocampus and cortex of APP^{swe}/PS1^{dE9} mice (Ruan et al., 2009). Microglia activation in APP^{swe}/PS1^{dE9} mice was shown to strongly express P2X7 purinergic receptor that mediates neuronal damage via activating NADPH oxidase enzyme and resulting ROS generation (Lee et al., 2011).

The role of T-cell induced inflammation is not well addressed regarding the pathogenesis of AD. Though, microglia activation, inflammation and oxidative stress have been shown to be significantly altered in these mice, no one has studied T-cell infiltration and activation in these transgenic mice. With the current study, we have demonstrated an active involvement of T-cells in the neuroinflammation associated with AD. Moreover, we also studied the biochemical, neurochemical and electrophysiological anomalies in these mice. Results from this study exhibits the potential functionality and reasonable validation of this transgenic animal model for testing developmental interventions targeting both the amyloid as well as inflammation cascade involved in the pathophysiology of AD.

4.2. Materials and Methods

Subjects: Double transgenic B6.Cg-Tg (APP^{swe}, PSEN1^{dE9}) 85Dbo/J (APP^{swe}+PS1) male mice and C57Bl6 male mice (non-transgenic controls) were obtained from Jackson Laboratory (Bar Harbor, Maine) and were housed with a 12 h day/night light cycle with free access to food and water. All animals, both transgenic and non-transgenic control, were 2 months old with a

body weight of 20-25 g. The double transgenic APP^{swe}+PS1 mice were generated by mating Tg2576 mouse line carrying the “Swedish” APP mutation (APP_{K6070NM671L}) (Hsiao et al., 1996b) with a mutant presenilin-1 mouse line (PS-1_{M146L6.2}) (Duff et al., 1996). All procedures involving animals were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

Chemicals: The following chemicals/drugs were purchased from Sigma Chemical Company (St. Louis, MO., USA): 1-heptane sulfonic acid (HSA) sodium salt, HPLC-grade acetonitrile (ACN), HPLC-grade methanol, Triton-X 100, 4-hydroxyquinoline (4-HQ), bovine serum albumin (BSA), kynuramine, ethylenediaminetetraacetic acid (EDTA) sodium salt, O-phthaldialdehyde (OPT), Tris-buffered saline tablet, Phosphate buffered saline tablet, Reduced glutathione (GSH), Dimethyl sulphoxide (DMSO), hydrogen peroxide (H₂O₂) and . For HPLC analysis, double-distilled water was filtered and deionized using with Milli-Q system was used (Waters, Milford, MA).

Experiment design and Treatments: C57/BL-6 mice and APP^{swe}+PS1 mice were randomly divided into groups based on their body weight. Both groups were kept on powdered chow diet for 8 months. After completion of eight months animals were subjected to battery of behavioral tests before sacrificing.

Behavioral Assessment: As a part of general behavioral assessment, body weights of the animals were recorded in an interval of one month by using digital weighing scale. Animals were observed for general signs of toxicity and hyperactivity. In order to assess cognitive performance animal were subjected to Y-maze and object recognition tests to assess the spatial and recognition memory in the double transgenic mice at 10 months of age.

Open Field Test: Locomotor behavior of mice were recorded with open field test where each mouse was placed in an open field testing apparatus equipped with 16 photo beams (San Diego Instruments, San Diego, CA), for a 50 minute single session. The distance traveled by each mouse in either the central or peripheral area of the open field was analyzed for the first 5 minutes (initial), the last 5 minutes, and the total 50 minute session. Rearing and grooming behaviors were also monitored (Holcomb et al., 1998; Holcomb et al., 2006; Ahuja et al., 2008).

Y-maze Test: An index of spatial working memory, Y-maze test is based on the natural tendency of rodents to explore a novel arm. The Y-maze apparatus consists of three equal arms (40 cm long, 15 cm wide and 30 cm high) made of opaque polyvinyl chloride (PVC) polymer and was performed as described in our previous studies (Holcomb et al., 2006). Briefly, each mouse was allowed to freely explore the two sides of Y-maze for 8 minutes with the third arm closed by PVC door. Mice exhibiting fewer than 10 arm entries were excluded from further analysis. Animal's behavior was recorded by a video recorder (Sony DCR-DVD310E), and later analyzed by an experienced and unbiased observer. The total number of arm entries, number of entries to each arm and time spent in each arm was registered. An animal was considered to enter an arm whenever he had his four paws inside that arm. After 2 hour of inter-trial interval, mice were placed again in the Y-maze to explore all three arms for 8 minutes recognition trial. The percentage entry into the novel vs previously explored arms (% novel arm entry), and spontaneous alternation scores for each mouse were calculated as a measure of short-term working memory and spatial navigation (Holcomb et al., 1998; Holcomb et al., 2006).

Object Recognition Test: Object recognition test was performed in a squared arena (30 cm × 30 cm × 25 cm) with clear plexiglass walls and a black painted plastic-covered floor. The test was performed as previously described by Muralikrishnan (2007). For habituation animals were

allowed to freely explore the arena for 5 min before one day of experiment (habituation period). During habituation, general locomotor activity was evaluated by direct observation. On the test day, initially animals were exposed to two identical objects (two plastic toys, F1 and F2) during 5 min for a training session. Selection of objects was done keeping in mind that objects are not bigger than twice the size of a mice and they are available in set of three (F1, F2 and F3). Following an hour inter-trial interval, the test session was performed by exposing each animal to previously encountered object F3 and to a novel object (another toy, N1) for 5 min. The position of the objects was not changed between the training and the test session. Animal behavior was recorded with video recorder and analyzed by an experienced and unbiased observer. Parameters that were analyzed were time spent exploring the objects during the two sessions. Exploration of an object was defined whenever animals pointed their nose toward an object at a distance ≤ 1 cm, whereas playing, turning around, climbing and/or biting the objects were not considered as part of exploration (Barker et al., 2007). The discrimination index (DI), an index of cognitive assessment, was calculated as the difference between the time spent exploring the novel object (N) and the familiar one (F3) in relation to the total time spent exploring the objects $[(N - F)/(N + F)]$ in the test session. Exclusion criteria included less than 10 s exploration of an object by an animal during the test or the training session and less than 1 s exploration of one of the objects in the test session. Only data from the first minute of test session were employed for the statistical analysis because animal's capacity to discriminate between the novel and the familiar objects is reported to diminish with time (Mumby et al., 2002).

During every behavioral test, animals were placed in an unbiased portion of the arena (e.g. facing centre or opposite to the objects) so that there is not a specific pattern available for

the animals to follow. Animals were placed back into their home cages after each trial and to remove animal's odor both arenas and objects were thoroughly cleaned with ethanol (30% v/v).

Biochemical analysis

Tissue Preparation for biochemical assays: Animals from each group were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. Homogenate of the brain samples were prepared by homogenizing the brain tissue in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C and the supernatant were collected.

Protein estimation: Protein was assayed using the Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL) as per the instructional manual of the kit. The assay determines colorimetrically at 660 nm the binding of a proprietary dye-metal complex to protein in acidic conditions. Bovine serum albumin (BSA) dissolved was used as a standard for protein measurement and a standard curve was obtained for determining the linearity of the assay.

Beta-Secretase activity: β -site-APP cleaving enzyme (BACE) or β -Secretase activity was determined fluorimetrically with commercially available beta-Secretase activity kit (Biovision, California, USA) as per manufacturer's instructions. The kit utilizes Beta-Secretase-specific peptides conjugated to two fluorogenic reporter molecules EDANS and DABCYL. Briefly, cortical and hippocampal brain samples were homogenized in 2X ice cold extraction buffer, followed by 10 minutes incubation and centrifugation at 10,000 \times g for 5 minutes. Supernatant was collected and immediately processed for the beta-Secretase detection while kept in ice. Appropriate amounts of lysate and reaction buffer were added in to the wells of black 96-well plate in duplicates, followed by addition of fluorogenic substrate and incubated in the dark at 37 °C for 1 hour. Following incubation, samples were read in a fluorescent plate reader (Synergy

HT, BioTek, VT, USA) with an excitation wavelength of 335-355 nm and emission of 495-510 nm at 25 °C. Negative and positive controls were utilized to determine background values and working of the assay. Beta-Secretase activity was represented as relative fluorescence unit per mg of protein.

Determination of Total β -amyloid content: Levels of full length human amyloid β 1–40 and 1–42 were measured from brain homogenates of cortex and hippocampus from APP^{swe}+PS1 mice using commercially available fluorometric ELISA kits (Biosource International Inc., Camarillo, CA). The guanidine-HCl extraction technique was followed as per the manufacturer's instructions to maximize the recovery of amyloid β because guanidine-HCl (5 M final concentration) has been demonstrated to be more effective than formic acid in extracting low levels of amyloid β (Morishima-Kawashima et al., 2000). Duplicate samples for each animal were assayed and the results averaged to give a single value of amyloid β 1–40 and 1–42 levels for each animal that as then used to calculate the mean \pm SEM for each treatment group.

Mitochondrial P2 fraction preparation: The brain samples were weighed and homogenized with a glass-Teflon homogenizer in 0.32 M sucrose in ice-cold 10 mM potassium phosphate buffer (pH 7.2) to prepare a 10% homogenate. The homogenate was centrifuged at 1,000g for 10 minutes at 4°C and the supernatant obtained subjected to a second centrifugation at 10,000g for 60 minutes. The pellet obtained was re-suspended in cold 50 mM Tris buffer and was centrifuged again at 10,000g for 60 minutes. This step was repeated one more time to obtain the mitochondrial P₂ fraction. The pellet thus obtained was re-suspended in cold 10 mM potassium phosphate buffer (pH 7.2) and kept overnight at -20°C. Prior to analysis, the suspension was sonicated at low energy until the pellets were uniformly dispersed. The mitochondrial P₂ fraction formed was used for assessing complex I and IV activity.

Assay of MAO activity: Monoamine oxidase (MAO) is an enzyme that is responsible for the oxidation of biogenic amines. Spectrofluorometric method that determines the amount of 4-hydroxyquinoline formed due to oxidation of kynuramine by MAO was used for the determination of MAO. Briefly, assay mixture consisted of crude homogenate (100 uL), potassium phosphate buffer (pH 7.4, 800 uL) and kynuramine (1mg/ml in PBS buffer, 100uL) in a 2ml eppendorf tube. The reaction was then terminated by addition of ice-cold 0.4N perchloric acid (300 uL) after 1hour. The reaction mixture was centrifuged (14, 000 x g for 5 min) and 2 ml of 1 N NaOH was added to one ml supernatant of the reaction mixture. Fluorescence intensity was measured at activation/emission wavelengths of 315/380 nm. A standard curve was obtained for commercially available 4-hydroxyquinoline. The specific enzyme activity of each sample was calculated as nmol of 4-hydroxyquinoline formed/h/mg protein (Muralikrishnan and Mohanakumar, 1998). Results are depicted as percentage control MAO activity.

Assay of Super oxide dismutase (SOD) activity: Super oxide dismutase (SOD) activity was measured spectrophotometrically by Marklund and Marklund method using pyrogallol as substrate (Marklund and Marklund, 1974). Briefly, 10 – 20 uL of P2 fraction was added to 890 uL of Tris-HCL buffer (0.05 M containing 1 mM DTPA, pH 8.5). The reaction was initiated by the addition of 100 µl of freshly prepared 2.6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0.13 mM in the assay mixture. The assay mixture was transferred to a 1.5 ml cuvette and the rate of increase in the absorbance at 420 nm was recorded for 2 min from 1 min 30 s to 3 min 30 s in a Shimadzu Model 200–20 double beam spectrophotometer with recorder. Results were expressed as percentage control.

Estimation of Catalase: Catalase activity was assayed by the method of Beers and Sizer (1952), where the disappearance of peroxide is measured spectrophotometrically at 240 nm with few

modifications to work with 96-well plate (Beers and Sizer, 1952; Dhanasekaran et al., 2007; Ahuja et al., 2008). Briefly, the assay mixture consisted of 260 μ L of 30 mM solution of hydrogen peroxide in phosphate buffer (0.05 M, pH 7.0), and 40 μ L of mitochondrial P2 fraction of brain tissue in a final volume of 300 μ L. Solution of hydrogen peroxide was added in the last as the reaction of hydrogen peroxide degradation is very fast. Hydrogen peroxide without tissue homogenate was used as blank. Change in absorbance was recorded at 240 nm for 2-3 minutes. Catalase activity was calculated as change in absorbance at 240 nm ($\Delta A_{240}/\text{min}$) from the initial (45 second) linear portion of the curve. Results were expressed as percentage control.

Glutathione (GSH) estimation: GSH was measured fluorimetrically as described earlier by Cohn and Lyle (Cohn and Lyle, 1966). Principally, this method employs o-phthalaldehyde (OPT) condensation reaction with GSH to yield a fluorescent product at pH 8.0. Briefly, assay mixture consisted of 0.1% OPT solution in methanol (100 μ L), 0.01M phosphate buffer (1.8 ml) and supernatant of tissue (100 μ L) after precipitation of protein by 0.1M phosphoric acid treatment. The assay mixture was incubated for 20 min and readings were taken at excitation and emission wavelengths of 340 and 420 nm respectively. A standard curve was prepared from commercially obtained GSH. The GSH content was calculated as nmol of GSH/mg protein and reported as percentage control levels.

Glutathione peroxidase assay (GPx): Colorimetric estimation of glutathione was performed according to the method of Lawrence and Burk (Lawrence and Burk, 1976), with few modifications. Glutathione activity is measured indirectly by a coupled reaction with glutathione reductase where recycling of oxidized glutathione, produced as a result of reduction of hydrogen peroxide by GPX, to its reduced state by glutathione reductase and NADPH is measured. The oxidation of NADPH to oxidized NADP⁺ results in decrease in absorbance at 340 nm, which is

directly proportional to GPx activity. The reaction mixture consisted of 0.01 M phosphate buffer solution (650 uL, pH 7.4), P2 fraction (50 uL) containing GPx to be estimated, 2.4 U/ ml of glutathione reductase enzyme (GRx, 10 uL), 10 mM GSH (100 uL), 1.5 mM t-butyl peroxide (100 uL) and 1.5 mM NADPH (100 uL) prepared in 1% sodium bicarbonate solution. NADPH was added in the last to start the reaction and immediately absorbance readings were recorded at 340 nm. The activity was calculated as μmol of glutathione oxidized/mg protein.

Determination of ROS generation: Reactive oxygen species generation in the mitochondrial P2 fractions was estimated by spectro-fluorometrically detection of conversion of non-fluorescent chloromethyl-DCF-DA (2',7'- dichlorofluorescein diacetate, DCF-DA) to fluorescent DCF at an excitation wavelength of 492 nm and an emission wavelength of 527 nm. The method has been described in detail earlier (Dhanasekaran et al., 2008). Briefly, 0.0005% w/v solution of DCF-DA in ethanol (10 uL), phosphate buffer (280 uL) and homogenate (10 uL) obtained from control and transgenic animals were incubated for 1 hour at 37°C. DCFH reacted with ROS to form the fluorescent product DCF. Intensity was analyzed by BioTek Synergy HT plate reader (BioTek, VT, USA). The generation of ROS was measured, normalized to tissue protein content and reported as relative fluorescence intensity/mg protein.

Estimation of cyclooxygenase (COX) activity: Enzymatic activity of COX was analyzed by commercially available Cayman COX assay kit (Cayman Chemical Company, Michigan, USA) that monitors the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) colorimetrically at 590 nm. The assay was performed as per the manufacturer instructions. Briefly, the assay buffer, solution of heme, and homogenized sample was added to well of 96-well plate in triplicates. Every sample had a background well where inactivated homogenized sample (samples boiled for 5 minutes) were mixed with assay buffer and heme solution. The

plate with respective standard, tissue and background sample was incubated for 5 minutes at room temperature. After incubation colorimetric substrate was added to each well and reaction was initiated by adding arachidonic acid solution to each well. Carefully plate was agitated on a plate shaker and absorbance was read at 590 nm using a BioTek Synergy HT plate reader (BioTek, VT, USA). Total COX activity was normalized to tissue protein levels and expressed as the percentage control activity.

Estimation of lipid peroxidation: The index of lipid peroxidation was estimated by measuring the malondialdehyde (MDA) content in the form of thiobarbituric acid-reactive substances according to the method of Wills (Wills, 1965; Ahuja et al., 2008; Dhanasekaran et al., 2008). Briefly, 100 μ L of cytosolic fraction of the whole brain samples and 200 μ L ice cold TCA (10% w/v in double distilled water) were incubated for 15 minutes on ice. Assay mixture was centrifuged at 2200 \times g for 15 min at 4°C temperature. Place 200 μ L supernatant of standards and samples into new labeled screw top 1.5 ml tubes and equal volumes (200 μ L) of 0.67% TBA was added. The reaction mixture was incubated for 20 minutes in boiling water. Samples after incubation were cooled down to room temperature and placed in 96-well plate. A standard curve of TBARS reactive substances was also prepared by employing different concentrations of MDA. Absorbance was read in plate reader at 532 nm and MDA levels were calculated as TBARS reactive substances per mg protein. Results expressed as percentage control.

Electrochemical detection of cortical Glutamate and gamma-amino butyric acid (GABA) levels:

Tissue preparation: Animals were sacrificed by cervical dislocation in the morning before 11.00 AM in order to avoid any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. For the analysis of neurotransmitters, the whole brains were dissected out

within thirty seconds, rinsed in ice cold normal saline, blotted dry on ash-free filter paper and each left and right cortex were dissected out separately. The cortical sections were weighed and homogenized by sonication in 1ml of chilled homogenization buffer (0.1M citric acid, 0.1M sodium dihydrogen phosphate monohydrate, 5.6mM octane sulfonic acid, 10 μ m EDTA in 10% (v/v) methanol solution, pH 2.8 with 4M NAOH). After homogenization samples were centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant stored at -80 °C until final HPLC analysis.

Measurement of Glutamate and GABA: The amino acids were estimated by employing HPLC-electrochemistry detection as described by Clarke et al. (2007). Briefly, the method utilizes the chemical properties of primary amines to react with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide to produce 1-cyanobenz-isoindole (CBI) derivatives that can be detected by either electrochemical or fluorescent detectors. After derivitization of both standards and tissue samples, 20 μ L of injection of each was injected in the HPLC system.

HPLC Equipment: The HPLC system (shimadzu) consisted of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 μ l injection loop, and C18, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 μ m and pore size of 80 Å. The flow rate was 0.65 ml/min and the electro-detection was performed at + 0.8 V for the analyses of glutamate and GABA.

Derivitization protocol and HPLC procedure: The derivitization of GABA and glutamate was done according to method published by Clark et al., (2007) with some modifications. Briefly, 10 μ l of either standard mix or sample supernatant, 90 μ l of borate buffer (0.1 M, pH 9.5), 10 μ l of potassium cyanide (10 mM) and 10 μ l of NDA (6 mM) were added to a single reaction tube,

vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20 µl of the derivative was injected into the appropriate HPLC system. The composition of mobile phase was 0.1M di-sodium hydrogen orthophosphate/50 µM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). Mobile phase was filtered through 0.45 µm and vacuum degassed prior to use. The samples were eluted isocratically over a 30 min runtime at a flow rate of 0.65 ml/min after a 20µl injection. A standard solution containing glutamate and GABA was run immediately prior to and following sample injections. Results are presented as pmol/mg tissue.

Estimation of hippocampal and cortical inflammation:

Multiplex analysis of hippocampal and cortical cytokines: Brain tissue lysates from hippocampus and cortical regions were used for determination of inflammatory cytokines and chemokines involved in inflammatory pathways according to the manufacturer's instructions. Briefly, diluted equal amounts of soluble protein lysates were added to set of microspheres (1x beads suspension) covalently conjugated to antibodies for the respective analytes in a 96-well filter plate. Plates were incubated overnight at 4°C on a plate shaker. Lysate was removed and beads were washed and again incubated for 1 hour at room temperature with Milliplex MAP biotinylated Reporter solution (a solution containing cocktail of biotinylated antibodies). Reporter solution was removed by vacuum filtration and solution of streptavidin–phycoerythrin was added to the plate and left for incubation on a plate shaker for 15 min in the dark. Following streptavidin–phycoerythrin conjugate binding, the fluorescent signal was amplified by an amplification solution. The beads were then resuspended in assay buffer 2 and fluorescent signal was measured with a Luminex 200 system (Millipore Corp.). Average median fluorescence

intensity (MFI) was measured for the standard and sample analytes and was normalized to protein concentrations of the samples. Data were reported as pg/mg protein for each sample.

ELISA determination of levels Inflammatory cytokines: The cytokine levels present in the brain tissue lysate were measured using commercially available sandwich ELISA kit and by following the manufacturers protocol. Three different cytokines relevant to present study were selected including IFN- γ (eBiosciences, USA), IL-17a (R&D Systems, USA) and IL-10 (BD Biosciences, USA). The results were presented in pg/ml.

Isolation of brain leukocytes and Flow-cytometric determination of activated T-cells: Leukocytes were isolated from APPswe+PS1 and wild type brains using a previously described method (Marten et al., 2000; Marques et al., 2008). Briefly, brain tissues from each group were finely homogenized in RPMI (2 g/L D-glucose and 10mM HEPES). Digestion of these homogenized samples was performed in 0.0625% trypsin (in Ca/Mg free HBSS) at room temperature for 20 min. Single cell preparations thus obtained were resuspended in 30% Percoll and banded on a 70% Percoll cushion at 900 \times g at 15°C. Brain leukocytes obtained from the Percoll interface were treated with Fc Block (anti-CD32/CD16 in the form of 2.4G2 hybridoma culture supernatant with 2% normal rat and 2% normal mouse serum) to inhibit non-specific antibody binding. Staining was done with anti-mouse immune cell surface markers for anti mouse-CD45-PerCP (557235), anti-CD4-FITC (561088), anti-IFN-g-APC (554413) and anti mouse IL-17A-PE (559502) and analyzed by flow cytometry. Control isotype antibodies were used for all isotype and fluorochrome combinations to assess nonspecific antibody binding. Live leukocytes were gated using forward scatter and side scatter parameters on a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo software (TreeStar, Inc.).

Quantitative PCR analysis of TNF-alpha and IL-1b: Total RNA was prepared from dissected cerebellar and hippocampal tissue of eight APPswe+PS1 transgenic mice and eight nontransgenic mice that were 10-11 months old. The homogenates from rotor-stator emulsification (Tissuemizer) of all tissues used were applied to RNeasy mini-spin columns (Qiagen, USA) with on-column DNase (Qiagen, USA) treatment followed by elution with RNase-free water, according to the manufacturer's specifications. All, total RNA samples were then reverse transcribed using Maxima First Strand cDNA Synthesis Kit (K1642, Thermo-Scientific, USA), as per the manufacturer's instructions using 5µg of RNA template in a Biorad iQ5 Thermocycler (Bio-Rad, USA). Primer pairs for qRT-PCR were generated to amplify 100 bp fragments of the gene of interest using the web-based application PrimerQuest (IDT, USA) and were obtained commercially from the IDT (IDT, USA). PCR primers were designed for two inflammatory markers TNF-alpha and IL-1β and their sequences are provided in the table. RT-qPCR was performed with Sso-Fast Evergreen Supermix (Bio-Rad, USA) utilizing CFX96 real-time PCR cycler (Bio-Rad, USA). Enzyme activation was done for 30 seconds at 98°C followed by 39 cycles at 98°C for 5 s (denaturing), at 58 °C for TNF-α, IL-1b and β-Actin for 10 seconds (annealing/extension) were carried out. Melting curve analysis was performed beginning at 65°C with increment of 0.5°C every 10 sec till it reaches 95°C, with fluorescence measured at every interval. No primer-dimer were observed as melting curve only revealed one peak of fluorescence as obtained from CFX Software, indicating a single gene product. Relative fold expression after normalizing with housekeeping gene β-Actin was obtained from the CFX Software, which were then statistically analyzed in prism software (version 4) with two tailed unpaired student's t-test.

Table 4.1 Primers used for Real-time PCR analysis		
Gene	Forward Primer	Reverse Primer
NR1	AATACGCTTCAGCACCTCGGACAG	AAAAGCAGGGCGAATGTCAGCAG
NR2b	GCCTCTTGGGTTTCTCCTCCTGAAC	CAGAAGGAAATGTATTCGGATGCCAG
NR2a	CGTTATTGATGAACCGCACTGACC	ATCTGAGCCACAGCCTCTTGGTC
GluR1	CCGTTGACACATCCAATCAGTTTGTC	AGAATGTTGACAGCCGTCACCTGC
GluR3	TGGGTCATTCTCACGGAGGATTC	GGCTTCTCAGTGGTGTCTGGTTG
GluR4	TTAGCAAGAGTGAGAGAAAGAGAGGAGAGC	CTATTTGAACGCTGCTCGGAAAGG
TNF-alpha	TCTCATGCACCACCATCAAGGACT	ACCACTCTCCCTTGCAGAACTCA
IL-1b	AAGGGCTGCTTCCAAACCTTTGAC	ATACTGCCTGCCTGAAGCTCTTGT
β-Actin	GTCCACACCCGCCACCAGTTCG	TTCTGACCCATTCCCACCATCACAC

Assessment of mediators of synaptic strength:

LTP assessment of hippocampal slices:

Slice preparation and extracellular field recordings: Male C57/BL6 and APP^{swe}+PS1 mice aged 10 months were decapitated under CO₂ anesthesia. After quick dissection, the brain was immersed in dissection buffer (cutting solution) consisting of (in mM): NaCl 85, KCl 2.5, MgSO₄ 4.0, CaCl₂ 0.5, NaH₂PO₄ 1.25, NaHCO₃ 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5, saturated with 95% O₂/5% CO₂ at a temperature of 1-4 °C. Coronal hippocampal slices (300 μ m) were obtained using a vibratome. These slices were perfused for an hour at 30 °C in artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 119, KCl 2.5, MgSO₄ 1.3, CaCl₂ 2.5, NaH₂PO₄ 1.0, NaHCO₃ 26, dextrose 11.0, and saturated with 95% O₂/5% CO₂.

All electrophysiological recordings were performed in a submersion chamber perfused with aCSF (30 °C) saturated with 95% O₂/5% CO₂. Slice was transferred to submerged chamber and field excitatory postsynaptic potentials (fEPSPs) were recorded. fEPSPs were evoked by stimulating Shaffer collateral afferents in the stratum radiatum with a bipolar electrode (MPI,

Gaithersburg, MD) and recordings were made in the axons of CA1 hippocampal region with a glass pipette filled with aCSF (1–4 M Ω). Signals were digitized, amplified and acquired using LTP program. Stimulation command was provided through the LTP program and was regulated by a stimulus isolator. Input–output curves were recorded by varying stimulus intensity between 0 and 200 μ A in increments of 50 μ A and measuring the slope and amplitude of the fEPSPs. Baseline fEPSPs were recorded at 50% of maximal response, as determined from an input–output curve for each experiment. Following stable baseline recordings of at least 20 minutes, LTP was induced by delivering 5 trains of theta burst stimulation (TBS) separated by 20 sec. Each TBS consisted of 10 trains of bursts, each of which had 4 pulses at 100 Hz, with an inter burst interval of 200 ms. LTP was measured as the percentage of the baseline fEPSP slope. During the 20-minute baseline and 1 hour following the TBS, fEPSP peak amplitude and slope were analyzed online using WinLTP acquisition software (Anderson and Collingridge, 2007). The data are presented as means \pm SEM. A two-tailed unpaired t-test was used to determine significance for all basal measures.

Quantitative PCR analysis of glutamatergic receptors: Cortical and hippocampal brain samples from each group were assessed for mRNA analysis of glutamatergic receptors, especially, NMDA subtype of NR1, NR2a, NR2b, and AMPA subtype of glutamatergic receptors mainly GluR1, GluR3 and GluR4. Total RNA extraction, cDNA synthesis and amplification was done as explained above in section 2.7.4. Primers designed for glutamatergic receptors are presented in table 1, which were obtained from IDT (IDT, USA). Quantification and amplification was done using Sso-Fast Evergreen Supermix (Bio-Rad, USA) in CFX96 real-time PCR cycler (Bio-Rad, USA). Enzyme activation was done for 30 seconds at 98°C followed by 39 cycles at 98°C for 5 s (denaturing), annealing and extension at different respective temperatures for NR11,

NR2a, NR2b, GluR1, GluR3 and GluR4 and β -Actin for 10 seconds (annealing/extension) were carried out. Melting curve analysis was performed beginning at 65°C with increment of 0.5°C every 10 sec till it reaches 95°C, with fluorescence measured at every interval. No primer-dimer were observed as melting curve only revealed one peak of fluorescence as obtained from CFX Software, indicating a single gene product. Relative fold expression after normalizing with housekeeping gene β -Actin was obtained from the CFX Software, which were then statistically analyzed in prism software (version 4) with two tailed unpaired student's t-test.

Western blot for glutamatergic NMDA receptors: Total protein was isolated from cells using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) containing protease inhibitor cocktail (P8340, Sigma, St. Louis, MO) and phosphatase inhibitors (P 5726, Sigma, St. Louis, MO). Protein concentration was measured using the Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Protein samples were aliquoted into 50 ug fractions and were stored in -70 °C for storage. On the day of experiment samples were boiled in invitrogen sample loading buffer for 5 minutes and then loaded onto a gradient 4-12% NuPAGE Bis-Tris Gel (Life Technologies, Grand Island, NY) and electrophoretically transferred onto nitrocellulose membrane through semi-dry transfer method. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline plus 0.1% Tween-20 at pH 7.4 (TBST). The membranes were incubated overnight at 4 °C with specific antibody constituted in 5% BSA in TBST. Primary antibodies used in the current study were anti NR2A (Cell Signaling), anti NR2B (Cell Signaling), along with anti β -Actin and anti-GAPDH, which were used as a loading control. Membranes were then washed with TBST (3 X, each for 10 min) and incubated with specific species dependent HRP-conjugated secondary antibodies for 60 min at room temperature. Membranes were again washed three times for 10 min with TBST after

incubation with each antibody. After washing membranes were incubated with chemiluminescent reagent (Enhanced ECL- substrate, Pierce, Rockford, IL) for 5 minutes. Membranes were wrapped in plastic wrap and placed in the x-ray film cassette with protein side oriented up. Chemiluminescence signal was measured as band density obtained on the X-ray film through utilization of Quantity One software (Bio-Rad, Hercules, CA). Band densities for each sample was normalized to its β -actin or GAPDH signal and reported as percentage control.

Multiplex analysis Multi-Pathway Signaling Phosphoprotein analysis: Brain tissue lysate from hippocampus and cortical regions were used for determination of phosphoproteins involved in various signaling pathways according to the manufacturer's instructions as explained earlier. The kit was used to detect changes in phosphorylated ERK/MAP kinase 1/2 (Thr185/Tyr187), p70 S6 kinase (Thr412), I κ Ba (Ser32), and CREB (Ser133), in the tissue samples. Results are expressed as average net MFI/mg protein.

Statistical analysis: Statistics was performed using the Prism-V software and results were expressed as mean \pm SE. The experimental data were analyzed by using Student's *t*-test for the comparison between two groups. Differences were considered significant at $p < 0.05$.

4.3. Results

Behavioral deficits in APP^{swe}+PS1 mice: General behavioral test such as locomotion is a mandatory for assessing the differences in the performance of animals in the memory evaluating behavioral tasks. Significant increased peripheral and central ambulation was observed in bigenic transgenic mice (Fig. 4.1c) during the 50 minute trial in an open-field apparatus. Hyperlocomotor activity has been observed by other investigators in the first 5 min trial of open-field test, which returns to control values on the third day of trial (Lalonde et al., 2004).

Using the Y-maze and two-trial recognition task, both spatial and recognition memory were assessed. At 10 months of age, both groups were evaluated for spatial and recognition learning and memory through Y-maze and object-recognition paradigms. APPswe+PS1 mice exhibited significant decline in spatial and recognition memory at 10 months of age. During the learning trial of Y-maze paradigm, mice from both groups did not show any difference in exploration behavior. When subjected to retention trial after 1 hour, APPswe+PS1 mice exhibited significant reduction of 15% in novel arm entries, representing spatial memory deficits (Fig. 4.1a, Control (42.37 ± 1.83) and APPswe+PS1 (27.54 ± 3.30); $p \leq 0.01$; $n=6$). Another outcome from the Y-maze trial, the alternation score was found not to differ significantly in both groups (Control 55.1 ± 13.8 and APPswe+PS1 52.8 ± 2.9 ; $n=6$), indicating no change in the working memory in the transgenic animals.

Similarly, APPswe+PS1 mice demonstrated a significant reduction in the time spent in exploring novel object (Fig. 4.1b, Control (0.63 ± 0.1038) and APPswe+PS1 (0.19 ± 0.026); $p \leq 0.01$; $n=6$), when assessed after 1 hour of learning trial with a novel object in the same arena. No significant changes in exploring the objects were observed in both groups during the learning trial of object-recognition behavioral test. APPswe+PS1 mice thus demonstrated significant memory impairment as compared to control C57/BL6 mice at 10 months of age.

Neurochemical alterations in APPSWE+PS1 mice: Glutamate and GABA are two predominant neurotransmitters that regulate excitatory and inhibitory neurotransmission in brain, respectively. Glutamate and GABA levels were determined in the homogenized lysate from the cortical regions of the brain tissue. In APPSWE+PS1 group, there was significant increase in the glutamate levels, as compared to control group (Fig. 4.2a, Control (0.00709 ± 0.002372) vs. APPswe+PS1 (0.02068 ± 0.001308); $p \leq 0.005$, $n=5$). Likewise, there was significant elevation in

GABA levels in the APP^{swe}+PS1 mice as compared to control group (Fig. 4.2b, Control (0.001542 ± 0.0006238) vs. APP^{swe}+PS1 ($0.003623 \pm 7.941e-005$); $p \leq 0.05$, $n=5$). The neurotransmitter levels were measured as pico moles/mg of protein of the cortical tissue.

Elevated Beta-Secretase activity and total Amyloid-beta levels in APP^{swe}+PS1 mice: ELISA assay for β -amyloid quantitatively determines total levels of human Amyloid beta 1-40 and 1-42. APP^{swe}+PS1 mice at 8 months of age had significantly higher amounts of both β -amyloid 1-40 and 1-42 in cortical and hippocampal regions of the brain (Fig. 4.3a, $n=5$, $p \leq 0.05$). Increase in the cortical region of the brain was more prominent than the hippocampal region implicating the cortex as the most affected area in AD. In concordance to Amyloid beta 1-40 and 1-42, activity of Beta-Secretase enzyme, known to catalyze the degradation of APP resulting in formation of amyloid beta 1-42, was found to be elevated in the cortical and hippocampal area of the APP^{swe}+PS1 mice. In APP^{swe}+PS1 mice beta-Secretase activity increased by 73% (Fig. 4.3b, $n=5$, $p \leq .001$) and 40% (Fig. 4.3b, $n=5$, $p= 0.042$) respectively, in cortical and hippocampal regions of the brain. The Beta-Secretase activity is expressed as relative fluorescence unit per mg of protein and amyloid-beta 1-40 and 1-42 content was represented as ng/mg of protein.

Oxidative stress in APP^{swe}+PS1 mice: Oxidative stress plays a substantial role in the etiology of Alzheimer's disease and other neurodegenerative diseases. In order to assess reactive oxygen species (ROS) and major players of oxidative stress, we analyzed levels of ROS species, lipid peroxides, glutathione (GSH), glutathione peroxidase (GSH-Px), Superoxide dismutase (SOD) and Catalase. In the double transgenic APP^{swe}+PS1 mouse, we observed evidential increase in oxidative markers such as ROS (Fig. 4.3c, Control (100 ± 7.42 , $n=6$) and PSPAP (308.61 ± 57.84 , $n=6$); $p \leq 0.01$) and LPO (Fig. 4.3c, Control (100 ± 9.45 , $n=6$) and PSPAP (139.33 ± 10.56 , $n=6$); $p \leq 0.05$). Pertaining to ROS and LPO, APP^{swe}+PS1 mice exhibited significant

increment in enzymatic activity of COX (Fig. 4.3c, Control (100 ± 16.12 , n=6) and PSPAP (187.25 ± 35.69 , n=6); $p \leq 0.05$) and GSH-PX (Fig. 4.3c, Control (100 ± 7.69 , n=6) and PSPAP (152.45 ± 8.085 , n=6); $p \leq 0.005$). Though insignificant, but substantial decrease of almost 50% was also observed in Catalase activity (Fig. 4.3c, Control (100 ± 32.02 , n=6) and PSPAP (55.36 ± 12.94 , n=6); $p = .086$) in APP^{swe}+PS1 mice as compared to control mice. The levels and activities of these markers were determined as absolute values obtained from the respective standard curves and expressed absolute value per mg of protein of the mitochondrial fraction obtained from the whole brain tissue lysate. These were converted to percentage control values for a satisfactory comparative depiction of results.

Increased inflammation in cortical and hippocampal regions of APP^{swe}+PS1 mice: chronically inflamed tissue is infiltrated by inflammatory cells, which releases enormously large amounts of cytokines and chemokines in the vicinity of the inflamed tissue. To assess the extent of chronic inflammation in the adolescent APP^{swe}+PS1dE9 mice, we analyzed the pro-inflammatory and anti-inflammatory cytokines in the brain of these mice. Multiplex analysis of the bigenic APP^{swe}+PS1dE9 mice revealed significant alterations in the amount of cytokines in tissue lysate from the cortical and hippocampal regions. Significant increase was observed in the cortical pro-inflammatory cytokines such as the IL-1b (Table 4.2, Control (38.05 ± 3.988) and APP^{swe}+PS1 (53.61 ± 1.090), n=6, $p \leq 0.005$), IL-2 (Table 4.2, Control (44.76 ± 3.381) and APP^{swe}+PS1 (57.48 ± 1.177), n=6, $p \leq 0.005$), IL-17a (Table 4.2, Control (16.42 ± 3.830) and APP^{swe}+PS1 (27.77 ± 1.213), n=6, $p \leq 0.05$), IP-10 (Table 4.2, Control (26.19 ± 1.845) and APP^{swe}+PS1 (32.33 ± 1.991), n=6, $p \leq 0.05$), MIG (Table 4.2, Control (75.87 ± 6.475) and APP^{swe}+PS1 (95.45 ± 4.195), n=6, $p \leq 0.05$) and Eotaxin (Table 4.2, Control (32.85 ± 2.729) and APP^{swe}+PS1 (42.44 ± 0.5328), n=6, $p \leq 0.005$). Also, the anti-inflammatory cytokines such as

IL-10 (Table 4.2, Control (6.728 ± 0.8910) and APP^{swe}+PS1 (3.264 ± 0.6475), $n=6$, $p \leq 0.05$) were found to be lowered in the cortex of APP^{swe}+PS1 mice. Hippocampal cytokines were altered but not to the same extent as the cortical cytokines. From the hippocampal sections we found a significant decrease in IL-13 (Table 4.2, Control (16.38 ± 2.179) and APP^{swe}+PS1 (6.450 ± 0.9767), $n=6$, $p \leq 0.005$) and contrary to cortex, a decrease in IL-17 (Table 4.2, Control (23.40 ± 5.729) and APP^{swe}+PS1 (6.757 ± 1.297), $n=6$, $p \leq 0.05$) and MIG (Table 4.2, Control (108.3 ± 15.53) and APP^{swe}+PS1 (60.93 ± 10.07), $n=6$, $p \leq 0.05$) was observed. Gene expression analysis was also performed to observe whether the change in protein expression is proportionate to the change in gene expression. Interestingly, we find significant increase in the gene expression of IL-1b in the cortex (Fig. 4.6D, Control ($1 \pm .1057$) and APP^{swe}+PS1 (2.94 ± 0.251), $n=3$, $p \leq 0.05$) though visible but insignificant increase in hippocampus (Fig. 4.6d, Control ($1 \pm .046$) and APP^{swe}+PS1 (1.68 ± 0.165), $n=3$) of APP^{swe}+PS1 mice. TNF-alpha gene, too was found to be significantly over-expressing both in cortical and hippocampal regions of brain of APP^{swe}+PS1 mice (figure 6d, $n=3$, $p \leq 0.001$) as compared to control mice. All these results points toward the region selective increment in the inflammatory response in transgenic mice at an age when the disease is progressing. Another alarming observation is the robustness of inflammatory response in cortical region which is suggestive of the involvement of inflammatory response in debilitating the cognitive cortical functions such as memory storage.

T-cell leukocyte infiltration and associated release of inflammatory markers: Inflammation, majorly the peripheral chronic inflammation, is considered to be modulated by leukocytes T-cells and B-cells. T-cell mediated immune response is more prominent and efficient in responding to chronic injuries. In addition, the neuro-inflammation which was thought to devoid of T-cell mediated response has recently been proved to be otherwise (Schneider et al., 2013).

Thus, to determine the participation and role of leukocytes in the pathogenesis of Alzheimer's in this particular animal model we analyzed two different types of T-cell population in the mice brain and their activation status. We extracted T-helper cells (identified with CD4 surface marker) from the brain and stimulated them to check their response via cytokine release through flow cytometry. APP^{swe}+PS1 mice were found to have significantly more number of CD4 positive T-helper cells that releases significantly higher amounts of pro-inflammatory cytokine IL-17a (Fig. 4.5b, Control (4.880 ± 0.7850) and APP^{swe}+PS1 (9.680 ± 0.7414), $n=3$, $p = 0.0113$). IL-17a releasing CD4⁺ cells are categorized as different types of T-cells, known as Thelper-17 cells. In addition to IL-17a, Interferon- γ (IFN- γ) releasing CD4⁺ cell population was also higher in bigenic mice suggesting a profound Thelper-1 cell mediated response. Regulatory T-cells are widely seen in cases of chronic inflammation, which is an underlying process of pathophysiology of AD and hence we tend to find out the activation status of these types of T-cells. The number of FOXP3⁺ CD4⁺ cell population in the brain of APP^{swe}+PS1dE9 mice was found to be higher as compared to wild type mice (Fig. 4.5a, Control (15.6 ± 1.767) and APP^{swe}+PS1 (26.30 ± 2.381), $n=3$, $p = 0.0226$).

Results from ELISA studies on the whole brain lysate excluding cerebellum also confirmed that IFN-gamma (Fig. 4.6 C, $n=5$, $p=.0011$) and IL-17a (Fig. 4.6A, $n=5$, $p< 0.0001$) are significantly elevated in the brain of APP^{swe}+PS1 mice. Interestingly, IL-10 was found to be elevated in APP^{swe}+PS1 mice, contrary to what was seen in cortical region (Fig. 4.6B, $n=5$, $p< 0.0001$). However, this was consistent with the increase in the population of FOXP3⁺CD4⁺ cells that secretes anti-inflammatory cytokine IL-10. Regional selectivity regarding the inflammation mediated response in the AD animal brain is another interesting feature we found in our results, but overall it is suggestive of the chronic inflammation in the APP^{swe}+PS1 mice.

Synaptic deficits as a result of alterations in post-synaptic proteins: Learning and memory deficits in the transgenic animal models of AD correlates very well with the synaptic deficits in the hippocampal and cortical brain regions. Synaptic plasticity in control and APP^{swe}+PS1 acute slices was examined by inducing long-term potentiation with theta burst stimulation (TBS), in the Schaffer-collateral pathway of hippocampus. TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ; with interburst interval 200 msec and is found to be the most effective and the most physiologically relevant as it induces the inherent discharge characteristic of hippocampal neurons (Albensi et al., 2007). Unpaired T-test showed that LTP was significantly impaired in bigenic transgenic mice with an average of $134.6\% \pm 0.9774\%$, compared with control slices in which LTP was induced with an average of $195.2\% \pm 3.239\%$ (Fig. 4.4a, ; $p < 0.0001$; $n = 6$ slices from 6 animals). The deficit in the LTP process represents the weakening of the synaptic plasticity and the processes involved in the LTP formation in 10 months old APP^{swe}+PS1 transgenic mice, which is in concordance with the impaired performance of these mice in the memory acquisition behavioral tasks.

Synaptic plasticity, as measured by LTP formation, is closely associated with glutamatergic transmission in the respective brain regions. Hence, western blots as well qRT-PCR were performed to examine the genetic as well as protein expression of the glutamatergic receptors. Genomic expression of NMDA and AMPA subtypes of ionotropic glutamate receptors in cortical and hippocampal brain regions of transgenic mice exhibited significant changes only in case of NMDA subtypes. Gene expression of NR2b was found to significantly decline in APP^{swe}+PS1 mice in the hippocampal region (Fig. 4.4d, Control (1 ± 0.15613) and APP^{swe}+PS1 (0.5117 ± 0.15613); $p = 0.016$; $n = 6$), and no change was observed in the genomic expression pattern of NR2a subtype of NMDA receptor. Whereas, NR1 subtype of NMDA

receptor mRNA expression was remarkably elevated in the cortical (Fig. 4.4d, Control and APP^{swe}+PS1 values 1 ± 0.55989 and 2.71561 ± 0.35878 , respectively; $p=0.027$; $n=6$) as well as hippocampal (Fig. 4.4d, Control and APP^{swe}+PS1 values 1 ± 0.3456 and 2.67882 ± 0.345 , respectively; $p=0.152$; $n=6$) regions of brain of bigenic transgenic mice. However, protein expression analysis of NR2b subtypes of NMDA receptor revealed, though insignificant, but a slight decrease in protein levels as compared to non-transgenic control mice. However, NR2a subtype of NMDA receptor protein expression in hippocampus was found to be significantly elevated in bigenic transgenic mice (Fig. 4.4e, $p=0.0401$; $n=6$). Interestingly, the mRNA expression studies showed NR2a to be unaltered in the transgenic mice. Post-translational modifications may be responsible for this irregularity in the mRNA and protein expression of NR2a receptors in the hippocampus. Protein expression of only hippocampal NR2 subtype of NMDA receptors were performed as NR2a and NR2b are accepted as the most important subtypes regulating Ca^{2+} influx and subsequently, governing the synaptic plasticity through Ca^{2+} dependent pathways.

Glutamate ionotropic receptors on activation lead to influx of cations such as Na^+ and Ca^{2+} resulting in depolarization of the target cell and stimulation of downstream second messenger pathways. NR2a and NR2b receptors modulate different types of protein kinases eventually resulting in different types of responses. LTP induction and maintenance is known to be a function of AMPA and NMDA subtypes of glutamatergic receptors (Lee and Kirkwood, 2011). Downstream protein kinases such as CAMKII, PKA, PKC and ERK/MAPK are well established mediators of LTP formation (Lee and Kirkwood, 2011). In addition to these mediators, a well known transcription factor CREB, in its activated state closely correlate with LTP formation and any deficit in its phosphorylated state leads to LTP impairment. We, so as to

examine the second messenger involved in alterations of LTP process associated with impaired memory formation in the bigenic transgenic mice, performed multiplex analysis of the phosphorylated states of these important signaling factors. We found significant decrement in phosphorylated states of ERK/MAP kinase 1/2 (phosphorylated at Thr185/Tyr187), and CREB (phosphorylated at Ser133) in hippocampal region of APP^{swe}+PS1 mice (Fig. 4.4f, ERK/MAPK 1/2; p=0.0315 and CREB; p= 0.0013; n=5), where this difference was not very well evident in the cortical regions of the transgenic mice. Activation of PI-3-kinase activates p70S6 kinase involved in protein synthesis (Raymond et al., 2002), we observed significant decline in activated state of p70S6kinase (Thr412) in both cortical and hippocampal regions of APP^{swe}+PS1 mice (Fig. 4.4f, Cortical and hippocampal p-values are 0.0027 and 0.0117, respectively; n=5). These results points toward the central pathological role of NMDA subtype glutamatergic receptors in causing memory impairment in these transgenic animal models of AD.

Figures and Figure Legends:

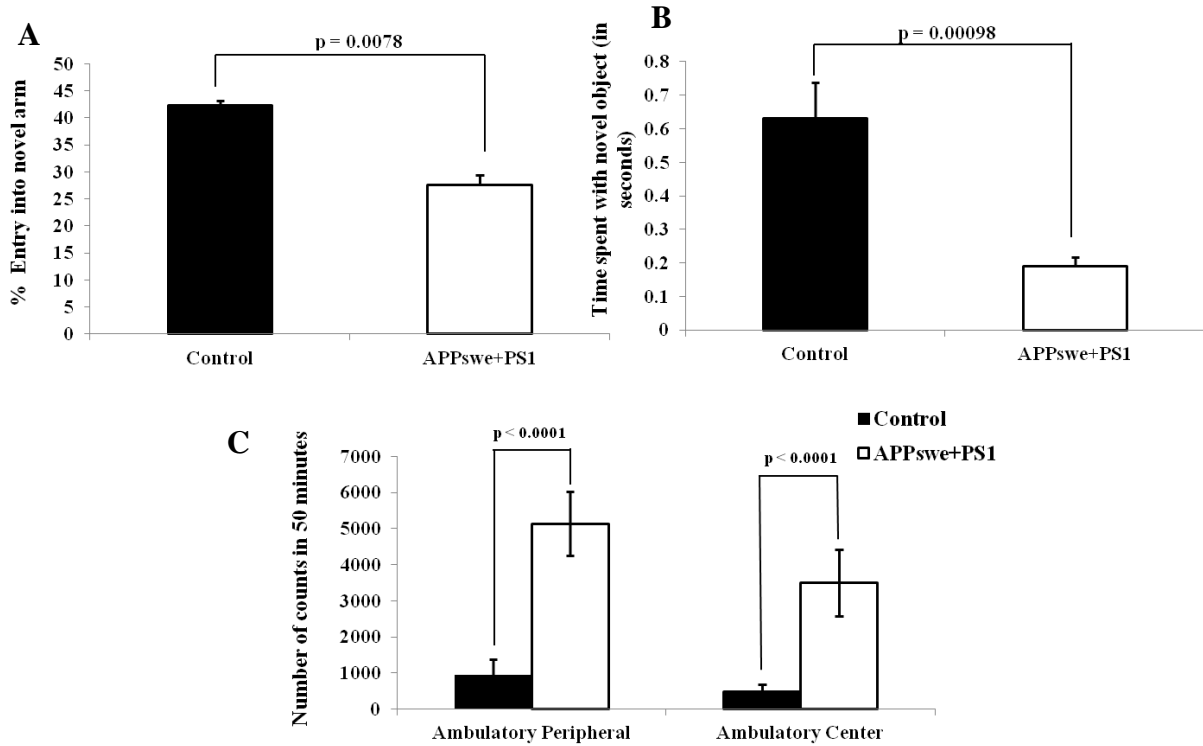


Figure 4.1. Bigenic APPswe+PS1dE9 mice show significant spatial working memory performances deficits. (A) Bar chart shows the significant reduction in the percentage of number of entries by APPswe+PS1dE9 mice in exploring the novel arm in the Y-maze than in the control non-transgenic C57/BL6 mice $p < 0.01$, $n=6$. (B) Bar plot showing drastic reduction of time spent to explore the novel object in object recognition task when compared to the familiar object by APPswe+PS1dE9 mice $p < .001$ as compared to non-transgenic control mice, $n=6$. (C) Bar plot depicts the significant increase in activity and general exploratory behavior, assessed in open field test, in APPswe+PS1dE9 mice relative to the control non-transgenic mice $p < .0001$ $n=6$.

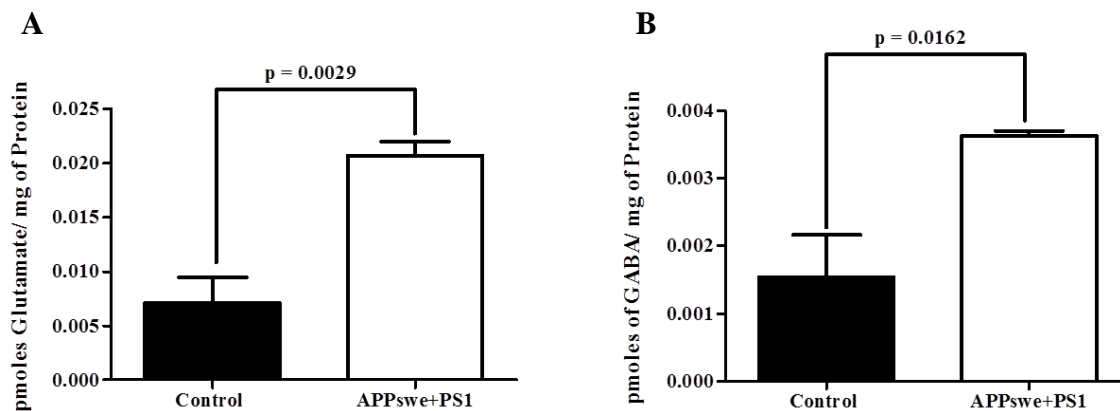


Figure 4.2. Marked increase in Glutamate and GABA levels in APPswe+PS1dE9 mice assessed by HPLC-ECD. A) Bar chart shows the significant increase in the level of glutamate neurotransmitter measured in cortical regions of brain of APPswe+PS1dE9 mice relative to control non-transgenic mice ($p < 0.01$; $n = 6$). Levels of neurotransmitter are expressed as picomoles/mg of protein B) Bar chart shows the significant increase in the cortical GABA content as detected by HPLC in APPswe+PS1dE9 mice in comparison to non-transgenic C57/BL6 mice ($p < 0.05$, $n = 6$). Results are expressed as picomoles/mg of protein.

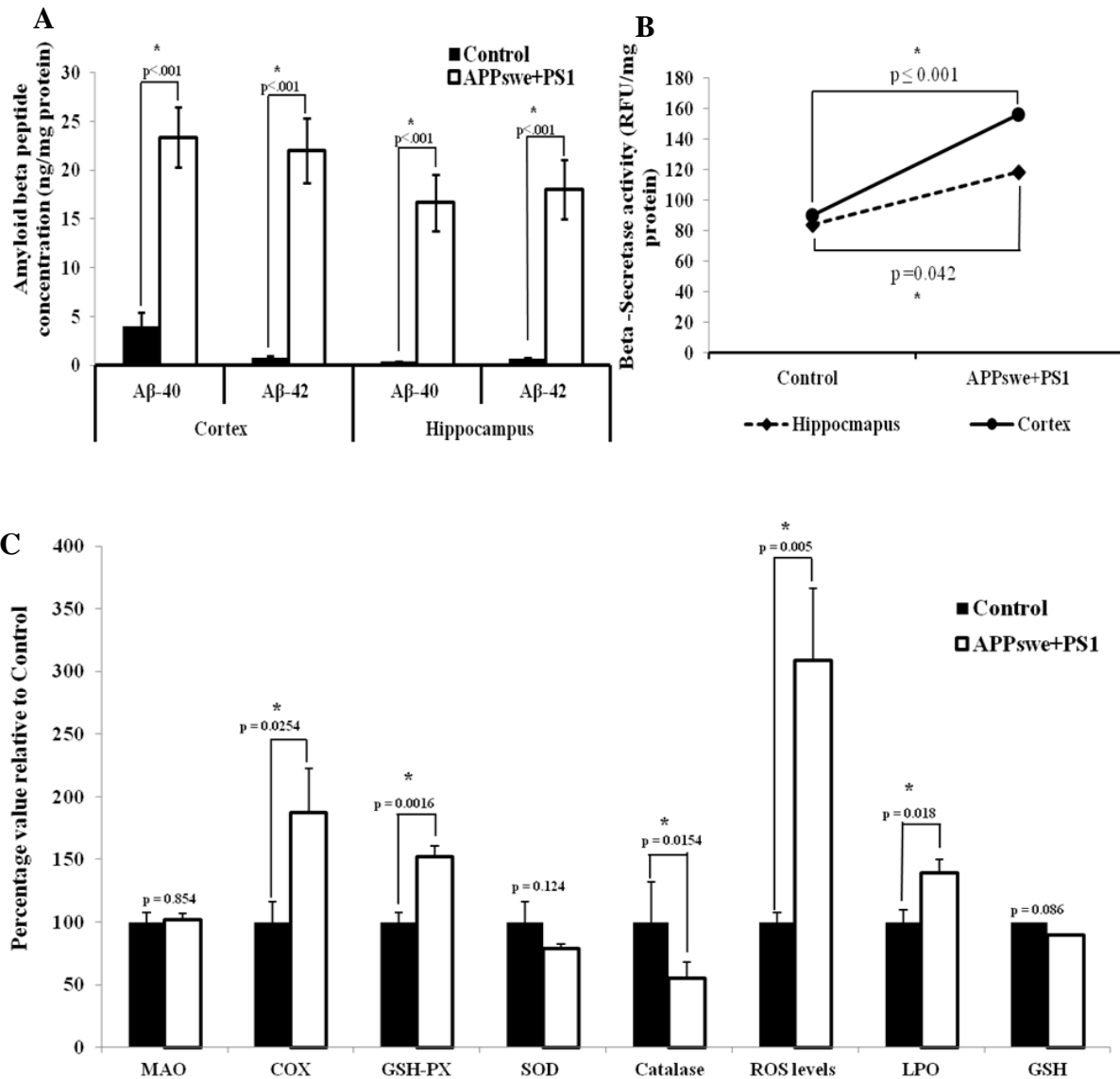


Figure 4.3. Excessive A β levels leads to oxidative stress in APPswe+PS1dE9 transgenic mice. A) Bar plot depicts the significant increase in A β_{40} and A β_{42} levels in cortex as well as hippocampus of APPswe+PS1dE9 bigenic mice compared to control mice ($p < 0.001$; $n = 6$). B) Line graph illustrates the significant increase in β -Secretase enzymatic activity in cortical and hippocampal regions of the brain of bigenic APPswe+PS1dE9 mice relative to control mice ($p < 0.01$, $n = 6$; $p < 0.05$, $n = 5$). C) Bar plot shows the alterations in biochemical oxidative markers in APPswe+PS1dE9. There was significant increase in COX-2 ($p < 0.05$, $n = 6$), Glutathione Peroxidase (GSH-PX) ($p < 0.005$, $n = 6$) activity and significant decrease in Catalase ($p < 0.05$, $n = 6$) enzymatic activity in transgenic mice compared to control mice. Reactive oxygen species (ROS)

($p=0.05$, $n=6$) and lipid peroxidation (LPO) ($p<0.05$, $n=6$) levels were significantly increased in transgenic mice.

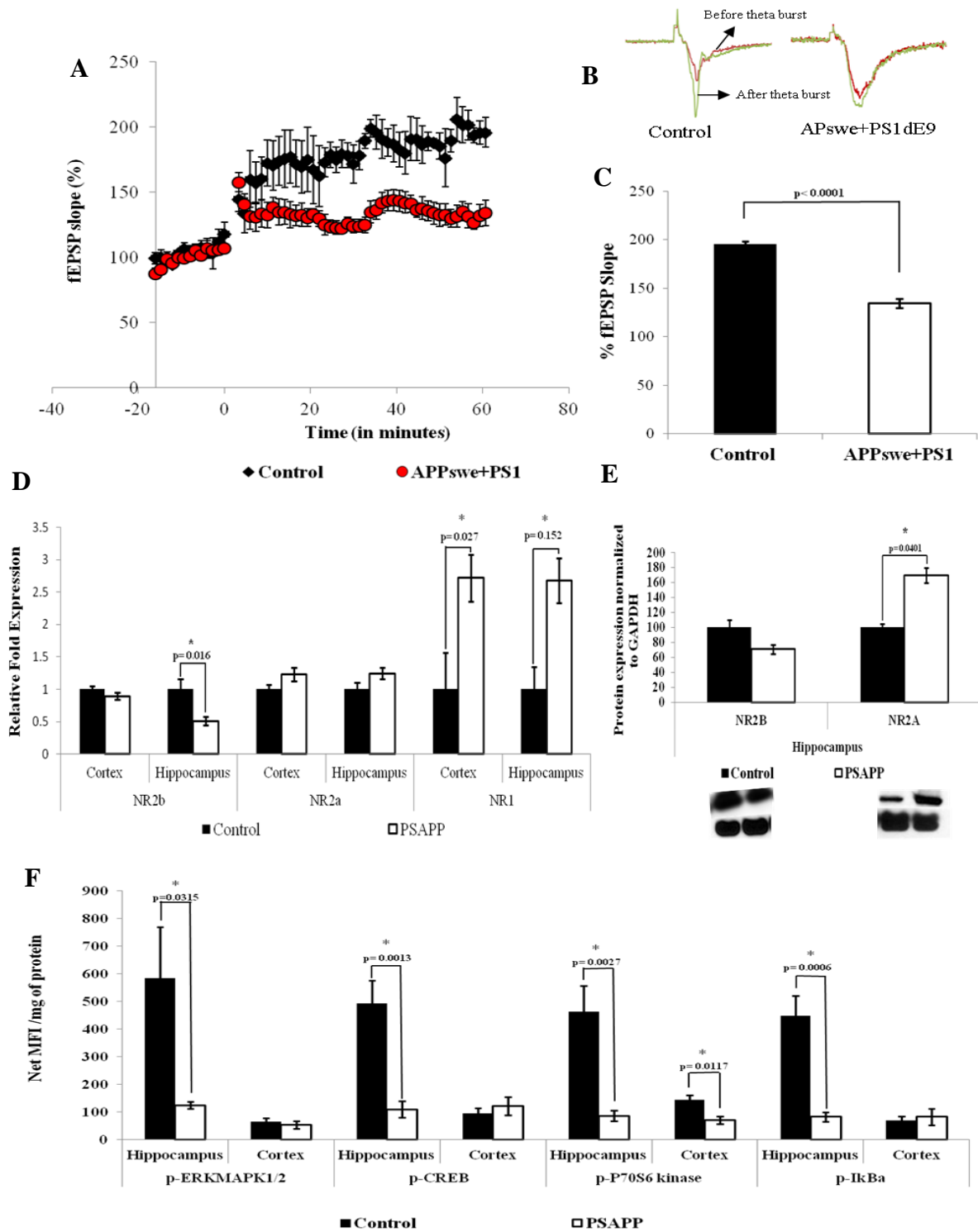


Figure 4.4. Synaptic deficits in APP^{swe}+PS1^{dE9} transgenic mice. (A) LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS. LTP was significantly impaired in acute hippocampal slices from APP^{swe}+PS1^{dE9} mice, normalized fEPSPs slopes of 60 min post-TBS averaged $134.6\% \pm 0.9774\%$ for APP^{swe}+PS1^{dE9} mice and $195.3\% \pm 4.510\%$ for control mice, ($p < 0.001$; $n=6$, 6 slices from 6 animals). (C). Bar plot showing drastic reduction of LTP in APP^{swe}+PS1^{dE9} mice compared to control mice. (D) Bar plot depicting the relative fold expression in mRNA of different subunits of NMDA receptors in cortex and hippocampus of brain of APP^{swe}+PS1^{dE9} mice compared to control transgenic mice. E) Bar plot depicting the relative change in the protein expression (after normalization with β -actin band density) of different subunits of NMDA receptors in cortex and hippocampus of brain of APP^{swe}+PS1^{dE9} mice compared to control transgenic mice. F) Bar plot depicting the relative change in the levels of the phosphorylated proteins of different cellular signaling markers determined through Millipore multiplex analysis. Phosphorylation of ERK 1/2, CREB, P70S6Kinase and I κ Ba was assessed in the cortical as well as hippocampal area of the brain of APP^{swe}+PS1^{dE9} mice compared to control transgenic mice.

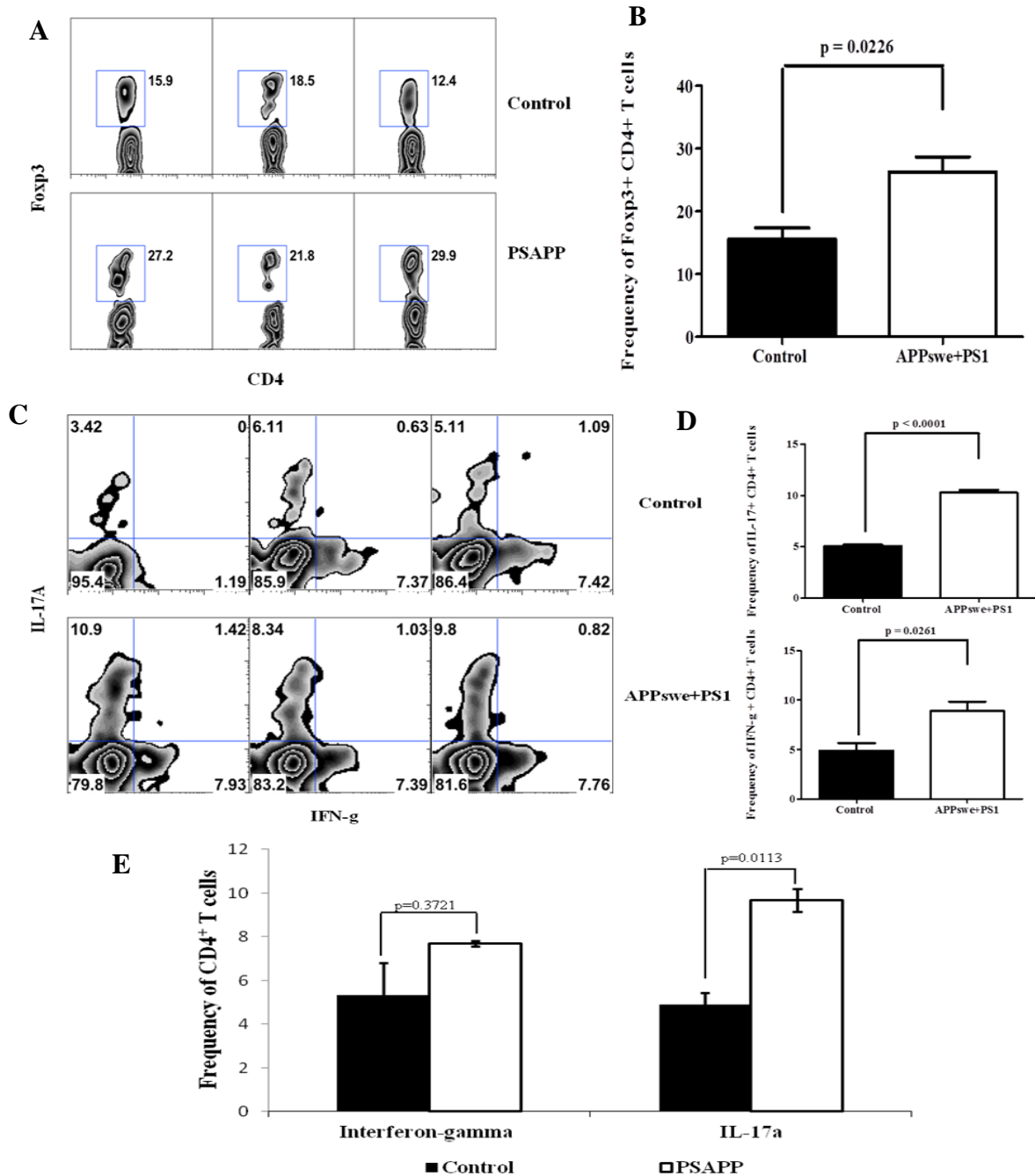


Figure 4.5. Frequency of T-helper cells in APPswe+PS1dE9 mice. (A) Flow cytometric illustration of frequency of CD4+FoxP3+ cells present in the whole brain lysate of the APPswe+PS1dE9 mice. (B) Bar plot shows the significant increase in the frequency of FOXp3+CD4+ T-cells in the APPswe+PS1dE9 mice compared to control non-transgenic mice ($p < 0.05$, $n = 3$). (C) Flow cytometric plot illustrating the frequency of IL-17a +CD4+ and IFN- γ +

CD4⁺ T-cells in the whole cell lysate of brain of transgenic mice relative to control mice. (D) and (E) Bar plot depicting the significant increase in the IL-17a secreting CD4⁺ T-cells ($p < 0.0001$, $n=3$) and and IFN- γ secreting CD4⁺ T-cells ($p < 0.05$, $n=3$) in APP^{swe}+PS1^{dE9} mice.

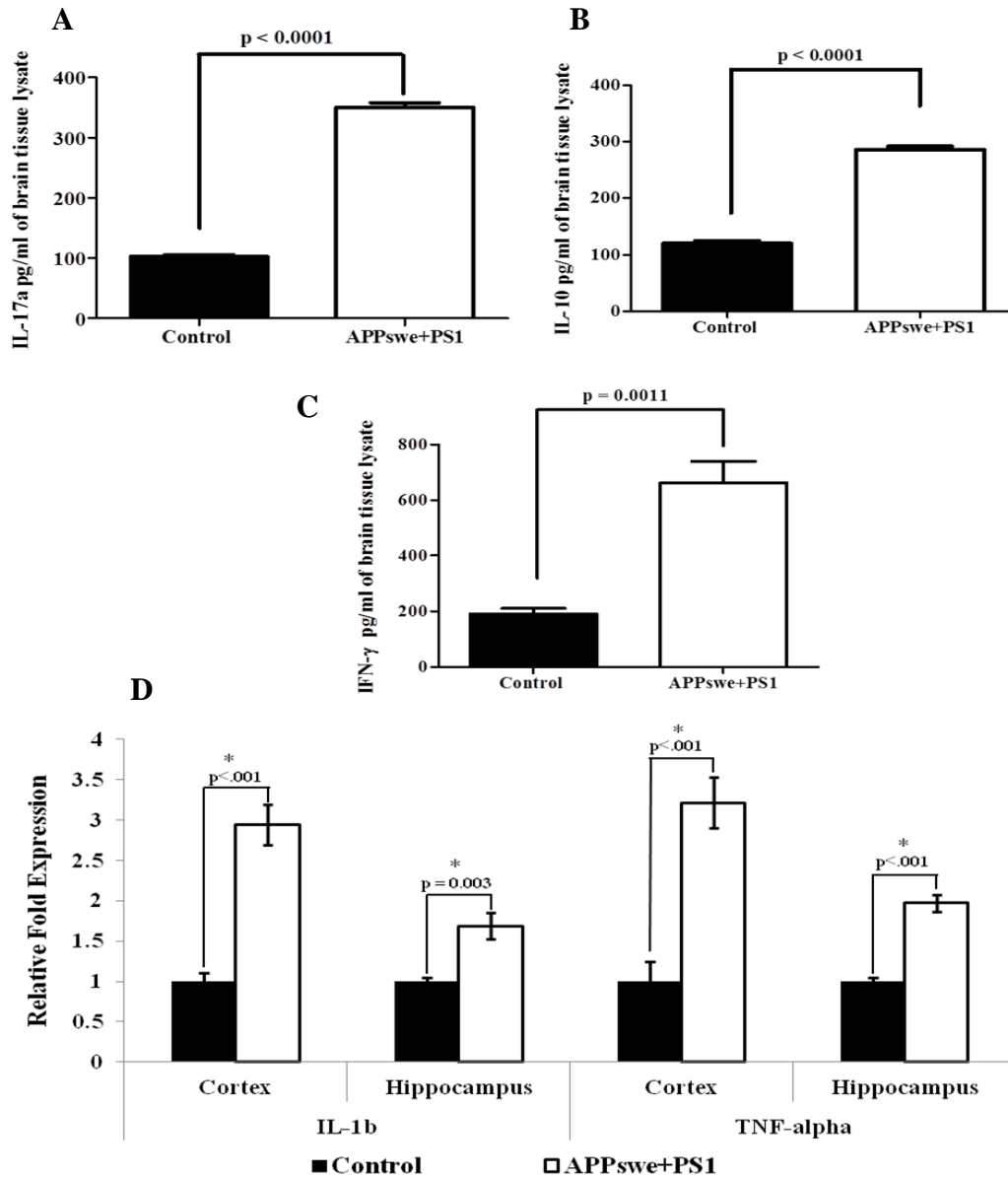


Figure 4.6. Neuro-inflammation in APP^{swe}+PS1^{dE9} mice. (A) Bar plot depicts the significant increase in the IL17a cytokines estimated through ELISA in the whole brain lysate of APP^{swe}+PS1^{dE9} mice compared to control non-transgenic mice ($p < 0.05$, $n=6$). (B) Bar plot depicts the significant increase in the IL10 cytokine estimated through ELISA in the whole brain lysate of APP^{swe}+PS1^{dE9} mice compared to control non-transgenic mice ($p < 0.05$, $n=6$). (C)

Bar plot depicts the significant increase in the IFN- γ cytokine estimated through ELISA in the whole brain lysate of APPswe+PS1dE9 mice compared to control non-transgenic mice ($p < 0.05$, $n = 6$). (D) Bar plot depicting the relative fold expression in mRNA of IL-1 β and TNF- α in cortex and hippocampus of brain of APPswe+PS1dE9 mice compared to control transgenic mice. IL-1 β mRNA was significantly elevated in the hippocampal as well as cortical regions of APPswe+PS1dE9 mice relative to non-transgenic control mice (Cortex, $n = 3$, $p < 0.001$; Hippocampus, $n = 3$, $p = 0.003$). Alike IL-1 β , TNF- α mRNA was also found to elevated in the bigenic mice model of AD compared to non-transgenic mice (Cortex, $n = 3$, $p < 0.001$; Hippocampus, $n = 3$, $p < 0.001$).

	Cortex		Hippocampus	
	Control (pg/mg protein)	APPswe+PS1 (pg/mg protein)	Control (pg/mg protein)	APPswe+PS1 (pg/mg protein)
IL-1b	38.05 \pm 3.988	53.61 \pm 1.090**	36.93 \pm 3.731	35.23 \pm 7.377
IL-2	44.76 \pm 3.381	57.48 \pm 1.177**	42.07 \pm 5.911	36.75 \pm 8.663
IL-7	3.390 \pm 0.5389	2.953 \pm 0.2241	2.148 \pm 0.4879	2.087 \pm 0.4489
IL-10	6.728 \pm 0.8910	3.264 \pm 0.6475*	6.437 \pm 0.7827	8.488 \pm 1.525
IL-13	14.69 \pm 3.619	10.41 \pm 1.444	16.38 \pm 2.179	6.450 \pm 0.9767**
IL-15	6.463 \pm 0.9125	6.187 \pm 0.6150	2.593 \pm 0.7125	2.845 \pm 0.7570
IL-17	16.42 \pm 3.830	27.77 \pm 1.213*	23.40 \pm 5.729	6.757 \pm 1.297*
IP-10	26.19 \pm 1.845	32.33 \pm 1.991*	16.95 \pm 1.599	12.02 \pm 2.672
MCSF	12.68 \pm 1.102	10.92 \pm 0.4098	8.798 \pm 0.9597	7.760 \pm 1.114
MIG	75.87 \pm 6.475	95.45 \pm 4.195*	108.3 \pm 15.53	60.93 \pm 10.07*
Eotaxin	32.85 \pm 2.729	42.44 \pm 0.5328**	23.27 \pm 1.925	21.21 \pm 2.910
	*	$p \leq 0.05$	**	$p \leq 0.005$

Table 4.2: Levels of pro-inflammatory and anti-inflammatory cytokines. Levels of pro-inflammatory and anti-inflammatory cytokines were assessed through multiplex analysis in the cortical and hippocampal areas of the APPswe+PS1dE9 mice brain as compared to control non-transgenic mice.

4.4. Discussion:

The present study shed light on the inflammation and immune mediated responses in the double transgenic APP^{swe}+PS1^{dE9} mice line at 10 months of age, and enhance our understanding of the pathological alterations associated with Alzheimer's disease and other amyloid-beta or peptide associated neurodegenerative disorders. Our results demonstrates that at an adolescent age (10 months old), there is significant cognitive deficits with underlying neuro-physiological deficits in bigenic APP^{swe}+PS1^{dE9} mice model accompanied by imbalance in oxidative ROS and inflammatory mediators. Most importantly, our study for the first time demonstrates a T-cell mediated response in this mice line with an augmented Thelper-17 cell response most probably linked to significant microglial activation along with an heightened Thelper-1 response. These observations greatly pave the way of validating this model for amyloid-beta induced neurotoxicity, cognitive anomalies and most plausibly, strongly linked immune mediated abnormal response in well established, double transgenic APP^{swe}+PS1^{dE9} mice model of Alzheimer's disease. It can be reasonably stated that this model can be used for developing interventions for both familial as well as sporadic AD as it simulates most of the characteristic features, such as amyloid toxicity and inflammation associated with AD.

APP^{swe}+PS1^{dE9} mice has been well accepted as a suitable model for familial AD attributing to exhibition of extracellular amyloid deposits and associated cognitive deficits starting from an early age of 4 and 6 months, respectively (Savonenko et al., 2005; Garcia-Alloza et al., 2006; Ruan et al., 2009). In order to validate the cognitive deficits in the 10 month old double transgenic mice, we assessed the performance of the animal subjects in two different cognitive tasks, one was the object recognition task and the other was Y-maze task, so as to assess recognition as well as spatial reference memory, respectively. APP^{swe}+PS1^{dE9} mice

exhibited significant memory decline at 10 months age in both types of memory tasks as compared to their age matched control non-transgenic mice (fig.1a & b). There were no differences in locomotor behavior of the transgenic mice in comparison to control mice as observed in open field test (fig.1c). Our results were consistent with previous report where APP^{swe}+PS1^{dE9} mice aged 9-13 months showed severe deficits in object recognition memory (Melnikova et al., 2006), although the deficits in recognition memory in these transgenic mice emerges only in later ages and hence known to be dependent on age (Bonardi et al., 2011). Y-maze or spatial reference memory is shown to decline early in the double transgenic mice such as APP^{swe}+PS1 mice even at ages of 6 months (Holcomb et al., 2006). These results demonstrate the memory impairment in these transgenic mice and thus validate its use as AD model.

Glutamate and GABA, two most abundant excitatory and inhibitory neurotransmitters, respectively, in the CNS very systematically regulates synaptic plasticity and associated physiological CNS functions such as learning, attentiveness and memory (Reis et al., 2009). As expected, in our study we found an abnormal increase in levels glutamate and GABA in cortical regions of the brain of APP^{swe}+PS1^{dE9} mice as compared to control non-transgenic mice. Due to A β -induced microglial and astroglial activation, alteration in glutamate levels surfaces, which eventually results in excitotoxicity and neuronal cell death (Bleich et al., 2003; Francis, 2003). This increase can be ascribed to extensive formation and deposition of plaque forming amyloid-beta 40 and 42 peptides, which we also observed in the cortex and hippocampus of these transgenic mice. Swedish mutations in APP gene and deletion in PS1 gene strongly enhances the production of A β , and out of the two A β , these mutations favors A β ₄₂, fibrillogenic form of A β , while not affecting the A β ₄₀ (Garcia-Alloza et al., 2006). Interestingly, we also encountered the significant increase in BACE activity, which is markedly altered in AD patients (Fukumoto et

al., 2002). The increase in the BACE activity and expression is considered to play an important etiological role in accumulation of A β , and thus, in the progression of AD in familial as well as sporadic AD. In the current animal model used in the present study, increased BACE activity signifies that the mutations in APP and PS1 caused the APP protein more accessible to beta- and gamma-Secretase enzymes and thus resulted in more activity of BACE and hence, increased accumulation of A β_{40} and A β_{42} . Enhanced accumulation of A β and plaque deposition in the cortical and hippocampal regions leads to glutamate and GABA imbalances, which eventually leads to synaptic deficits, excitotoxicity, neuronal cell death and cognitive deficits in this transgenic animal model. Several reports depict robust decrease in expression of glutamate transporters and alterations in other regulators of glutamatergic system that leads to increased levels of glutamate in the CNS of animals and humans (Bleich et al., 2003; Francis, 2003). Glutamate and GABA levels are tightly regulated as glutamate can be converted to GABA by activity of glutamic acid decarboxylase (GAD) (Francis, 2003). Thus, significant increase in excitatory neurotransmitter and accompanying decrease in inhibitory neurotransmitter underlie the higher excitability in the cortical and hippocampal neuronal, microglial and astrocytic cell population, eventually leading to physiological alterations in them.

Oxidative stress is found to coexist with amyloid beta accumulation and deposition in almost all clinical cases of AD as well as in experimental animal models and is thus regarded as one of the etiological factors underlying sporadic and familial AD. To address the presence of oxidative stress in our animal model aged 10 months, we assessed ROS generation and extent of lipid peroxidation as measures of oxidative stress in the whole brain homogenate of both groups. Furthermore, to pinpoint the underlying molecular machinery of increased oxidative stress, we analyzed the activity of enzymes usually linked to oxidative stress generation such as GSH-Px,

Catalase, SOD, and MAO in the whole brain homogenate. Both, ROS and lipid peroxidation (LPO) levels were significantly elevated along with increased GSH-Px and Catalase activity, while no change was observed MAO and SOD enzymatic activity. The results suggests that A β accumulation resulted in imbalance of oxidative and anti-oxidative machinery that ultimately lead to increased ROS generation and lipid peroxide formation. Our results are in concordance with previous studies, which shows a causal link between increased A β accumulation and oxidative stress generation, and both as a function of age in Alzheimer's disease (Rowan et al., 2004). Altered activities of antioxidant enzymes such as GSH-Px and Catalase imply hydrogen peroxide as the major oxidative entity in these transgenic animals, and the possibility that these alterations could be secondary to the increased oxidative radicals generated due to the altered glutamate levels leading to substantial Ca²⁺ influx in the neuronal cells. Increased Ca²⁺ influx leads to induction of detrimental molecular cascade resulting in induction of nitric oxide synthase (NOS) enzymes resulting in extensive generation of nitric oxide (NO) and oxidative stress (Rowan et al., 2004). In our previous study with a different double transgenic model of AD, known as APP^{swe}+PS1 mice model, we have demonstrated robust increment in oxidative stress, which is supposed to leading cause of synaptic deficits and progression of AD. Hence, our current results establish the role of oxidative stress, which correlates very well with A β accumulation, in the pathogenesis of cognitive deficits and AD symptoms in the current double transgenic APP^{swe}+PS1dE9 mice.

In chronic neurodegenerative disorders such as AD, chronic inflammation is conceived to be one of the causative factors that emerge at a very early age and is shown to present in postmortem analysis of clinical cases. Vast evidence suggest microglia and astrocytes as the major players involved in the inflammatory process in AD, though, recently neurons also have

been reported to contribute to this process (Akiyama et al., 2000a; Akiyama et al., 2000b). However, these cellular components, in normal scenarios play critical roles in the homeostasis and function of the brain, which complicates the physiology in the disease process. Microglia supports and protects the neurons and act as immunoprotectant defense system in the CNS that is able to express major histocompatibility complex II, proinflammatory cytokines, chemokines, complement proteins and in an activated states also possess phagocytic and scavenger properties (Moore and O'Banion, 2002). Microglia has been found to cluster around A β plaque deposits in AD patients as well as double transgenic APP^{swe}+PS1^{dE9} and other animal models (Moore and O'Banion, 2002; Ruan et al., 2009), and are reported to express scavenger receptors and purinergic P2X₇ receptors, which mediates adhesion to A β fibrils and secretion of ROS (El Khoury et al., 1996; Lee et al., 2011). Accumulating evidence suggests that during CNS inflammation in neurodegenerative disorders, activated microglia express costimulatory molecules such as CD80, CD86, and CD40 in order to interact with T-cells and modulating T-cell mediated response by promoting either their proliferation, T-effector functions (e.g., cytokine secretion), or both (Aloisi, 2001; Magnus et al., 2005; Wirenfeltd et al., 2011). In order to find the coexistent T-cell stimulation, we collected T-helper cells (CD4 cells) and studied their release profile after stimulation by PMA. Cytokines trapped within the cells after application of golgi body stop reagent showed more pro-inflammatory response in the APP^{swe}+PS1^{dE9} mice as compared to control mice, with robust secretion of IL-17a and IFN- γ (figure 5). We also studied the FOXP3 labeled T-cells and found them to elevated, though not significantly (figure 5a). These FOXP3 labeled T-cells represents T-regulatory cells that mediate anti-inflammatory response in the CNS by producing anti-inflammatory cytokine IL-10. Role of T-cells in AD has been a dichotomous and controversial one, associated with both neuroprotection as well as

neurotoxicity (Town et al., 2005). Early as well as recent reports indicate the T-cell regulated inflammatory response is involved in either pathophysiology, progression or both of the most common dementia related neurodegenerative disorder (Togo et al., 2002; Panossian et al., 2003; Magnus et al., 2005). The brain, which used to be regarded as a immune privileged organ, has now shown evidence of T-cell infiltration and cytotoxic responses by cytotoxic T-cell lymphocytes (CTLs), reflecting immune preference as a function of immune response regulation at other cellular levels. Most important of these regulatory levels of brain is the usually impermeable blood brain barrier (BBB). However, recent investigations have depicted BBB permeability to activated T-cells, which increases dramatically during systemic infection or other neurodegenerative diseases (Ransohoff et al., 2003). Moreover, the cytokines and other secreted cellular products are also shown to orchestrate the neuronal immune response by modulating the neuronal cell viability or BBB permeability, examples of those would be cytokines such as IL-1, IL-2 and IL-17a (Huppert et al., 2010). Our results are in accordance with other studies, which provide evidence for T-cell infiltration and activation in the AD pathology. In the current study with our animal model, at an age of 10 months, the presence of T-helper cells in their activated state that secretes elevated IL-17a and IFN- γ represents augmented T-cell mediated immune response. This increased T-cell mediated cytotoxicity at an adolescent age implies the pathophysiological role of inflammation in progressing AD and its behavioral symptoms. A recent review of role of immune signaling in neuronal development, synaptic plasticity has emphasized the aberrant role of inflammation in neuro-developmental and neurodegenerative disorders (Boulangier and Shatz, 2004). A recent study demonstrated that CD40 deficiency induced by gene deletion in the APP and APP^{swe}+PS1 transgenic mice attenuates the inflammation response in these transgenic mice, indicating the importance of CD40 signaling in

inducing the full repertoire of AD-like pathology (Todd Roach et al., 2004; Laporte et al., 2006). Obregon et al., supported these observation by demonstrating that genetic or pharmacologic disruption of CD40-CD40L interaction enhanced A β 1–42 immunization efficacy and reduced cerebral amyloidosis in the APP^{swe}+PS1 and Tg2576 mouse models of AD (Obregon et al., 2008). Broader implications of the inflammation in AD is reported by other studies, which depicts the detrimental effects of heightened immune response such as increased cytokine release and increased ROS formation on synaptic transmission and different forms of synaptic plasticity, eventually impairment of learning and memory formation. For instance, increased ROS formation is reported to attenuate LTP formation in hippocampal slices of mice (Haus-Wegrzyniak et al., 2002).

Microglia activation and T-cell induced cytotoxic inflammatory response is interceded by augmented secretion and release of pro-inflammatory cytokines that induces several cellular pathways leading to heightened ROS formation and cytotoxicity. In order to validate the microglia and T-cell mediated immune response, we assessed the levels of different cytokines in our AD animal model through ELISA as well as Multiplex analysis. To our expectations, we found a potent increase in pro-inflammatory cytokines in both cortex as well as the whole brain lysate of the double transgenic animals used in the current study. Among pro-inflammatory cytokines, IL-17, IL-1b, and IL-2 were found to be significantly elevated in cortical regions along with marked decreased anti-inflammatory IL-10 cytokines levels in APP^{swe}+PS1dE9 mice as compared to control mice. Furthermore, potent chemokines such as IP-10, MIG and Eotaxin were also found to be elevated significantly in the cortex of transgenic mice in comparison to control mice. IFN- γ and IL-17a levels were also found to be elevated in whole brain lysate through ELISA analysis, which further confirmed our results obtained through

multiplex. As a general rule, any alteration in protein expression follows altered genetic transcription. Thus, we also assessed the mRNA expression level of two major pro-inflammatory cytokines such as TNF- α and IL-1 β in the hippocampus and cortex and found them to be significantly elevated in both regions of the brain. Increased TNF- α protein expression has been shown to tightly tie with inflammatory response in AD (Fillit et al., 1991; Blasko et al., 1999a; Perry et al., 2001). Recent report has demonstrated that TNF- α and IFN- γ favors amyloid beta deposition by inducing A β peptides production and decreasing the APPs secretion and thus shifting the balance towards augmented A β deposition and plaque formation (Blasko et al., 1999a). Moreover, these cytokines stimulates production and release of prostaglandins that may mediates the neurotoxic actions of these cytokines in the AD brain (Prasad et al., 1998). Our results also support these reports as we found marked increase in the activity of COX enzyme, which is key mediator in formation of prostaglandins. Thus, our results as well as other reports suggest strong inflammatory response, mediated by microglia and T-cell interaction in the etiology, pathogenesis and progression of AD.

APPswe+PS1dE9 mice exhibit significant synaptic deficits:

Synaptic deficits along with glutamatergic anomalies are characteristic features of AD pathology. To identify the synaptic deficits in our transgenic mouse model of AD, we assessed LTP, a well known form of synaptic plasticity in the hippocampal slices of these organisms and compared it with age matched control mice. There was a significant deficit in the induction as well as maintenance of LTP in the transgenic animal model in our study, implicating impaired synaptic plasticity as compared to control mice. In addition, we also found decreased mRNA for NR2b receptors, NMDA subtype of glutamatergic ion channel receptors, in the hippocampus with no change in cortex. On the other hand, NR2a receptors remain unchanged in both regions

as compared to control mice, indicating an increase in the total NR2a/NR2b ratio. However, we did not find any significant change in the protein expression of NR2b receptors in both regions, though there was a slight decrease in NR2b in hippocampus but was not significant. Increased NR2a/NR2b receptor ratio has been linked to decreased synaptic strength by several investigators. NR2b receptors are predominately expressed and functionally are pro-active in developmental stages and as a person reach adolescence they are replaced on the synapses by NR2a receptors. In synapses these NR2b subtype of receptors mediate Ca^{2+} influx that results in depolarization of neuronal cells indicating strong synaptic transmission (Cao et al., 2007) and thus facilitates LTP formation. Recent studies established the crucial role played by NR2b receptors in regulating LTP rather than LTD, which was initially considered to be major role of NR2b in hippocampus. Moreover, rats over-expressing NR2b outperformed control rats in a series of working memory tasks (Cui et al., 2011) and also maintain the superior learning and memory in these ageing transgenic NR2b over-expressing mice (Cao et al., 2007). To identify any alterations in the molecular signaling pathway that is strongly linked to LTP and glutamatergic receptor expression and translocation. We found significant decrease in the phosphorylated state of hippocampal CREB and ERK1/2 MAPkinase, both of which have been shown to activate transcription factors involved in learning and memory. Also, we found a decrease in hippocampal p-70S6 kinase phosphorylation in APP^{swe}+PS1^{dE9} mice, which is a serine/threonine kinase that acts downstream of PIP3/mTOR/Akt pathway and targets the S6 ribosomal protein eventually inducing protein synthesis at the ribosome. The p70S6Kinse pathway in neurons is a critical component of the late phase of LTP by inducing the protein synthesis much required to maintain LTP and is activated by glutamatergic stimulation in a calcium/calmodulin-dependent fashion through a calcium pool controlled by postsynaptic

voltage-dependent calcium channels. Thus, a decrease in the phosphorylation of p70S6kinase implicates impairment of protein synthesis machinery involved in learning and memory in hippocampus, which has already been shown to occur in the cortical areas of APP/PS1 mice (Lafay-Chebassier et al., 2005). Hence, synaptic deficits were very prominent in bigenic transgenic mice which proportionate very well with behavioral deficits.

Synaptic deficits and chronic inflammation: Interestingly, inflammation in the areas controlling higher cognitive functions has both direct and indirect effects on those functions. Role played by inflammation in AD is so important that it led to formulation of an inflammatory hypothesis that states involvement of immune mediated responses in pathogenesis and progression of AD (Akiyama et al., 2000b; Inflammation: a unifying theory of disease? Research is showing that chronic inflammation may be the common factor in many diseases, 2006). Dilapidation in synaptic plasticity, LTP formation and decrease in glutamatergic NR2b receptors in hippocampus were reported in a recent study examining the effect of peripheral and central inflammation on learning and memory (Di Filippo et al., 2013). The same study established the elevated levels of IL-1b playing a pathophysiological role in manifesting these effects. Moreover, the intracellular protein kinases and transcriptional factors have also been shown to be altered by cytokines such as IL-6 (Tancredi et al., 2000) and IL-1b (Imamura et al., 2011). For instance, substantial inhibition of short and long term memory was accompanied by a simulation of STAT3 tyrosine phosphorylation and an inhibition of MAPK/ERK dual phosphorylation in IL-6 treated hippocampal slices (Tancredi et al., 2000). Along the same lines, in our study the synaptic deficits and behavioral anomalies in bigenic APP^{swe}+PS1^{dE9} mice model of AD were accompanied by robust pro-inflammatory cytokine release in hippocampus and cortical areas, increased mRNA expression of both IL-1b and TNF-alpha in hippocampus and cortex.

Moreover, we also observed significant increase in activated T-cell infiltration in the brain of bigenic mice, especially Thelper-17 cells that have recently been strongly linked to CNS autoimmune disorders. Although, the current study lacks to establish the causative function of inflammation and Thelper-17 cells in mediating the synaptic and behavioral deficits in higher cognitive areas of brain, yet it provides the correlative evidence. Additionally, the overall increase in Thelper-17 cells and its associated cytokine in the brains of these transgenic mice has been demonstrated for the first time and implicates impregnably the involvement of these cells in progression of AD as they are known to secrete pro-inflammatory cytokines that can weaken the BBB, induce maturation and differentiation of Thelper cells (Huppert et al., 2010). Viewing in context of synaptic deficits induced by cytokines such as IL-1b, IL-6, TNF-alpha, and NF-kappa B, the current study established elevated levels of these cytokines by utilizing different techniques and thus, demonstrated that the synaptic deficits seen in APPswe+PS1dE9 may be mediated by the up-regulation of these pro-inflammatory cytokines together with the increased amyloid-beta deposition. Furthermore, inflammatory cytokines augments the APP processing leading to enhanced A β secretion and plaque deposition, further corroborates the indirect effect of immune mediated responses in progressing AD pathogenesis.

Conclusion: Results obtained from the current study conclusively indicates the infiltration and involvement of T-cell, especially T-helper cells in the CNS of APPswee+PS1dE9 mice in comparison to age matched control mice. Also, for the first time, through ELISA as well as flow cytometry, role of T-helper17 cells have been implicated in Alzheimer's disease. Furthermore, impairment in LTP, an underlying physiological process of memory formation, was observed in the bigenic mutated mice model of AD. Conclusively, it could reasonably be stated that AD has a

prominent chronic immune mediated pathogenetic component, which commensurate very well with the behavioral and physiological cognitive deficits exhibited in AD.

4.5. References

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5. Anti-inflammatory effects of *Centella asiatica* extract alleviates behavioral and synaptic deficits in a transgenic mice model of Alzheimer's disease

Abstract

Alzheimer's disease (AD) is the most common type of age related dementia that critically affects the quality of life. Inflammation holds a central position in the pathophysiology of AD and eventually, leads to Amyloid-beta ($A\beta$) deposition and progression of disease. *Centella asiatica* is a natural herbal drug that is traditionally used as a brain tonic to cure cognitive disorders. In our previous study, we have shown this drug to decrease $A\beta$ content in the brain of double transgenic mouse model of AD. However, the effectiveness and molecular mechanism of *Centella asiatica* extract (CaE) against AD associated cognitive deficits is unknown. Therefore, the current study aimed to elucidate the effectiveness as well as elaborate the molecular mechanism of 8 months treatment of CaE in bigenic APP^{swe}+PS1^{dE9} mouse model of AD. Learning and memory related deficits were assessed through battery of behavioral tests including Y-maze and object recognition test. Synaptic plasticity in hippocampus region was evaluated via electrophysiology and determination of different synaptic proteins associated with synaptic plasticity. Flow cytometry was used to investigate the effect of CaE on the T-cell activation and frequency. ELISA was utilized to evaluate the cytokines levels. Results from the current study showed significant improvement in the cognitive task after CaE administration for 8 months. LTP also improved in CaE treated group along with the other synaptic markers. There was significant amelioration of pro-inflammatory markers in the hippocampal and cortical regions of the brain of transgenic mice. We also found a dramatic attenuation in the frequency of T helper-1 and T helper-17 types which also correlated with significant decrease in the IFN- γ

and IL-17a levels. Concordantly, we found effective decline in the oxidative stress associated with A β and inflammatory induced ROS generation in the CNS of bigenic mouse model of AD. The results from the current study illustrate the efficiency of CaE in ameliorating cognitive deficits associated with AD. Furthermore, the anti-inflammatory action of CaE correlates strongly with the synaptic strengthening and antioxidant effects of CaE against transgenic animal model of AD.

5.1. Introduction

Age related dementia manifests itself in some cases as a fatal, progressive neurodegenerative cognitive disorder known as Alzheimer's disease (AD). AD is characterized by extracellular β -amyloid (A β) plaques (known as senile plaques, SPs) and intracellular neurofibrillary tangles (NFTs) together with severe atrophy in the cortical and hippocampal regions of brain (Cummings et al., 1998; Chu, 2012). These pathogenic hallmarks co-localize with activated microglia, astrocytes and inflammatory proteins and surround the dystrophic neurites leading to neuronal cell death (Akiyama et al., 2000b). Sequential cleavage of the membrane associated amyloid precursor protein (APP) by two different enzymes, β - and γ -secretase results in formation of A β , which likely is the pathogenic culprit of AD (Cummings et al., 1998). Toxic gain of function of beta-convoluted sheets of amyloid peptide includes generation of reactive oxygen species, lipid peroxidation, induction of neuro-inflammatory cascade, triggering of apoptotic stimuli and initiation of synaptic deficits, together which all sums up to neuronal injury and severe atrophy (Lue et al., 1996; Eikelenboom et al., 2010). The last two decades had witnessed numerous studies investigating therapeutic interventions targeting A β and expecting dramatic recovery in the experimental models of AD (Chu, 2012).

However, limited success has been obtained employing this approach. Recent studies implicate multiple factors orchestrating the pathogenesis and progression of AD, in a symbiotic fashion (Chu, 2012). Thus, a change of current single targeted approach to multi-targeted approach is urgently required to treat or prevent this highly morbid neurological age related disorder.

A β is known to initiate in the CNS numerous pathways that lead to neurodegeneration and progression of AD. In addition to acting as a toxic compound itself, it prominently stimulates the immune system of CNS resulting in building up of inflammation mediated response as a function of time (Lue et al., 1996). There are anecdotal evidences suggesting the early pathogenic role of inflammation in AD (Gupta and Pansari, 2003). Also, the inflammatory mediators have been shown to have positive modulatory effect on the secretion and deposition of A β through augmentation of APP processing in the neuronal cells (Blasko et al., 1999b). Thus, chronic inflammation, which itself is capable of self propagation acts as an indirect source of the main pathogenic agent, A β . Furthermore, inflammation as well as A β is well capable of inducing the ROS generation through disruption of various pathways and leads to oxidation of intracellular macromolecules including membrane lipids, proteins and receptors (Hu et al., 1998; Butterfield et al., 2001). Disruption of mitochondrial energy balance is another manifestation of one of the toxic effects of A β as well as ROS, which leads to further boosting up of ROS generation and cytotoxicity (Butterfield et al., 2001). To complicate the matters, mitochondrial potential imbalance leads to activation and release of apoptotic stimuli resulting in neuronal cell apoptosis, which is also supported by inflammation and ROS species. Moreover, A β indirectly by disrupting the synaptic glutamate level as well as directly acts on NMDA subtypes of glutamatergic receptors, which results in neuronal excitotoxicity due to excessive calcium influx (Parameshwaran et al., 2008; Ferreira et al., 2012). Keeping in view the above stated facts, we

hypothesize that by inhibiting various pathways by employing either single or cocktail of therapeutic interventions; AD could be successfully controlled or treated.

An herbal extract is considered to be nature's own handpicked cocktail of various bioactive compounds capable of affecting various physiologic pathways in synergetic and/ or antagonistic manner. Ancient medical scriptures of Indian and Chinese cultures reports various such therapeutically active herbal compounds effectively capable of treating various ailments of current human society. *Centella asiatica* is one of those well known ancient medical treasure that has found use in Ayurvedic and traditional Chinese medicine. *Centella asiatica* (Umbelliferae), an herbaceous creeping plant indigenous to almost all over the world including countries like China, Malaysia, VietNam, India, and southeastern United States. In India, the plant is found throughout the country and is commonly known as Brahmi in Sanskrit. Ayurvedic literature has prescribed its usefulness in treating various ailments including wound healing, albinism, anemia, asthma, bronchitis, cellulite, cholera, measles, constipation, and as an antipyretic, analgesic, anti-inflammatory, and "brain tonic" agent (Appa Rao et al., 1973). One of the most popular exploitation of *Centella asiatica* is its use as "Medhya Rasayan", a term used to describe agents that helps to improve memory and the power to recall facts from the brain. Recently, pharmacological studies have demonstrated beneficial therapeutic effect of the Titrated Extract of *Centella asiatica* (TECA) in wound healing, microangiopathy, and gastric ulcers (Shukla et al., 1999; Cesarone et al., 2001a; Cesarone et al., 2001b; De Sanctis et al., 2001; Incandela et al., 2001; Paocharoen, 2010; Belcaro et al., 2011). Over the past few decades, investigational studies has shown the herb to possess antitumor activity (Babu et al., 1995), anti-inflammatory (Punturee et al., 2005), CNS depressant activity (Sakina and Dandiya, 1990), anti-apoptotic (Xu et al.,

2012), antioxidant properties and neuroprotective activity (Dhanasekaran et al., 2009; Krishnamurthy et al., 2009).

Animal studies exhibits the outstanding performances of *Centella asiatica* treated animals in various memory tasks such as active and passive avoidance, object recognition, and water maze. Clinical studies performed with healthy elderly volunteers have shown positive modulation of cognition and mood along with improvement in physical performance and health-related quality of life (Wattanathorn et al., 2008; Mato et al., 2011). Moreover, in case of mentally retarded children it is reported to improve general mental ability (Appa Rao et al., 1973). Though, there is great deal of clinical as well as animal experimental evidence, but there is still paucity of studies shedding light on the specific mechanism of *Centella asiatica* with respect to the most common form of dementia i.e. AD. Hence, the current study aimed to establish the therapeutic potential of *Centella asiatica* extract (CaE) as well as decipher the underlying cellular mechanisms of action of CaE against AD in a very well established double transgenic AD mice model. Results from the current study, indeed establish the significant neuroprotective, cognition enhancing effects of CaE, which attribute to its modulatory effects on various inflammatory, apoptotic, oxidative and synaptic plasticity pathways. Thus, CaE acts as a cocktail of therapeutic compounds capable of relieving amyloid beta deposition and improving cognitive function of brain.

Transgenic mice currently represents almost the most widely used animal models for various disorders, and in case of neurological disorders, they are especially the investigator's preferences to analyze a specific aim. Though, they have considerable disadvantageous, their use in AD for understanding the pathophysiology of the disease and screening of novel therapeutic effective compounds is not uncommon. The current study also employed double transgenic mice

harboring familial AD linked Swedish mutant human APP (APP^{swe}) and mutant human presenilin (PS1-dE9) gene. Commonly referred as APP^{swe}+PS1dE9 mice, they display senile plaques (SPs) in hippocampus and cortex at six to seven months along with spatial learning deficit at seven months of age, (Savonenko et al., 2005; Garcia-Alloza et al., 2006). The amyloid beta plaques, in these mice, accumulate in the regions of the brain largely resembling the pattern of accumulation of senile plaques in human AD (Garcia-Alloza et al., 2006; Kim et al., 2012). At nine months of age they exhibit dystrophic neurites with very subtle neuronal cell loss along with relatively milder cerebral amyloid angiopathy (CAA) (Garcia-Alloza et al., 2006; Bonardi et al., 2011; Darvesh et al., 2012). Recent investigations have revealed profound inflammation at early and later stages of this animal model, which especially included robust microglia activation and clustering surrounding senile plaques (Garcia-Alloza et al., 2006) and elevated hippocampal levels of GFAP and GFAP-positive astrocytes (Ruan et al., 2009). A study by Wirz and colleagues (Wirz et al., 2012), demonstrated striking resemblance in the alteration of three inflammatory related genes in human and APP^{swe}+PS1dE9 associated AD at early and later ages of this bigenic mutated mice. The results from above mentioned study posit the usability of APP^{swe}+PS1dE9 model in studying the immune mediated response in pathology of AD. Moreover, in agreement to human AD pathophysiology, this mice model also presents higher oxidative stress and cholinergic dysfunction (Zhang et al., 2012). In a nutshell, the APP^{swe}+PS1dE9 mice model effectively represents the behavioral deficits and the underpinning pathophysiological alterations associated with chronic neurodegenerative cognitive disorder AD and hence can be used for the screening of novel therapeutic compounds.

In the current study we investigated the therapeutic potential of the concentrated herbal extract of *Centella asiatica* (CaE) against the amyloid beta induced myriad of pathophysiological

alterations leading to cognitive deficits. We expect CaE, assigning to its anti-oxidative, anti-apoptotic and anti-inflammatory effects, to prevent APP^{swe}+PS1^{dE9} mice against the pathophysiological alterations. Also, ascribing to its direct effect on synaptic strengthening, CaE is expected to ameliorate the impaired LTP formation in these mice most probably by activation of the transcriptional factors related with LTP formation and modulating the glutamatergic transmission in the hippocampus.

5.2. Materials and Methods:

Animals and Treatment groups: Double transgenic B6.Cg-Tg (APP^{swe}, PSEN1^{dE9}) 85Dbo/J (APP^{swe}+PS1) male mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and were housed with a 12 h day/night light cycle with free access to food and water. All animals were 2 months old with a body weight of 20-25 g. The double transgenic APP^{swe}+PS1 mice were generated by mating Tg2576 mouse line carrying the “Swedish” APP mutation (APP_{K6070NM671L}) (Hsiao et al., 1996b) with a mutant presenilin-1 mouse line (PS-1_{M146L6.2}) (Duff et al., 1996).

APP^{swe}+PS1 mice were randomly divided, based on their body weight, into three treatment groups. Three groups included 1) Transgenic control group; 2) transgenic low dose CaE treated group and 3) transgenic high dose CaE treated group. *Centella asiatica* powdered extract was uniformly mixed with powdered chow. The low dose group received 2.5 g/kg body weight of CaE and the high dose group received 5 g/kg body weight. Drug doses were calculated after estimating the daily feed intake of the each group. All animals were kept on powdered chow diet for 6 months. After completion of 6 months animals were subjected to battery of behavioral tests before sacrificing.

All procedures involving animals were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

Behavioral Assessment: As a part of general behavioral assessment body weights of the animals were recorded in an interval of one month by using digital weighing scale. Animals were also observed for general signs of toxicity and hyperactivity. In order to assess cognitive performance animal were subjected to Y-maze and object recognition tests to assess the spatial and recognition memory in the double transgenic mice at 10 months of age.

Y-maze Test: The Y-maze task is used to determine an index of spatial working memory. It is performed as explained in Chapter 4 section 4.2.

Object Recognition Test: Performed as described in Chapter 4 section 4.2.

Biochemical analysis:

Tissue Preparation for biochemical assays: Animals from each group were sacrificed by decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes, and other antioxidant molecules. Homogenate of the brain samples were prepared by homogenizing the brain tissue in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon homogenizer. Tissue homogenates were centrifuged at 10,000 g for 60 min at 4°C and the supernatant were collected.

Protein estimation: Protein was assayed using the Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL) as per the instructional manual of the kit. Explained in Chapter 4 section 4.2

Beta-Secretase activity: Please refer chapter 4 section 4.2 for methodology.

Determination of Total β -amyloid content: Please refer chapter 4 section 4.2 for methodology.

Mitochondrial P2 fraction preparation: Please refer chapter 4 section 4.2 for methodology.

Assay of Super oxide dismutase (SOD) activity: Please refer chapter 4 section 4.2 for methodology.

Estimation of Catalase: Please refer chapter 4 section 4.2 for methodology.

Glutathione (GSH) estimation: Please refer chapter 4 section 4.2 for methodology.

Determination of ROS generation: Please refer chapter 4 section 4.2 for methodology.

Estimation of lipid peroxidation: Please refer chapter 4 section 4.2 for methodology.

Electrochemical detection of cortical Glutamate and gamma-amino butyric acid (GABA) levels:

Tissue preparation: Please refer chapter 4 section 4.2 for methodology.

Measurement of Glutamate and GABA: Please refer chapter 4 section 4.2 for methodology.

HPLC Equipment: Please refer chapter 4 section 4.2 for description of HPLC instrument.

Derivitization protocol and HPLC procedure: Please refer chapter 4 section 4.2 for methodology.

Estimation of hippocampal and cortical inflammation:

Multiplex analysis of hippocampal and cortical cytokines: Please refer chapter 4 section 4.2 for methodology.

ELISA determination of levels Inflammatory cytokines: Please refer chapter 4 section 4.2 for methodology.

Isolation of brain leukocytes and Flow-cytometric determination of activated T-cells: Please refer chapter 4 section 4.2.7.3 for methodology.

Quantitative PCR analysis of TNF-alpha and IL-1b: Please refer chapter 4 section 4.2 for methodology.

Assessment of mediators of synaptic strength:

LTP assessment of hippocampal slices: Please refer chapter 4 section 4.2 for methodology of slice preparation and extracellular field recording.

Western blot for glutamatergic NMDA receptors: Please refer chapter 4 section 4.2 for methodology.

Multiplex analysis Multi-Pathway Signaling Phosphoprotein analysis: Please refer chapter 4 section 4.2 for methodology.

Statistical analysis: Statistics was performed using the Prism-V software and results were expressed as mean \pm SE. Column statistics was run along with Kolmogorov–Smirnov test to check for the normal distribution of the data. Experimental data that pass the normal distribution test was subjected to one way ANOVA followed by Tukey test for the comparison between different treatment groups. Failing the normality test, non-parametric Kruskal –Wallis test was performed on the experimental data set, which was followed by the Dunn’s multiple comparison post-hoc analysis to test for statistical significance among different treatment groups. Differences were considered significant at $p < 0.05$.

5.3. Results

CaE ameliorated behavioral deficits in APP^{swe}+PS1 mice: All animals from each group were tested for general behavioral signs of toxicity. None of the treatment groups exhibited any significant change in the body weight (Fig. 5.1A, n=10). Also, we did not find in any of the treatment groups any toxicological signs or symptoms including excessive salivation, tremors, straub tail, piloerection etc.

Clinically, AD represents itself majorly as an age related cognitive disorder that affects centrally memory along with other cognitive domains of brain including executive functioning, language, visuospatial functioning, attention and affect (Gold and Budson, 2008). Episodic memory is the most earliest and most prominent of the memories that are impaired in AD patients along with semantic memories, which lies under a broader classification into declarative

type of memory. On the same lines, bigenic APP^{swe}+PS1^{dE9} mice exhibits profound deficiency in declarative type of memories related to visuospatial assessment and object recognition. In order to assess cognitive enhancement in the double transgenic APP^{swe}+Ps1^{dE9} mice, we analyzed all groups of animals in these two well known memory related tasks. We evaluated the spatial memory of all animals in the different treatment groups by employing Y-maze task. During the learning trial, subjects from each group did not show any significant difference in exploration behavior. When subjected to retention trial after 1 hour, the 10 month old APP^{swe}+PS1 mice exhibited significant reduction in number of entries into novel arm, which recovered significantly due to the CaE treatment for 8 months (Fig. 5.1B, n=6, p< 0.05). Both low and high dose CaE treatment thus exhibited improvement in the already impaired spatial memory in APP^{swe}+PS1^{dE9} mice. Another outcome from the Y-maze trial, the alternation score was found not to differ significantly in both groups (Control 55.1±13.8, APP^{swe}+PS1 52.8±2.9, CaE 2.5 g/kg 54.8±1.9 and CaE 5 g/kg 58.1±10.8; n=6), indicating no change in the working memory in the transgenic animals.

Similarly, CaE treatment at both lower and high doses for 8 months significantly ameliorated the profound decline in the performance of 10 month old transgenic mice in the object recognition task. Recognition index in both CaE 2.5g/kg as well as CaE 5g/kg group improved profoundly as compared to bigenic APP^{swe}+Ps1^{dE9} group (Fig. 5.1C, n=6 , p< 0.05). Thus, CaE treatment for 8 months profoundly improved the declarative component of Y-maze as well as object recognition task in transgenic model of AD.

CaE treatment diminishes A β load in APP^{swe}+PS1 mice: Amyloid-beta peptide holds central position in the pathophysiology of AD (Farlow, 1998). The total amyloid load dramatically increases in AD brain especially in hippocampal and cortical areas of the forebrain. In our

current study, we measured the total amyloid content of both A β 40 and A β 42 as the guanidine soluble fractions, which most closely reflect A β deposits detected by immunohistochemistry. In our previous study we did observe an increase in the total amyloid content of both A β 40 and A β 42 in the hippocampal and cortical areas of the 10 month old APP^{swe}+PS1dE9 mice. Similarly, in the current study, we found significant increase in the hippocampal as well as cortical A β 40 and A β 42 content (Fig. 5.2A, n=6, p< 0.005). CaE treatment for 8 months at low and high doses effectively attenuated the hippocampal increase in A β 42 content as compared to transgenic group (Fig. 5.2A, n=6, p<0.05). The cortical A β 42 levels were found to be remarkably reduced by CaE 5g/kg, though the A β 42 levels were still significantly higher than the non-transgenic control. We also reported significant decrease in the cortical and hippocampal A β 40 levels, though the effect was not dose dependent as only CaE 5g/kg was effective in controlling the A β 40 levels in the cortex. Moreover, trend of reduction in A β 40 content followed A β 42 as the levels were still higher than the non-transgenic control group. To account for this observation, two possibilities explanations could be suggested. First, the CaE is modulating the A β 40 and A β 42 levels indirectly by modulating the other pathological factors known to exacerbate APP conversion to A β 40 and A β 42 species. Secondly, being 10 month old transgenic mice, the A β production in these adolescent animals would be much higher than the age-matched non-transgenic control mice and the significant decrease exhibited by CaE is of paramount clinical importance.

CaE treatment resulted in reduction in β -Secretase activity: The β -amyloid is formed as a result of the proteolytic processing of β -APP (β -amyloid precursor protein), which involves sequential cleavage by various proteases named α -, β -, and γ -secretase (Cummings et al., 1998). β -, and γ -secretase are involved in amyloidogenic processing of β -APP and α -secretase results in non-

amyloidogenic processing. In our earlier chapter, we reported the significant increase in the β -secretase activity of APP^{swe}+PS1^{dE9} mice model that correlate very well with increased A β 40 and A β 42 levels. In the current study, as expected we did find significant increase in the activity of β -secretase enzyme, which was significantly alleviated by CaE 5g/kg treatment (Fig. 5.2B, n=6, p<0.05). The resultant change in the β -secretase activity possibly reflects the overall change in the β -secretase protein expression as a result of feedback signaling by A β species and other known mediators induced by A β . Previous studies have shown A β and other inflammatory mediators such as TNF- α and IFN- γ to regulate the expression of β -secretase enzyme through feedback mechanism and thus, eventually facilitate the A β plaque deposition (Yamamoto et al., 2007; Faghihi et al., 2008). Moreover, a recent study has reported the *Centella* extract major constituent, Asiaticoside to beneficially modulate the expression of different cellular targets of APP processing pathway that included α - and β -secretase. They have shown asiaticoside to dose dependently decrease the β -secretase expression and increase the α -secretase protein expression in the rat cortical neurons (Patil et al., 2010). The mechanism of modulation of synergistic action of CaE on different targets was reported to be unclear. Consistently, in our study we have confirmed the previous report of asiaticoside modulating β -secretase expression by exhibiting a significant decrease in the β -secretase activity by employing a CaE extract that comprises 20% asiaticoside content. Thus, our study suggests that one of the mechanisms by which CaE attenuated A β levels would be the down-regulation of β -secretase expression.

Amelioration of pro-inflammatory immune mediators led by CaE treatment: Inflammation is known to appear as one of the earliest to appear in the affected areas of the brain of AD patients (Eikelenboom et al., 2010) and corroborates prominently with extracellular amyloid plaques (Senile plaques, SPs) and intracellular neurofibrillary tangles (NFTs) (Lue et al., 1996). A body

of evidence has shown A β to induce earlier mediators of innate immune response by activating complement pathway and acting as a host-derived danger signals (danger associated molecular patterns, DAMPs) which is recognized by first line of innate immune cells in the CNS such as microglia (Rubio-Perez and Morillas-Ruiz, 2012). Though, there are various confusing reports about the type of inflammatory and anti-inflammatory cytokines profile found in the CNS as well as plasma of AD patients, but the common consensus about IL-1 β , IFN- γ and TNF- α is unquestionable and consistent (O'Barr and Cooper, 2000; Ruan et al., 2009; Rubio-Perez and Morillas-Ruiz, 2012). Moreover, in our earlier study we reported significant elevation in the inflammatory cytokines such as IL-1 β , IFN- γ and IL-17 in the CNS of APP^{swe}+PS1^{dE9} mice, which has also been shown by other investigators (Ruan et al., 2009; Browne et al., 2013). Based on the above evidence, it can be reasonably suggested that inflammation plays a central role in pathophysiology and progression of AD. Being a potent anti-inflammatory herbal drug, we evaluated the effect of CaE on the immune mediated response in the APP^{swe}+PS1^{dE9} bigenic mouse model of AD. In the current study, concordant to our previous results there was marked elevation in the IL-1b protein as well as mRNA expression in both cortex and hippocampus of double transgenic APP^{swe}+PS1^{dE9} group (Fig. 5.3A, B, C, and D, n=5-6, p<0.05). CaE treatment (5 g/kg for 8 months) exhibited remarkable attenuation of augmented IL-1b protein expression in both cortical and hippocampal regions of the treated APP^{swe}+PS1^{dE9} mice (Fig. 5.3A and B; n=6, p<0.05). Reduction in IL-1b protein expression by CaE 5g/kg dose was well in proportion with the significant reduction in the mRNA expression of IL-1b in the cortex and hippocampus as compared to transgenic APP^{swe}+PS1^{dE9} group (Fig. 5.3C and D; n=5, p<0.05). In case of lower dose CaE treatment though there was a slight decrease in the both protein as well as mRNA expression of IL-1b, the decrease was not significant except in the cortical IL-1b

protein expression (Fig. 5.3B, n=6, p<0.05). Previous reports support our findings of CaE treatment to suppress IL-1b expression eventually acting as a potent anti-inflammatory herbal drug (Hartog et al., 2009).

We also evaluated the TNF- α mRNA expression in the hippocampus and cortical regions of all treatment groups. To our expectation, there was significant increase (almost 2 fold) in the TNF- α mRNA expression in the double transgenic APP^{swe}+Ps1^{dE9} mice group as compared to non-transgenic control group (Fig. 5.3E and F, n=5, p<0.05). CaE treatment at 5 g/kg dose significantly counteracted the increase in TNF- α mRNA levels in the cortical regions (Fig. 5.3F, n=5, p<0.05), which was a 2 fold decrease in TNF- α levels. Although, the CaE treatment in the hippocampus resulted in a decrease in the TNF- α level, the effect was not significant. Also, CaE at lower doses i.e. 2.5 g/kg was not able to effectively attenuate the increase in the TNF- α mRNA expression in both cortical and hippocampal regions of the brain. Our results are in line with the previous studies reporting the effective anti-inflammatory effect of CaE exhibited by inhibiting the pro-inflammatory markers such as TNF-alpha, IL-2 and IL-1b (Punturee et al., 2005).

Great deal of evidence indicates the involvement of INF- γ and associated cytokines such as IL-17a in double transgenic animal model of AD (Del Bo et al., 1995; Ruan et al., 2009) and have shown its coexistence with IL-1b. The inhibitory effect of CaE treatment on IL-1b mRNA and protein expression implicates the possible inhibition of other pro-inflammatory cytokines that are known to be the outcome of early central cytokine release i.e. IL-1b. In order to account for this hypothesis, we evaluated the effect of high dose treatment of CaE (i.e. CaE 5g/kg) on the IFN- γ and IL-17a levels present in the whole brain lysate through ELISA. In line with our expectations, we found significant up-regulation in the both INF- γ (Fig. 5.3H, n=6, p< 0.01) and

IL-17a (Fig. 5.3G, n=6, p<0.0001) levels in the APP^{swe}+PS1^{dE9} bigenic mouse model of AD. CaE at 5g/kg for 8 months prominently counteracted the increase in IL-17a (Fig. 5.3G, n=6, p<0.0001) and IFN- γ (Fig. 5.3H, n=6, p<0.05) levels in the whole brain lysate of treated double transgenic group. Our results, for the first time have shown a significant decrease in the potent pro-inflammatory mediators of adaptive immunity in the CNS. Thus, the above results indicates that CaE attributing to its potent anti-inflammatory effects blocks the initial steps of immune mediated response to β -amyloid in the CNS of the double transgenic mice and subsequently inhibits the well known inflammatory signaling cascade that results in prominent cell death and progression of synaptic deficits in the APP^{swe}+PS1^{dE9} bigenic mouse model of AD.

Reduced activation of T-helper cell associated response in APP^{swe}+PS1^{dE9} mouse model of

AD: Innate immune response against A β in CNS comprises robust microglia activation and recruitment in the vicinity of extracellular fibrillar A β plaques leading to release of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-18, TNF- α and IFN- γ (Eikelenboom et al., 2010). IL-1 β plays a key role in chronic neuroinflammation associated with AD and induces microglia, astrocytes and macrophages to release pro-inflammatory cytokines, which further induces release of inflammatory cytokines and chemokines resulting self perpetuating chronic neuroinflammation in the CNS. Our previous work with APP^{swe}+PS1^{dE9} and a recent study has shown robust activation and recruitment of T-helper cells in the CNS of these double transgenic mouse model as a result of microglia activation and IL-1 β and IFN- γ release (Browne et al., 2013). Furthermore, the study also reports the pathophysiological role of T-helper 1 type of leukocytes in progression of AD by facilitating amyloid plaque deposition and associated synaptic deficits. In order for CaE to effectively curb the neuro-inflammation and exert neuroprotective action in CNS of transgenic animal, we expect it to weaken the T-helper cell

induced neuroinflammation by decreasing their activation. Therefore, we extracted the T-helper cells, which express CD4 marker on their surface, from the brain of each animal and stimulated them with phorbol myristate acetate (PMA). PMA stimulation resulted in activation and exaggerated release of inherent cytokines from these T-cell fraction. Application of Golgi body stop chemical trapped these cytokines within these cells, which were then analyzed after employing respective antibodies in the Flow cytometry. We found a significant increase in the frequency of CD4+ cells expressing IFN- γ (Fig. 5.4C, n=5, p<0.05) and IL-17a (Fig. 5.4B, n=5, p<0.05) cytokines in APP^{swe}+PS1dE9 group, supporting our previous results. As anticipated, we observed significant decrease in the frequency of the IL-17a (Fig. 5.4B, n=5, p<0.05) expressing CD4+ as well as IFN- γ (Fig. 5.4C, n=5, p<0.05) expressing CD4+ cells in the CaE 5g/kg treated double transgenic group as compared to APP^{swe}+PS1dE9 group. Earlier during our evaluation of neuro-inflammation in CNS of APP^{swe}+PS1dE9 mouse model, we observed robust increase in the CD4 + Foxp3+ regulatory T cells, which release anti-inflammatory cytokine such as IL-10, to keep a check against the T-cell mediated pro-inflammatory response (Holaday et al., 1993). On the same lines, we found in the current study, significant increase in the frequency of CD4 + Foxp3+ T-reg cells in APP^{se}+PS1dE9 group (Fig. 5.4D, n=5, p<0.05) compared to non-transgenic control group. The dramatic increase in the CD4 + Foxp3+ T-reg cells is suggestive of the CNS response to fight against chronic inflammation, which is expected to further intensify with the CaE treatment. To our surprise, we found CaE 5g/kg to significantly suppress the frequency of these T-reg cells in the CNS of treated APP^{swe}+PS1dE9 bigenic mouse model. The resultant decrease in the CD4 + Foxp3+ T-reg cells population in the CNS of treated transgenic mouse is implicating the prevention of building of chronic inflammatory state as is the case in the untreated APP^{swe}+Ps1dE9 mouse. These observations implicates that by

modulating the inflammatory response, as shown by diminished pro-inflammatory cytokine profile in the CNS of APP^{swe}+PS1^{dE9} bigenic mouse, CaE exerted neuroprotective and cognition enhancing effect.

CaE ameliorates synaptic deficits associated with A β induced neurotoxicity in the CNS of APP^{swe}+PS1^{dE9} mice: Synaptic deficits correlate strongly with amyloid pathology and behavioral symptoms observed in AD patients (Akiyama et al., 2000b). In addition to β -amyloid, neuroinflammation especially the inflammatory cytokines such as IL-1 β , TNF- α and IL-6 exerts the most deleterious effects on the synaptic plasticity in the CNS (O'Connor and Coogan, 1999; Imamura et al., 2011). Inflammatory cytokines are known to deregulate the synaptic glutamate levels by disrupting the microglia governed strictly regulated glutamate levels (Zhao et al., 2012). In the current study we found significant increase in the cortical glutamate levels in the APP^{swe}+PS1^{dE9} mice compared to non-transgenic control mice (Fig. 5.5A, n=5, p<0.001). Increase in the glutamate levels is in consistence with the previous reports showing increased synaptic glutamate levels in the different cortical regions of the brain (Schallier et al., 2011). CaE treatment at lower dose 2.5 g/kg decreased glutamate levels to control values (Fig. 5.5B, n=5, p<0.001). CaE 5g/kg also decreased the glutamate levels but the decrease was not found to be significant. Similarly, we observed significant elevation in the levels of the prominent inhibitory neurotransmitter, GABA in the cortical regions of the APP^{swe}+PS1^{dE9} bigenic mice group compared to control group. The current result is in line with previous results where increased GABA levels as well as molecular markers of GABAergic system were found to be elevated in AD clinical as well as experimental animal models (Reinikainen et al., 1990; Schwab et al., 2012). CaE treatment both CaE 2.5 g/kg and CaE 5g/kg prevented the significant elevation in the glutamate and GABA levels in the APP^{swe}+PS1^{dE9} mice model of AD. Implications of this

results signifies the increased excitotoxicity as well as augmented inhibition in the CNS of experimental AD cases which associates with elevated inflammatory response. CaE efficiently counteracts the inflammatory response and thus, also prevents the associated perturbation in the excitatory and inhibitory neurotransmitter systems in the brain of double transgenic APP^{swe}+PS1^{dE9} mouse model of AD.

We evaluated synaptic plasticity in the hippocampus of the APP^{swe}+PS1^{dE9} mice model by analyzing long term potentiation, a physiological correlate of memory formation in the CNS (Izquierdo and Medina, 1995). Synaptic plasticity in acute slices from all was examined by inducing long-term potentiation with theta burst stimulation (TBS), in the Schaffer-collateral pathway of hippocampus. TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ; with interburst interval 200 msec and is found to be the most effective and the most physiologically relevant as it induces the inherent discharge characteristic of hippocampal neurons (Albensi et al., 2007). Results showed that LTP was significantly impaired in bigenic transgenic mice with an average of $134.6\% \pm 0.9774\%$, compared with control slices in which LTP was induced with an average of $195.2\% \pm 3.239\%$ (Fig. 5.5C, ; $p < 0.05$; $n = 6$ slices from 6 animals). CaE treatment at high dose for 8 months recovered impaired LTP partially to an average of $156.93\% \pm 4.53\%$ (Fig. 5.5D; $p < 0.05$, $n = 6$ slices from 6 animals). Reason for testing only high dose CaE in the LTP experiment was because of higher effectiveness of high dose in attenuating inflammatory response in APP^{swe}+PS1^{dE9} mice model. The molecular mechanism of LTP formation involves several protein kinases and one of the central protein kinases is the extracellular signal-regulated kinases mitogen-activated protein kinases 1/2 (ERK-MAPK1/2). Various protein kinases such as protein kinase A (PKA), Ca²⁺/calmodulin-dependent protein kinase (CAMKII) and ERK-MAPK1/2 are known to phosphorylate cAMP-response element-

binding protein (CREB) at Serine 133 residue so as to activate it and stimulate the gene transcription required for the protein synthesis and early response elements for early and late phase of LTP formation. Impaired synaptic plasticity in the Schaffer-collateral pathway is suggestive of the decreased phosphorylation of CREB and its upstream mediator. In the current study, we evaluated CREB and ERK 1/2 phosphorylation through multiplex analysis. We found a significant decrease in phosphorylation of ERK MAPK (Fig. 5.5E, n=6, p< 0.005) and CREB (Fig. 5.5F, n=6, p< 0.005) in the hippocampal and cortical areas of the brain of APP^{swe}+PS1^{dE9} mice model compared to wild type control group. CaE treatment at 5 g/kg significantly increased the phosphorylation of ERK 1/2 (Fig. 5.5E, n=6, p< 0.005) and CREB (Fig. 5.5F, n=6, p< 0.005) both in the hippocampus and cortex of the brain of APP^{swe}+PS1^{dE9} mice. The increase in the phosphorylation of ERK1/2 and CREB was profoundly higher in the CaE 5 g/kg group, though lower dose was unable to recover the declined phosphorylated ERK1/2 and CREB levels to control values both in hippocampus and cortex of the brain of APP^{swe}+PS1^{dE9} mice. This suggests that either by activating the kinases or by indirectly interacting with upstream receptors or signaling mediators CaE exerts mnemonic effects in the CNS. CREB activation is known to regulate the post-synaptic NMDA and AMPA subtypes of glutamatergic receptor surface expression by governing the expression of Brain-derived neurotrophic factor (BDNF) protein. Our previous *in-vitro* results have shown CaE capability to modulate the expression of glutamatergic receptors in rat hippocampal neuronal cell culture, H19-7 cells (Chapter 3, section 3.3.5). Therefore, we evaluated the expression of NMDA subtypes of receptors in the hippocampus of the brain of different treatment groups. We found an increase, though insignificant, in the expression of NR2a receptors in the hippocampus of APP^{swe}+PS1^{dE9} group compared to control non-transgenic group (Fig. 5.5G, n=6). CaE treatment at lower doses

i.e. at 2.5 g/kg resulted in significant decrease in the NR2a expression (Fig. 5.5G, $p < 0.05$, $n = 6$). In case of NR2b, there was trend of increase in the hippocampus of APP^{swe}+PS1dE9 group, which tend to decrease with increasing dose of CaE (Fig. 5.5H, $n = 5$). Although, these results are insignificant but the trend of effect of CaE treatment is maintained, which implies that CaE is affecting the glutamatergic receptor surface expression. The trend of increase in the NR2a and NR2b expression in the APP^{swe}+PS1dE9 mice signifies the deregulated expression of these receptors due to the altered synaptic glutamate levels that could lead to increased influx of Ca^{2+} ions and eventually results in excitotoxicity and neuronal cell death. Thus, it would be logical to assume that by decreasing the expression of NMDA subtypes of glutamatergic receptors, CaE protects the hippocampal neurons from $A\beta$ associated excitotoxicity and cell death (Ferreira et al., 2012). CaE by virtue of its potent anti-inflammatory action protects the CNS from synaptic deficits and cognitive deficits.

CaE ameliorated the inflammation associated oxidative stress: Inflammation is one of the secondary reactions of the β -amyloid toxicity. Another aspect of β -amyloid toxicity includes excessive generation of reactive oxygen species and imbalance of mechanisms regulating oxidative stress generation and removal (Nunomura et al., 2001). Inflammation itself can lead to generation of oxidative stress in the different immune reactive defensive cells. Different cytokines, chemokines and other immune mediated signaling molecules are capable of elevating excessive metabolism leading to ROS generation (Du Yan et al., 1997; Mohsenzadegan and Mirshafiey, 2012). In order to evaluate the effect of CaE treatment on the oxidative stress generation, we evaluated the ROS formation as well as the levels of different metabolic enzymes involved in ROS generation. There was significant increase in the ROS levels in the APP^{swe}+PS1dE9 mice group compared to control group (Fig. 5.6A, $n = 5$, $p < 0.005$), which was

efficiently attenuated by CaE treatment for 8 months by both CaE 2.5 g/kg (Fig. 5.6A, n=5, p<0.005) and CaE 5 g/kg (Fig. 5.6A, n=5, p<0.05) dose in APP^{swe}+PS1dE9 mice. In accordance with ROS level, there was significant elevation in lipid peroxide levels in APP^{swe}+PS1dE9 mice model (Fig. 5.6B, n=5, p<0.005), which was attenuated by CaE 2.5 g/kg (Fig. 5.6B, n=5, p< 0.005) as well as CaE 5g/kg (Fig. 5.6B, n=5, p< 0.001) treatment for 8 months in double transgenic APP^{swe}+PS1dE9 mice model of AD. We also assessed the glutathione levels in the brain and found it to be depleted in the APP^{swe}+PS1dE9 mice (Fig. 5.6C, n=5, p<0.05), which recovered with CaE 2.5g/kg dose treatment for 8 months (Fig. 5.6C, n=5, p<0.05). To test the underpinning metabolic enzymes affected by the CaE treatment to protect from the ROS stress, we evaluated different metabolic enzymes such as SOD and Catalase. We only found significant decrease in the Catalase activity, an enzyme known to neutralize hydrogen peroxide formed in the cellular metabolism, in the APP^{swe}+PS1dE9 (Fig. 5.6 D, n=5, p< 0.05) mice relative to control mice group. CaE 2.5 g/kg for 8 months competently increased the Catalase activity in transgenic mice relative to transgenic group alone (Fig. 5.6D, n=5, p<0.05). CaE treatment at 5g/kg dose increased the Catalase activity though the increase was insignificant relative to double transgenic APP^{swe}+PS1dE9 mice group alone. The above results implicate the effectiveness of CaE in preventing the ROS generation in APP^{swe}+PS1dE9 mice model of AD and most probably by increasing the Catalase activity. There is also a possibility of CaE to exert anti-oxidative effect as a secondary effect of decreasing the overall immune mediated response in the brain of bigenic APP^{swe}+PS1dE9 mice model of AD.

Figures and Legends:

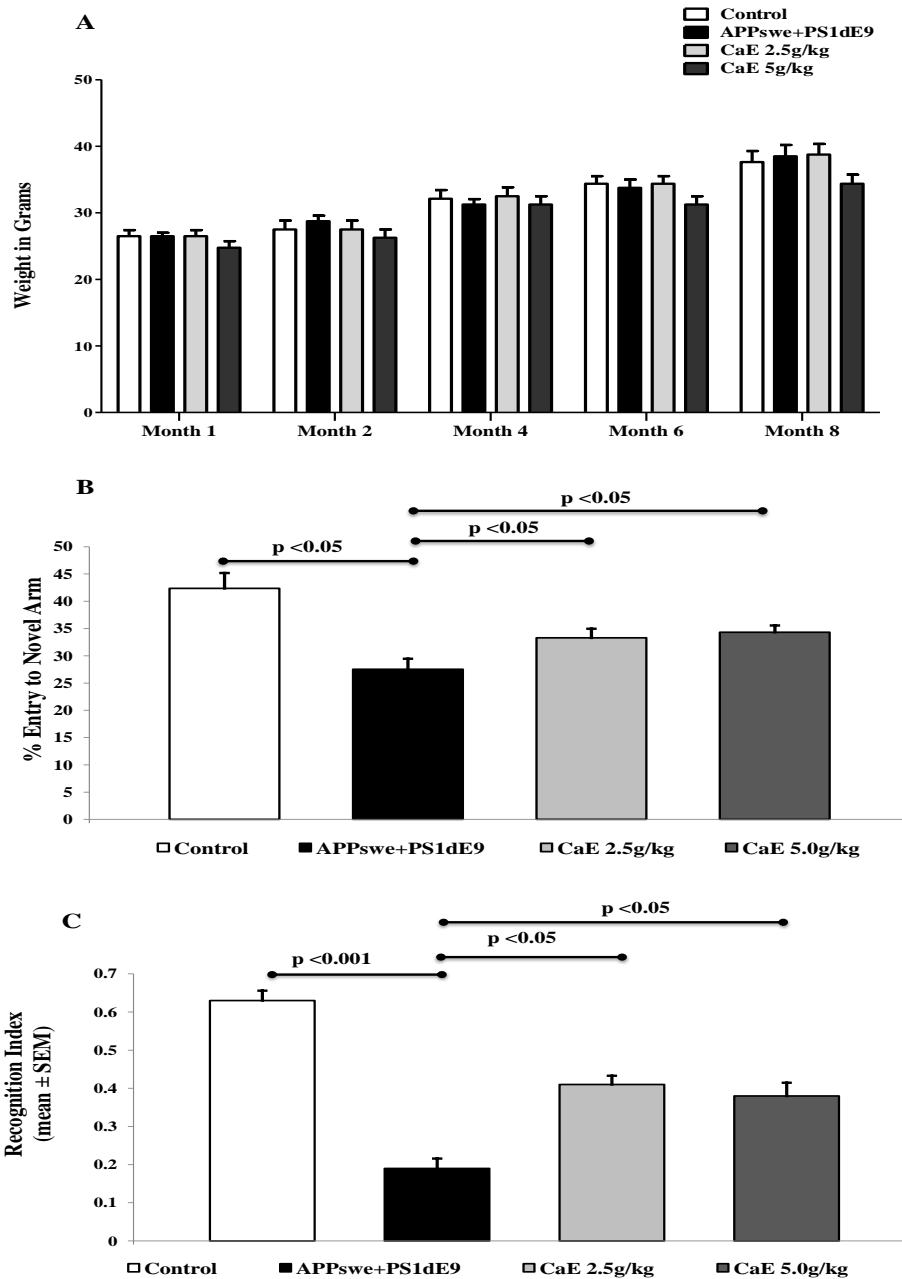


Figure 5.1: CaE ameliorates behavioral deficits in APPswe+PS1dE9 mice. A) Bar plot illustrates no change in the body weight of animals from different treatment group measured at 5 time points, which are 1 month, 2 month, 4 month, 6 month and 8 month. Body weight is expressed as mean \pm SEM, n=10. B) Bar plot depicts the percentage entry into novel arm of the Y-maze on the day of learning trial. CaE at both 2.5g/kg and 5g/kg doses ameliorated the decline

in the % entries into novel arm observed in the APP^{swe}+PS1^{dE9} mice. Data is expressed as mean \pm SEM, n=6. Statistical significance defined as $p < 0.05$. C) Bar plot represents the recognition memory expressed as recognition index assessed through object recognition task. CaE treatment significantly prevented the recognition deficits in bigenic APP^{swe}+PS1^{dE9} mice model of AD. Data is expressed as mean \pm SEM, n=6. Statistical significance defined as $p < 0.05$.

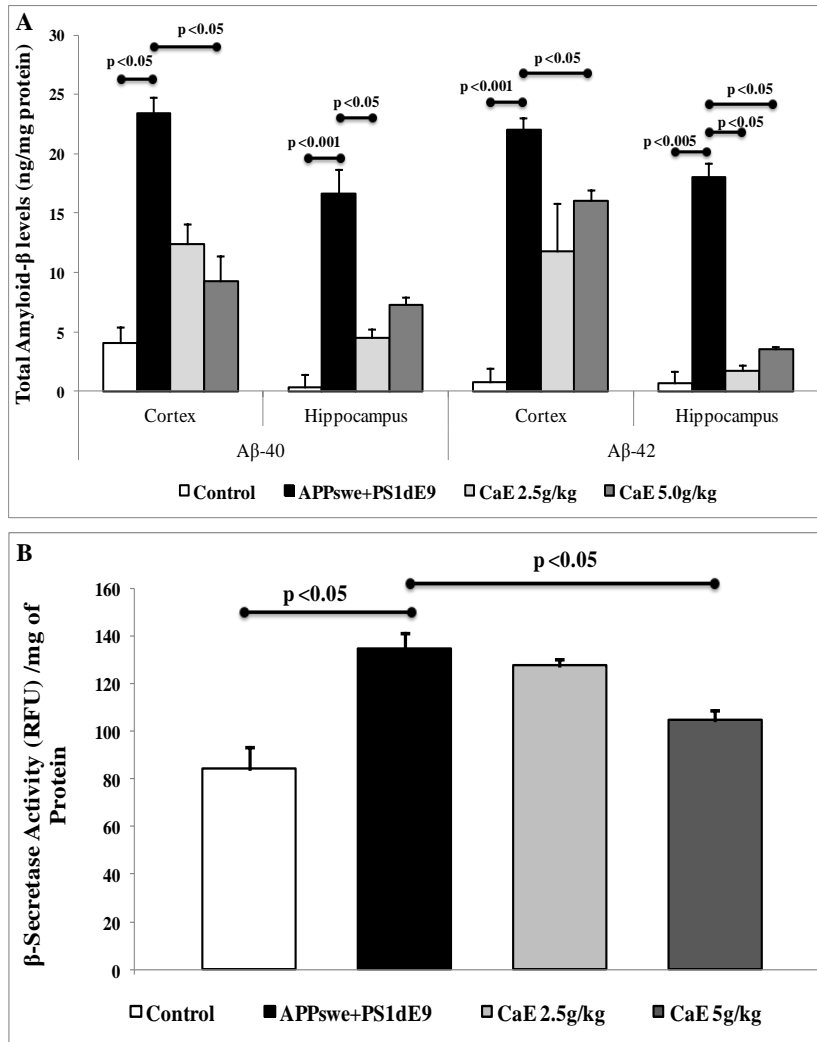


Figure 5.2: CaE attenuates deposition of A β load in double transgenic APPswe+PS1dE9 mice. A) Bar plot depicts the changes in the A β_{40} and A β_{42} levels in cortex as well as hippocampus of different treatment groups. CaE at both lower and higher doses prevented the deposition of A β_{42} in the hippocampus of APPswe+PS1dE9 mice. A β_{40} and A β_{42} levels were expressed as ng/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as p<0.05. B) Bar plot illustrates the significant increase in β -Secretase enzymatic activity in cortical regions of the brain of bigenic APPswe+PS1dE9 mice relative to control mice (p<0.05, n=6). CaE at higher dose 5g/kg, counteracted the increase in the β -

Secretase activity in APP^{swe}+PS1^{dE9} mice. The β -Secretase enzymatic activity was expressed as RFU/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as p<0.05.

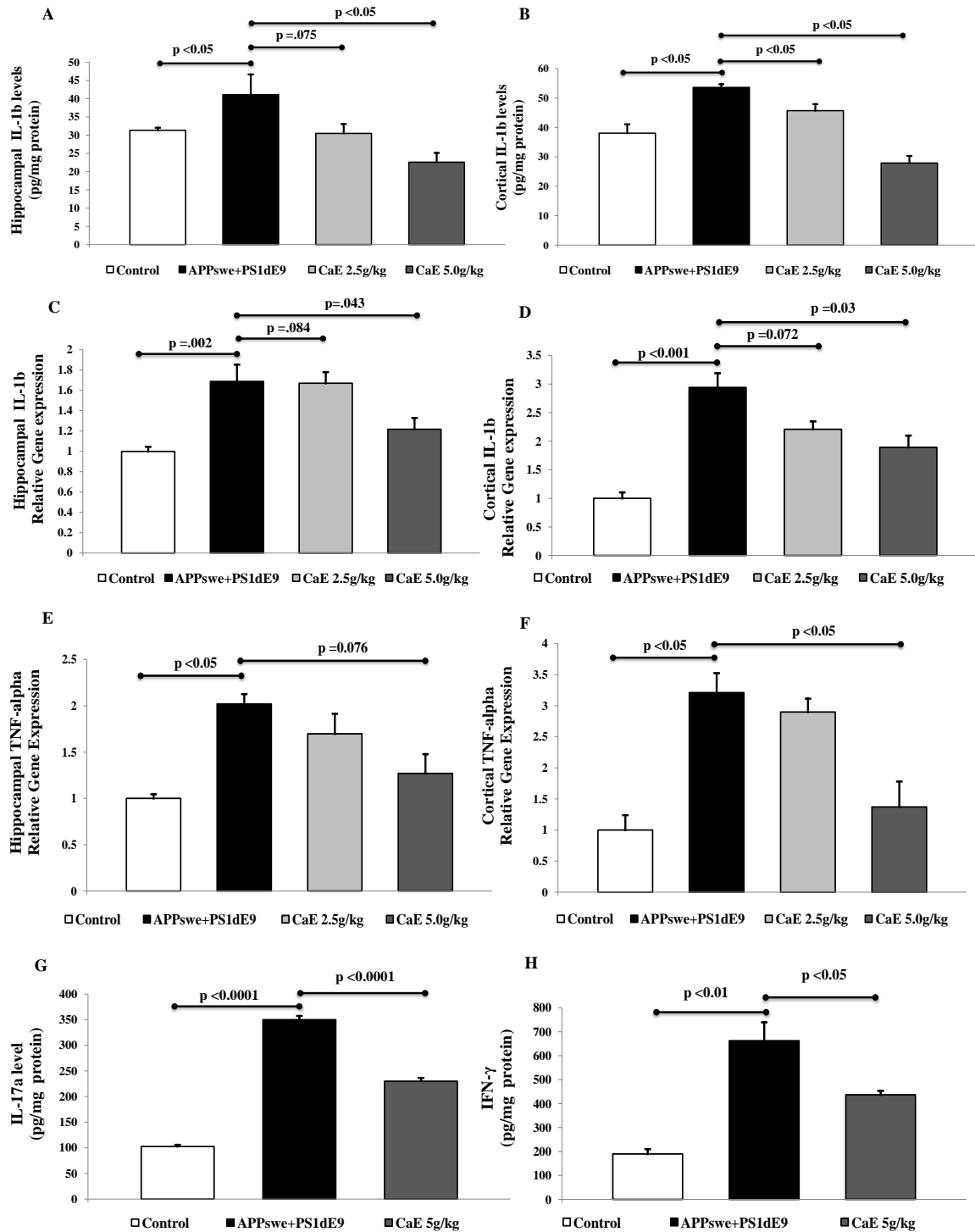


Figure 5.3: CaE ameliorated pro-inflammatory immune mediators in APPswe+PS1dE9 mice model of AD. A) Bar plot depicts the changes in the levels of IL-1 β cytokine in

hippocampus of the brain of different treatment groups measured through Multiplex analysis. IL-1 β levels were expressed as pg/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as p<0.05. B) Bar plot depicts the changes in the levels of cortical IL-1 β cytokine in the brain of different treatment groups measured through Multiplex analysis. IL-1 β levels were expressed as pg/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as p<0.05. C) Bar plot represents the relative fold change in the mRNA levels of IL-1 β cytokine in hippocampus region of the brain of different treatment groups after normalization with the β -actin mRNA levels. IL-1 β levels were plotted as relative mean \pm SEM, n=5 for each group. Statistical significance defined as p<0.05. D) Bar plot represents the relative fold change in the mRNA levels of IL-1 β cytokine in cortical region of the brain of different treatment groups after normalization with the β -actin mRNA levels. IL-1 β mRNA levels were plotted as relative mean \pm SEM, n=5 for each group. Statistical significance defined as p<0.05. E) Bar plot illustrates the relative fold change in the mRNA levels of TNF- α in hippocampus region of the brain of different treatment groups after normalization with the β -actin mRNA levels. TNF- α mRNA levels were plotted as relative mean \pm SEM, n=5 for each group. Statistical significance defined as p<0.05. F) Bar plot illustrates the relative fold change in the mRNA levels of TNF- α in cortical region of the brain of different treatment groups after normalization with the β -actin mRNA levels. TNF- α mRNA levels were plotted as relative mean \pm SEM, n=5 for each group. Statistical significance defined as p<0.05. G) ELISA dependent analysis of IL-17a levels in the whole brain lysate of different treatment groups. Only CaE at higher doses was assessed. IL-17a levels were expressed as pg/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as p<0.05. H) ELISA dependent analysis of IFN- γ levels in the whole brain lysate of different treatment groups. Only CaE at higher doses was assessed in comparison to other treatment groups. IFN- γ levels were expressed as pg/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as p<0.05.

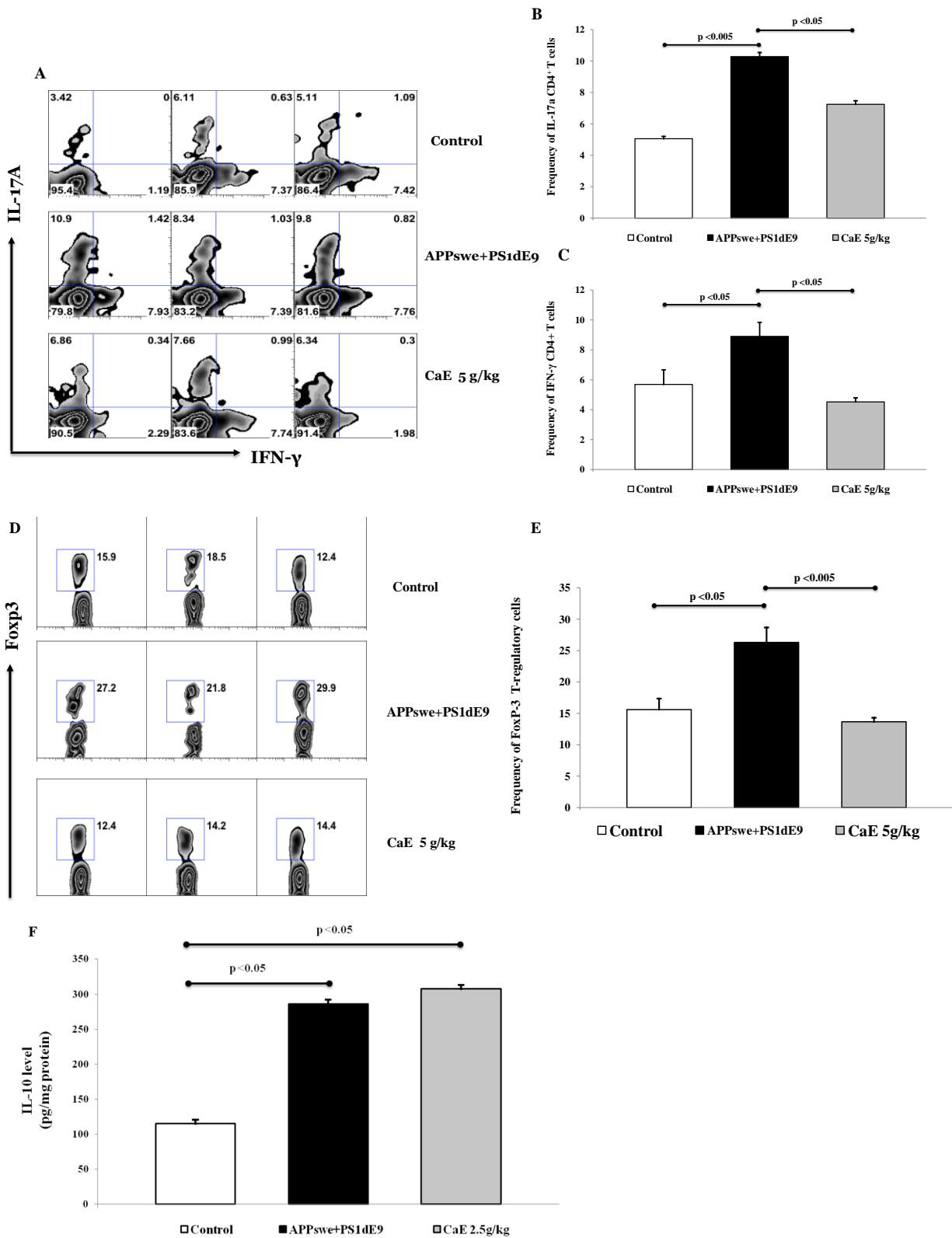


Figure 5.4: CaE ameliorated pro-inflammatory immune mediators in APP^{swe}+PS1^{dE9} mice model of AD. A) Flow cytometric contour plot illustrating the frequency of IL-17a +CD4+ and IFN- γ + CD4+ T-cells extracted from the whole brain of different treatment groups. The four quadrants represent the different intensity of number of IFN- γ and IL-17a labeled CD4+ T-cells. B) Bar plot depicting the change in the frequency of IL-17a secreting CD4+ T-cells. Data expressed as mean \pm SEM of frequency of CD4+ cells obtained from 5 animals from each group (n=5). Statistical significance defined as $p < 0.05$. C) Bar plot depicting the change in the frequency of IFN- γ secreting CD4+ T-cells in different treatment groups. Data expressed as mean \pm SEM of frequency of CD4+ cells obtained from 5 animals from each group (n=5). Statistical significance defined as $p < 0.05$. D) Flow cytometric contour plot illustrating the frequency of CD4+FoxP3+ cells extracted from the whole brain of the animals of the different treatment groups. (E) Bar plot shows the change in the frequency of FOXP3+CD4+ T-cells in the in different treatment groups. Data expressed as mean \pm SEM of frequency of CD4+ cells obtained from 5 animals from each group (n=5). Statistical significance defined as $p < 0.05$. F) ELISA dependent analysis of IL-10 levels in the whole brain lysate of different treatment groups. IL-10 levels were expressed as pg/mg protein and plotted as mean \pm SEM, n=6 for each group. Statistical significance defined as $p < 0.05$.

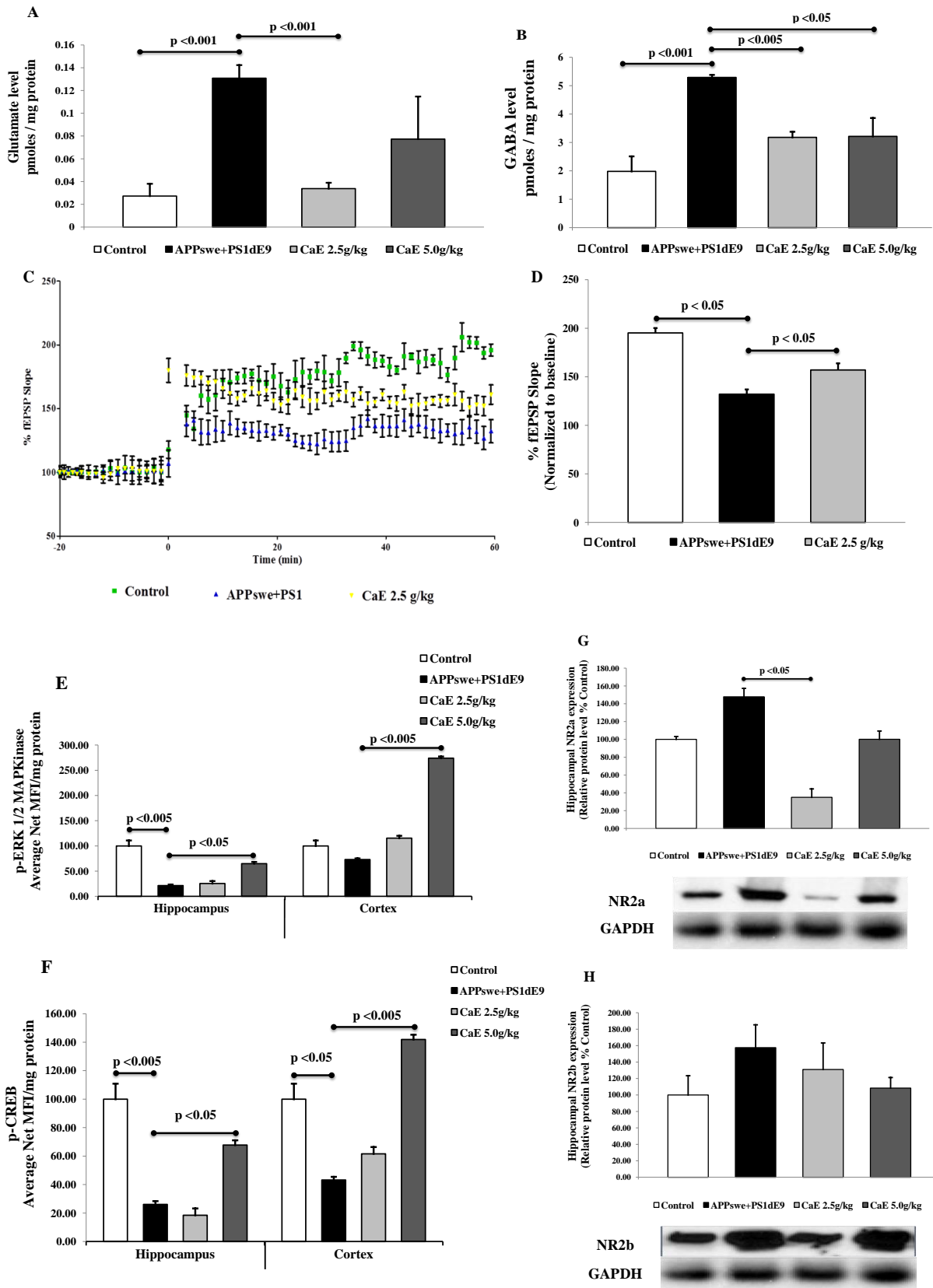


Figure 5.5: CaE ameliorates synaptic deficits in APP^{swe}+PS1^{dE9} transgenic mice. A) Bar plot shows the change in the cortical glutamate levels in the different treatment groups. Glutamate levels are expressed as pmoles/mg protein and plotted as mean \pm SEM, n=5 from each treatment group. Statistical significance defined as $p < 0.05$. B) Bar plot shows the change in the cortical GABA levels in the different treatment groups. GABA levels are expressed as pmoles/mg protein and plotted as mean \pm SEM, n=5 from each treatment group. Statistical significance defined as $p < 0.05$. C) LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS. LTP was significantly impaired in acute hippocampal slices from animals of different treatment groups. Percentage field excitatory post synaptic potential (fEPSP) slope was expressed as mean \pm SEM of 6 slices from 6 animals from each treatment group. D) Bar plot showing change in the percentage slope of LTP in different treatment groups. Statistical significance defined as $p < 0.05$. E) Bar plot depicting the change in the phosphorylated ERK1/2 protein levels in the cortical and hippocampal areas of the brain of animals from each group determined through Millipore multiplex analysis. Phosphorylated protein were normalized with mg protein and expressed as mean \pm SEM, n=6. Statistical significance defined as $p < 0.05$. F) Bar plot depicting the change in the phosphorylated CREB protein levels in the cortical and hippocampal areas of the brain of animals from each group determined through Millipore multiplex analysis. Phosphorylated protein were normalized with mg protein and expressed as mean \pm SEM, n=6. Statistical significance defined as $p < 0.05$. G) Bar plot depicting the relative change in the protein expression (after normalization with β -actin band density) of NR2a subunit of NMDA receptors in hippocampus of the brain of different treatment groups. Data is expressed as percentage relative density of each band to control group and plotted as mean \pm SEM, n=6. Statistical significance defined as $p < 0.05$. H) Bar plot depicting the relative change in the protein expression (after normalization with β -actin band density) of NR2b subunit of NMDA receptors in hippocampus of the brain of different treatment groups. Data is expressed as percentage relative density of each band to control group and plotted as mean \pm SEM, n=6. Statistical significance defined as $p < 0.05$.

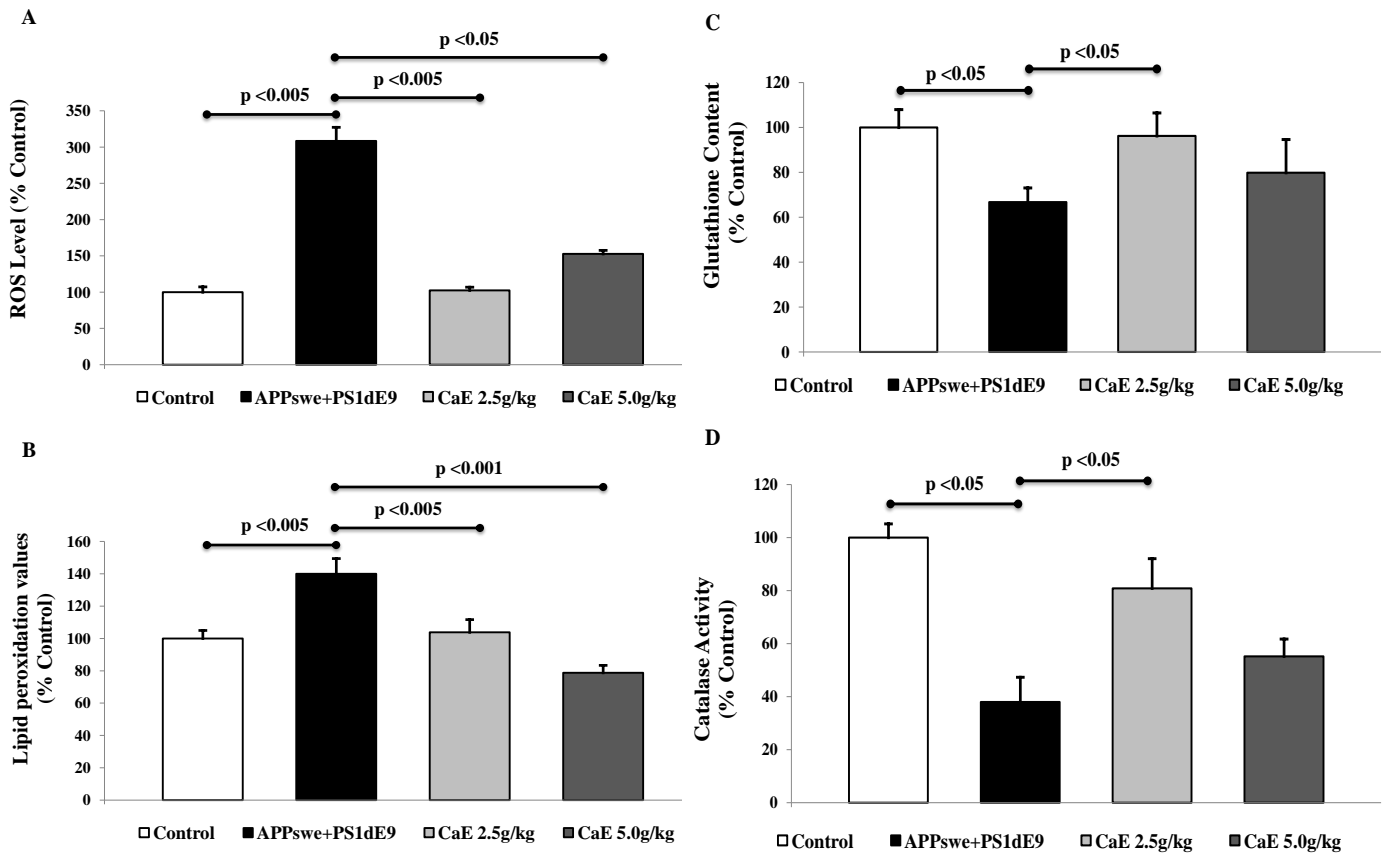


Figure 5.6: CaE attenuates oxidative stress in APPswe+PS1dE9 transgenic mice. A) Bar plot depicts the change in the reactive oxygen species (ROS) levels in the whole brain lysate of the different treatment groups. Data is expressed as percentage control and plotted as mean \pm SEM, n=5 from each treatment group. Statistical significance defined as $p < 0.05$. B) Bar plot depicts the change in the lipid peroxidation levels in the different treatment groups. Data are expressed as percentage control and plotted as mean \pm SEM, n=5 from each treatment group. Statistical significance defined as $p < 0.05$. C) Bar plot depicts the change in the total glutathione levels in the different treatment groups. Data are expressed as percentage control and plotted as mean \pm SEM, n=5 from each treatment group. Statistical significance defined as $p < 0.05$. D) Bar plot depicts the change in the total Catalase activity in the different treatment groups. Data are expressed as percentage control and plotted as mean \pm SEM, n=5 from each treatment group. Statistical significance defined as $p < 0.05$.

5.4. Discussion

CaE mediated suppression of inflammation, a major mechanism underpinning the cognition enhancing and neuro-protective effect of CaE: Numerous reports from the other investigators have successfully revealed state of chronic inflammation in the CNS of bigenic APP^{swe}+PS1^{dE9} mouse model of AD, especially in the cortical and hippocampal regions. These inflammatory responses include CD11b-positive activated microglia, activated astrocytes (as shown by Clustered glial fibrillary acidic protein (GFAP)-positive astrocytes) at an early age of 4 and 6 months, respectively (Garcia-Alloza et al., 2006; Ruan et al., 2009). Accompanying this chronic inflammation was the appearance of robust pro-inflammatory cytokine and chemokines release especially comprising of TNF- α , IL-1 β , IL-6 and MCP-1 at 8 to 10 months old APP^{se}+PS1^{dE9} mice. These observations entails the modulation of the immune mediated inflammatory response, as shown by diminished pro-inflammatory cytokine profile in the CNS of APP^{swe}+PS1^{dE9} bigenic mouse, as the major mechanism by which CaE exerts neuroprotective and cognition enhancing effect. Inflammation is considered as a secondary process to A β toxicity, though completely capable of accomplishing the AD pathophysiology and progression of AD. Previous reports have implicated A β to stimulate NLRP3 inflammasome via P2X 7 receptor and a cathepsin B-sensitive pathway (Niemi et al., 2011). The inflammasome activity leads to excessive IL-1 β and IL-18 release from different immune responsive defensive cells present in the CNS such as microglia, astrocytes and oligodendrocytes (Halle et al., 2008). Inflammatory release of pro-inflammatory cytokines, chemokines and other chemical mediator results in recruitment of macrophages and phagocytes as well as it activates the innate T-cell population in the CNS to A β . T-cell activation further facilitates self-perpetuated release of inflammatory cytokines and building up of a chronic state of inflammation in the CNS of AD

experimental animal models. CaE treatment for 8 months significantly decreased the pro-inflammatory cytokine levels, especially the IL-1 β levels. Attenuation in the inflammatory cytokine levels especially the IL-1 β levels seems to be the central key factor of CaE in mediating neuro-protection and improving learning and memory in the APP_{swe}+PS1dE9 mice model of AD. AS reported earlier IL-1 β along with other inflammatory mediators modulates the β -secretase enzyme activity and facilitates the A β secretion and deposition (Yamamoto et al., 2007), which appear at the same age implicating the strong association. We observed CaE treatment to decrease the β -secretase enzyme activity and attenuate the A β levels in the double transgenic animal model. In addition, we observed CaE to ameliorate the synaptic deficits which were observed in the bigenic animal model. The synaptic deficits are suggested to be tightly tied together with chronic inflammation seen in the brain of AD patients. Proving the fact is the body of evidence provided by the previous reports where the appearance of inflammatory response and synaptic deficits occur at the same age in the bigenic APP_{swe}+PS1dE9 mice (Savonenko et al., 2005; Reiserer et al., 2007; Ruan et al., 2009; Bonardi et al., 2011). Also, inflammasome activity seems to be perturbed by A β levels in the APP_{swe}+PS1dE9 mice via P2X7 receptor in the microglia (Lee et al., 2011). CaE treatment for 8 months has shown effective control on the inflammatory response in the APP_{swe}+PS1dE9 mice model. A recent report has suggested the involvement of T-helper cell induced inflammatory response in the 6-7 month old APP_{swe}+PS1dE9 mice (Browne et al., 2013). Concordantly, we have shown increased T-helper cell activation and maturation to Th-1 and Th-17 type of cells, which are maintaining the chronic inflammation in the transgenic animal. CaE treatment at higher dose effectively attenuated the frequency of Th-1 and Th-17 type of CD4⁺ cells in the brain of bigenic APP_{swe}+PS1dE9 mice model in the current study. This implicates that by decreasing the activation and maturation of T-

helper cells in the brain of APP^{swe}+PS1^{dE9} mice, CaE attenuates the chronic inflammation and exerts neuroprotective effects. The anti-inflammatory and neuroprotective effect of Centella is in accordance with the previous reports of CaE mediated neuroprotection against glutamate and other toxins such as Amyloid-beta (Dhanasekaran et al., 2009; Krishnamurthy et al., 2009; Prakash and Kumar, 2012; Xu et al., 2012). However, the current study for the first time describes in detail the anti-inflammatory effects of CaE to be the central factor for neuroprotection and improving behavioral deficits by employing a double transgenic model of AD.

Our results shows the potent anti-inflammatory effects of *Centella asiatica* concentrated extract (20% concentrated with asiaticoside) against A β -induced neuro-toxicity and behavioral deficits in the double transgenic mouse model of AD. CaE anti-inflammatory properties mediate the anti-oxidative, anti-apoptotic and synaptic strengthening actions in the APP^{Swe}+PS1^{dE9} mice model. This ensures the future employment of the *Centella* extract as an innovative herbal drug intervention against AD induced dementia and neurotoxicity.

5.5. References

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6. Summary and Conclusion

The current study investigated the therapeutic potential and molecular mechanisms of a novel traditional herbal drug, *Centella asiatica* in strengthening cognitive abilities and ameliorating synaptic deficits in the most common neurodegenerative cognitive disorder, which is AD. We utilized *in-vitro* as well as *in-vivo* models, which simulate various pathophysiological factors associated with cognitive dysfunction commonly witnessed in AD patients, to investigate the molecular mechanism of *Centella asiatica* extract of whole plant. The conclusion from this study is presented in form of the salient findings, which are as follows.

- I. Our *in-vitro* studies have shown *Centella asiatica* extract to possess anti-oxidative, anti-caspase and anti-inflammatory activities *per se*.
- II. We found *Centella asiatica* extract *per se* in the hippocampal neuronal population to effectively modulate the NMDA receptors and increase the phosphorylation of CREB, CAMKII and ERK1/2 signaling proteins. Thus, results from this study established *Centella asiatica* extract to modulate the expression of glutamatergic receptors via modulation of PKA/CAMKII/ERK1/2/CREB pathway. Implications from this study indicate the possible utilization of *Centella asiatica* extract in improving synaptic deficits and cognitive dysfunction various neurodegenerative disorders.
- III. *Centella asiatica* extract at different concentrations in two different differentiated neuronal populations exhibited significant neuroprotective activities against Hydrogen peroxide induced neurotoxicity.
- IV. *Centella asiatica* extract significantly decreased the ROS generation in hydrogen peroxide challenged neuronal cells and thus antioxidant activity of *Centella asiatica* extract is considered to be one of the underlying neuroprotective mechanisms.

- V. Against oxidative stress, *Centella asiatica* extract was found to modulate the NR2b and NR2a receptors significantly. NR2b receptors were found to be significantly down regulated due to CaE treatment and NR2a receptors were up regulated. Previous reports have shown NR2b to mediate excitotoxicity and NR2a to induce neuronal survival and enhance synaptic plasticity.
- VI. Conclusively from the *in-vitro* study, we found CaE to possess three important neuroprotective properties, which were antioxidant, anti-apoptotic and anti-excitotoxic activities.

We next tend to validate our *in-vivo* animal model, which is a well established double transgenic mice model of AD. We aim to employ an animal model that has synaptic deficits along with other underlying factors such as oxidative stress, chronic inflammation and robust apoptosis. The bigenic APP^{swe}+PS1^{dE9} mice model of is well accepted animal model of AD that has shown to exhibit all above mentioned pathophysiological factors. In order to confirm the usefulness of selected *in-vivo* mouse model, we evaluated the inflammatory, oxidative, apoptotic anomalies as well as the synaptic deficits in the APP^{swe}+PS1^{dE9} mice. The salient findings from this study were:

- I. We found extensive behavioral deficits in the APP^{swe}+PS1^{dE9} mice model and without any signs of neuro-toxicological symptoms such as tremors, epilepsy, piloerection and straub tail etc.
- II. Bigenic APP^{swe}+PS1^{dE9} mice model of AD exhibited severe synaptic deficits as shown by significant LTP deficits. In addition, APP^{swe}+PS1^{dE9} mice showed marked alterations in the NDMA types of glutamatergic receptors along with the deficits in the ERK phosphorylation, CREB phosphorylation and p70S6 kinase activation in the

hippocampus and cortical regions of the brain. All these observations implicate prominent synaptic deficits in the APP^{swe}+PS1^{dE9} mice model of AD.

- III. We also found extensive immune mediated response leading to chronic inflammation in the different areas of the brain of APP^{swe}+PS1^{dE9} mice. We found significant increase in the pro-inflammatory cytokine levels especially IL-1 β , IL-17a and IFN- γ . Also, we found significant increase in the levels of the activated T-helper cells. In the brain of APP^{swe}+PS1^{dE9} mouse, we found significant elevation in the IL-17a secreting T-helper cells and IFN- γ secreting T-helper cells, which indicate T-helper 1 cell mediated inflammation.
- IV. Along with the prominent inflammation, we found marked elevation in the oxidative stress. There was marked elevation in the levels of ROS species as well as lipid peroxidation in the APP^{swe}+PS1^{dE9} mice.
- V. Conclusively, APP^{swe}+Ps1^{dE9} mice model is the best suitable *in-vivo* model for our investigative study with CaE as we found behavioral and synaptic deficits along with the prominent inflammatory response as well as profound oxidative stress.

Our next goal was to find the effectiveness of CaE in combating the behavioral and synaptic deficits in the APP^{swe}+PS1^{dE9} mice model of AD. We also intended to discern the molecular mechanism of CaE against A β mediated toxicity in the brain of APP^{swe}+PS1^{dE9} mice. CaE was orally administered to APP^{swe}+PS1^{dE9} mice for 8 months to simulate the regimen of food supplement and prevent the development or progression of AD. We found following salient findings from the study.

- I. CaE at both doses prevented the behavioral deficits as well as attenuated the A β deposition in the APP^{swe}+PS1^{dE9} mice. We did not find any signs of general neuro-

toxicity even after eight months of *Centella asiatica* extract administration in the APPSwe+PS1dE9 transgenic mice.

- II. The improvement in the cognitive performance of the APPswe+PS1dE9 mice was found to be strongly proportionate with the amelioration of the synaptic deficits observed in the APPswe+Ps1dE9 mice. In addition, NR2b receptor level were found to be down-regulated and NR2a to be up regulated by CaE treatment.
- III. Interestingly, the beneficial effects of CaE were found to be due to the strong anti-inflammatory action of *Centella asiatica* extract. Evidently, *Centella asiatica* extract decreased the frequency of T-helper cells both IL-17a+CD4+ and IFN- γ +CD4+ cells in the brain of APPswe+PS1dE9 mice, which was accompanied by the drastic improvement in the cytokine and chemokines profile of the immune mediators in the brain of APPswe+PS1dE9 mice.
- IV. Oxidative stress was found to be attenuated in the *Centella asiatica* extract treated group, along with the strong elevation of the antioxidant enzymes system eventually boosting up the oxidative stress resistance of the brain. Anti-inflammatory effect of *Centella asiatica* extract is reasonably believed to be the central factor for exerting and strengthening the antioxidant effect in the brain and protecting the neuronal cells against the A β induced cell death.
- V. Summing up, our overall results evidently reveal the neuroprotective potential of CaE, if used as a supplement in prevention of age related cognitive disorders. In addition, we for the first time have shown CaE to act by ameliorating the T-cell mediated response in the brain of aAPPswe+PS1dE9 mice. Our results also imply the involvement of an upstream

mediator of inflammation being modulated by *Centella asiatica* extract to execute its anti-inflammatory properties.

Our overall findings support pleiotropic mechanism of *Centella asiatica* extract working coherently to improve the cognitive performance of the 10 month old APP^{swe}+PS1^{dE9} mice. Among these mechanisms, the most important is the anti-inflammatory effect of CaE, which holds up the central position. As *Centella asiatica* extract in *in-vitro* studies has exhibited modulation of several other important mediators of neuronal cell death, therefore the pleiotropic effects of *Centella asiatica* extract cannot be completely neglected. Thus, our study establish the *Centella asiatica* extract to be a suitable candidate for the palliative therapy as well as prototype for future studies that can yield better therapeutic interventions for neurological cognitive disorders.